

**An investigation of the functions of leaf surface modifications in the
Proteaceae and Araucariaceae.**



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Abstract

Plant leaves exhibit a remarkable diversity of size, shape, developmental patterns, composition, and anatomical structures. Many of these morphological variations are assumed to be adaptations that optimize physiological activity and thus assist plants to survive in a range of different habitats. This study aimed to investigate the function of some of these leaf modifications, including leaf wax, stomatal plugs and stomatal crypts.

Investigations using *Leucadendron lanigerum* (Proteaceae) indicated that the amount of waxy coverage and the shape of wax crystals varied with the age of the leaves and the season. Wax coverage was found to significantly lower cuticular water loss but had no impact on reflectance. There was a significant increase in photosynthesis and transpiration rates in leaves from which wax had been removed. This increase was most likely due to an increase in stomatal conductance of the leaves after removing epicuticular wax. Despite the lack of effect on leaf reflectance, removal of wax prior to exposure to high light resulted in significant decreases in efficiency relative to control leaves. Overall, these results suggest that the presence of wax on the epidermis and at the entrance of stomata of *L. lanigerum*, in addition to restricting water loss, may also provide some protection against photodamage.

The impact of stomatal plugs on gas exchange in *Agathis robusta*, a rain forest tree from the Araucariaceae was investigated. Under saturating PFD, leaves with plugs had significantly lower transpiration rates, stomatal conductance and photosynthetic rates, but higher leaf temperatures than unplugged leaves. Water loss in detached leaves kept in the dark was significantly greater in unplugged than plugged leaves. In contrast, plugs had no impact on water film formation and

both plugged and unplugged leaves had similar electron transport rates when wet. These results suggest that stomatal plugs in *Agathis robusta* present a significant barrier to water loss but do not prevent water films from forming.

It was also demonstrated that the establishment of stomatal plugs in *Agathis robusta* occurs annually and, unlike trichomes in other species, stomatal plugs could be replaced at least during the first two years of leaf life. Investigation of leaves infected by fungi showed that waxy plugs blocked the penetration of stomata by fungal hyphae. Hyphae penetrated the leaf tissue either through stomata that lacked waxy plugs or at later stages of infection, directly through the cuticle. This suggests that stomatal plugs in *Agathis robusta* present a significant barrier to fungal penetration through stomata, and so help to prevent fungal infection of leaves. This function is important for trees living in rain forest environments where fungal attack is common.

Finally, investigation into the impact of stomatal crypts on cuticular water loss in *Banksia* species indicated that, contrary to previous speculation, stomatal crypts play little or no role in increasing resistance to water loss. No relationship was found between crypt depth and rates of transpiration over a range of VPDs, in the 14 *Banksia* species studied. A strong positive relationship between leaf thickness and crypt depth was found, while a negative relationship was observed between leaf thickness and stomatal density.

Dedication

This thesis is dedicated to Prof. Russell Baudinette, the former head of the Department of Environmental Biology and interim head of the School of Earth and Environmental Sciences.

Russ was not only a remarkable administrator and researcher, he was a great friend. I learned many things from him that will be a huge benefit in my future life.

I feel very sad that I will not be able to see him again while I am leaving here, and I will always miss his kind manner, humour and compassion.

One of the most important lesson that I learned was that reaching the peak of the mountain is not the greatest pleasure in life, but enjoying the climb is the real pleasure of our lifetime. I think that few people embodied this lesson like Russ.

Declaration

I declare that this thesis contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution.

To the best of my knowledge and belief, this thesis contains no material previously published or written by another person, except where due reference is made in the text of this thesis.

I give my consent to the copy of my thesis, when deposited in the University library, being made available for loan or photocopying.

March, 2005

Signed

Mansour Afshar Mohammadian

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1. Introduction

Variation in several leaf morphological parameters may serve as adaptations for plants living in different habitats (Smith and Nobel 1977; Poblete *et al.* 1991; Jordaan and Kruger 1992; Soliman and Khedr 1997; Mauseth 1999; Villar-de-Seoane 2001; Waldhoff and Furch 2002; Waldhoff 2003). Although fully open stomata occupy only approximately 0.5% to 5% of the leaf surface, more than 95% of the water lost by plants, and almost all of the CO₂ gained, passes through stomata (Jones 1983). Therefore, stomata and the modifications of stomata are of great interest in understanding how plants regulate gas exchange, including CO₂ uptake and water loss. However, little quantitative discussion and experimental research has been conducted on the impact of various stomatal modifications, e.g. stomatal plugs, stomatal crypts and waxy coverage, on gas exchange. Therefore, despite numerous assumptions about the impact of different stomatal modifications on physiological activity of leaves, there is a lack of empirical evidence to support these assumptions.

Leaf Modification and Water Stress

Genotype and environmental factors are responsible for variation in leaf characteristics in plants (Hardy *et al.* 1995; Hovenden and Schimanski 2000; Hovenden 2001; Gomez-del-Campo *et al.* 2003). Water stress is one environmental factor that has been invoked as a major selective force in the evolution of leaf traits (Jeffree *et al.* 1971; Ehleringer 1981; Robinson *et al.* 1993; Turner 1994; Hardy *et al.* 1995; Taiz and Zeiger 1998, P 726; Ockerby *et al.* 2001; Gomez-del-Campo *et al.* 2003). In general, drought tolerance is the capacity of a plant to withstand periods of dryness. This can be achieved through improved

water uptake from the soil, reduced water loss, and better water conservation (Seddon 1974). In this regard, xerophytes are those species that are adapted to meet the conditions of strongest transpiration and most insecure water supply.

Xerophytes have a number of leaf traits that have been linked to drought tolerance, these include: stomatal crypts, sunken stomata, low stomatal density, epicuticular wax, a well developed coating of trichomes, large epidermal cells, thick epidermal cell walls, compact mesophyll, thick cuticles, leathery leaves, and low leaf area (Seddon 1974; Smith and Nobel 1977; Weiglin and Winter 1991; Jordaan and Kruger 1992; Carpenter 1994; Soliman and Khedr 1997; Villar-de-Seoane 2001; Waldhoff and Furch 2002; Gomez-del-Campo *et al.* 2003; Torre *et al.* 2003; Waldhoff 2003). As evaporation from leaves is dependent on the stomata, boundary layer and cuticular conductance (Wei and Wang 1994), any modification of leaf properties that impacts on these parameters is likely to influence water loss.

Furthermore, in dry habitats natural selection would favor any morphological response or other mechanism that reduces water loss (Hill 1998a). However, there is no common agreement on the possible function of different leaf modifications. The leaf characteristics mentioned for plants living in arid climates can be found in plants that are not xerophytes and even in plants living in moist conditions. This poses questions about the primary functions of these leaf traits in plants that live in different habitats.

The Leaf Cuticle

A major challenge for most plants, especially those living in arid climates, is developing a barrier against water loss, and the cuticle that is present on all leaf

surfaces, is the major barrier against uncontrolled water loss from leaves (Nobel 1991; Riederer and Schreiber 2001; Barber *et al.* 2004). Although cuticular water loss accounts for only 5 to 10% of total leaf transpiration, it can be significant when stress is severe and even a small reduction in water loss could be vital for some plants (Kerstiens 1997; Taiz and Zeiger 2002).

The thickness of the cuticle can vary from 0.1-10 μm (Riederer and Schreiber 2001). Although a thick cuticle has been suggested as a xeromorphic adaptation (Carpenter 1994; Reynoso *et al.* 2000; Riederer and Schreiber 2001), permeability of the cuticle to water does not always decrease with increasing thickness of the cuticle (Mickle 1993; Riederer and Schreiber 2001). Kersteins (1996) stated that a thick cuticle does not necessarily increase the resistance of a leaf to water transfer. However, Hajibagheri (1983) observed an inverse relationship between cuticle thickness and epidermal water loss in *Suaeda maritima*. Also, research conducted on *Zea mays* (maize) revealed an inverse relationship between epidermal water loss and cell wall and cuticle thickness (Ristic and Jenks 2002). Overall, it has been frequently assumed that a thicker cuticle may protect plants against water loss (Grubb 1977; Fahn and Cutler 1992; Turner 1994; Wirthensohn and Sedgley 1996; Reynoso *et al.* 2000; Ristic and Jenks 2002). Although, the cuticle is a barrier to water loss (DeLucia and Berlyn 1984), it is the epicuticular wax that has been identified as the major component of plant cuticles responsible for reducing water loss (Nobel 1991; Takamatsu *et al.* 2001).

Leaf Cuticular Wax

Some leaf properties e.g. cuticular wax and trichomes, occur in both dry and wet environments. Therefore, it is likely that they have multiple functions. It has been

suggested that epicuticular wax, in addition to increasing resistance to water loss and reflecting light, creates a hydrophobic surface, beading off water as well as removing pathogens and pollutants from leaf surfaces (McNeilly *et al.* 1987; Neinhuis and Barthlott 1997; Gordon *et al.* 1998; Beattie and Marcell 2002).

The endoplasmic reticulum is thought to be involved in the synthesis of wax and these waxes are transported out of the epidermal cells by exocytosis (Jenks *et al.* 1994). Wax, which is often embedded in the cuticle and sometimes coats the cuticle, is a heterogeneous polymer of long chain fatty acids (up to C₃₄), aliphatic alcohols and alkanes. Thus it is more complicated chemically than cutin, which contains mostly shorter chain fatty acids (C₁₆, C₁₈) (Kolattukudy 1980; Baker 1982; Holloway 1982; Walton 1990). The chemistry and diversity of surface waxes has been extensively reviewed (Baker 1982; Holloway 1982; Walton 1990; Bianchi 1995; Jeffree 1996). However, in general, there are two distinct classes of wax: (1) epicuticular wax on the surface of the cuticle, and (2) intracuticular wax within the cutin layer that is composed of shorter chain fatty acids than epicuticular wax. Physical shape of wax crystals also varies in different plant species. For example, in citrus trees epicuticular wax crystals are in plate form, in *Picea* and *Ginkgo* they are rodlets and in *Eucalyptus* they are granular. The micromorphology of epicuticular waxes has been well investigated (Baker 1982; Barthlott 1990; Barthlott *et al.* 1998; Meusel *et al.* 1999), however, the impact of different forms of epicuticular wax crystals on gas diffusion is not clear yet.

Cuticular waxes essentially establish barriers, whereas cutin forms a mechanically stable matrix supporting the waxes (Leng *et al.* 2001). Baur (1997) suggested that waxes fill spaces within the lamellae of the cuticle (intra cuticular wax) and increase resistance to vapour flow. Numerous studies have been conducted to

investigate the relationship between the amount of epicuticular wax and cuticular transpiration. However, the hypothesis that the quantitative and qualitative characters of epicuticular waxes determine their permeability to water has not been supported so far (Schreiber and Riederer 1996a; Riederer and Schreiber 2001). For example, Jordan (1983) found no correlation between cuticular transpiration and the quantity of epicuticular wax beyond a critical thickness in sorghum. The authors indicated that epicuticular wax greater than 0.067 g m^{-2} provides an effective barrier to water loss through the cuticles of sorghum leaves under most conditions. Bengtson (1978) found similar results in seedlings of six oat cultivars. Also, the results of research conducted on *Zea mays* (maize) revealed that the amount of cuticular wax did not correlate inversely with epidermal water loss (Ristic and Jenks 2002). In contrast, Clarke (1993) reported that the quantity of epicuticular waxes on crops such as wheat influences water relations, wettability of the leaf, and resistance to insects and diseases. It may be that epicuticular wax only impacts water loss if its thickness approaches a certain threshold, and above this the impact on cuticular transpiration does not change. Nevertheless, there is no common agreement about the impact of cuticular wax on water loss.

Considering the low thickness of the wax layer on leaf surfaces, it is unlikely that this layer could significantly increase the thickness of the boundary layer and as a consequence increase the resistance to gas diffusion through stomata. For example, Reicosky and Hanover (1976) calculated the boundary layer thickness of *Picea pungens* and concluded that the epicuticular waxy layer of *P. pungens* is not deep enough to impact on the boundary layer and so is unlikely to affect gas exchange rates from *P. pungens* leaves. However, some researchers suggested that

a thick wax layer might increase the thickness of the boundary layer and further decrease water loss (McKerie and Leshem 1994; Hill 1998a).

Presumably, in addition to the thickness of the waxy layer, the shape of wax crystals, their physical arrangement and the chemical composition of the wax may all affect leaf gas exchange (Riederer and Schneider 1990; Reynhardt and Riederer 1994). However, it has been suggested that the physical arrangement of wax crystals has a greater effect on the diffusion properties of cuticles than the chemical properties of wax (Riederer and Schneider 1990; Reynhardt and Riederer 1994; Hauke and Schreiber 1998).

Water repellency, beading of water by forming droplets on leaf surfaces, is another function that has been suggested for epicuticular wax (Mauseth 1988; Grant *et al.* 1995; Gordon *et al.* 1998). Because CO₂ diffuses 10,000 times more slowly through water than air (Weast 1979), it is advantageous for plants to prevent the formation of water films on leaves (Smith and McClean 1989; Brewer *et al.* 1991). Consequently, epicuticular wax may help to maintain photosynthesis even in very wet environments (Smith and McClean 1989).

Leaf surface wetness may also influence pathogen invasion. By reducing leaf surface wettability, a waxy leaf surface facilitates the removal of foreign particles (e.g. dust, pollutants, spores and salt residues) by rain, fog or dew (Martin and Juniper 1970; Juniper and Southwood 1986; McNeilly *et al.* 1987; Smith and McClean 1989; Neinhuis and Barthlott 1997; Gordon *et al.* 1998; Beattie and Marcell 2002). For example, Rutledge and Eigenbrode (2003) found that epicuticular wax on pea plants reduced the attachment of *Hippodamia convergens* larvae to leaf surfaces. Also, removal of dust from the leaf surfaces can be important in warm habitats with high light. Dust particles deposited on leaf

surfaces can absorb light and as a consequence significantly increase leaf temperature (Eller 1977). Dust can also increase transpiration by mechanically holding open the stomatal pore, preventing it from closing to regulate water loss (Beasley 1942; Ricks and Williams 1974; Hirano *et al.* 1995). Thus, a waxy coverage helps to keep leaves free of contaminants and pathogens.

On the other hand, it has been reported that surface waxes increase the reflection of incoming radiation and thus help to keep the leaf cool (Seddon 1974; Ehleringer *et al.* 1976; Barthlott 1990; Schulze and Caldwell 1994; Taiz and Zeiger 1998). Johnson (1983) found that light reflectance in *Triticum turgidum* increased linearly with the amount of epicuticular wax, and also that the quantity of wax was greatest in the driest environment. In contrast, Jefferson *et al.* (1989) found that epicuticular wax had no beneficial or detrimental effects at saturating light and optimum temperature for photosynthesis of wheat (*Triticum spp.*) lines. However, the authors found that surface reflectance in the 400-700 nm wavelength regions was 8-15% higher in glaucous (waxy covered leaves) than non-glaucous leaves of wheat lines.

The inhibition of photosynthesis by excess light, which occurs when excess excitation arriving at the PSII reaction centre leads to its inactivation and/or damage is called photoinhibition (Rohacek and Bartak 1999; Waldhoff *et al.* 2002; Baker and Rosenqvist 2004; Bartak *et al.* 2004). It has been suggested that wax on the epidermis, by reflecting light from leaf surfaces, may confer significant photoprotection to plants exposed to high solar radiation environments (Robinson *et al.* 1993). If epicuticular waxes reflect light, it could benefit plants, especially those living in arid zones exposed to high solar radiation, by preventing photoinhibition.

Therefore, it is likely that cuticular wax may have multiple functions in plants living in both dry and wet environments. These functions may include increasing the resistance to water loss, removing water, pollutants and pathogens from leaf surfaces and reflecting radiation to reduce photoinhibition. However, few experimental studies have tested the above functions of epicuticular waxes.

Stomatal Plugs

Waxy stomatal plugs occur in the stomatal antechamber of many plants, particularly conifers (Stockey and Ko 1986; Schmitt *et al.* 1987; Mickle 1993; Stockey and Atkinson 1993; Brodribb and Hill 1997; Burrows and Bullock 1999). However, they do not occur in all conifers. For example, *Pinus halepensis* (Boddi *et al.* 2002) and the genera *Actinostrobus*, *Callitris* and *Widdringtonia* (Brodribb and Hill 1998) all produce wax but do not possess wax plugs. It has been assumed that waxy plugs reduce the cross sectional area and thus reduce gas diffusion through stomata (Brodribb and Hill 1997; Jimenez *et al.* 2000). However, Brodribb and Hill (1997) concluded that wax plugs are probably not primarily an adaptation to restrict water loss for the following reasons. First, some conifer species in arid areas of Australia produce wax but lack stomatal plugs. Second, amongst species with waxy stomatal plugs, the size and form of the plugs are not related to the rate of maximum leaf conductance. Third, since the presence of plugged stomata pre-dates the spread of aridity in Australia during the Tertiary (Hill 1990), the authors suggested that wax plugs might not have evolved as an adaptation to life in arid habitats. In contrast, Jimenez *et al.* (2000) suggested wax plugs evolved as an adaptation to restrict water loss.

Unfortunately, very few studies have investigated the physiological impacts of stomatal plugs. Jeffree *et al.* (1971) demonstrated by theoretical calculations that occlusion of the stomatal antechamber by waxy plugs may reduce transpiration by two thirds and photosynthesis by one-third relative to leaves lacking plugs, and suggested that stomatal plugs act as an antitranspirant. In contrast, Feild *et al.* (1998), investigated the function of stomatal plugs in *Drimys winteri* and concluded that stomatal plugs do not act to decrease water loss but instead prevent formation of a water film on leaf surfaces. This is very important in the wet environments where *D. winteri* occurs because the chance of water film formation on leaves is high. However, since waxy plugs decrease the entrance area of stomata, it seems likely that they should increase stomatal resistance, and as a consequence affect gas exchange rates including transpiration and photosynthesis. On the other hand, stomatal plugs may prevent the penetration of water and pollutants into stomatal pores (Hasemann *et al.* 1990), as well as preventing fungal invasion.

Foliar fungal invasion is mostly a mechanical process. Following spore attachment on the leaf surface and the germination of spores, penetration by the germ tube takes place through natural openings such as stomata and cracks on the leaf surface (Gallardo and Merino 1993; Mendgen *et al.* 1996; Canhoto and Graca 1999). During later steps of germination, cutinase activity might facilitate fungal penetration through the cuticle as well (Boulton 1991; Mendgen and Deising 1993; Dean 1997; Canhoto and Graca 1999). However, stomata, as natural discontinuities in the leaf surface, have been suggested as a major route for fungal penetration into leaf tissues (Lucas 1998; Canhoto and Graca 1999).

However, studies have shown that fungal infection and also the extent of hyphal penetration vary for different species of both plants and fungi. For example, Wu and Hanlin (1992) found that the hyphae of *Leptosphaerulina crassiasca* directly penetrated the cuticle and epidermal cell walls of *Arachis hypogaea* (peanut). They suggested that the lack of any physical break in the cuticle at the infection site indicated possible enzymatic activity by hyphae. Das *et al.* (1999) showed that the hyphae of *Drechslera sorokiniana* penetrated the leaves of wheat through stomata. The waxy cuticle has also been considered as a key physical factor delaying fungal penetration into leaf tissue of *Eucalyptus globulus* (Jenkins and Suberkropp 1995; Canhoto and Graca 1999). The latter authors found that, at least in the early stages, stomata are the main access pathway for fungal penetration. However, two weeks after inoculation, digestion activity by the fungi facilitated penetration through cuticle and cell walls as well. In contrast, Mims and Vaillancourt (2002) infected leaves of maize with *Colletotrichum graminicola* and found that the hyphae penetrated maize leaves directly through epidermal cells and observed that the host cells frequently formed papillae in response to the infection, but these were not usually successful in preventing fungal penetration. On the other hand, Akhtar Khan (1999) reported that the hyphae of *Ascochyta rabiei* penetrate through all surface structures of the chickpea leaf including epidermal cells, between the epidermal cells, between the guard cells and subsidiary cells, and through stomata. However, the seedlings in this study were just 15 days old, and if older seedlings with thicker cell walls had been used, the results might have been different. Also, there was no comparison of the proportion of hyphae that entered through the various pathways mentioned.

There has been much speculation concerning the function of stomatal plugs as a means of preventing fungal invasion, but no experimental studies have been performed to substantiate these speculations.

Stomatal crypts

Three major adaptive functions of stomata in any environment include optimizing the trade off between taking up CO₂, losing water and regulating temperature by evaporative cooling (Jones 1998). Although stomata must be open for the plant to obtain CO₂, all plants need to minimize water loss to reduce the risk of dehydration and/or catastrophic xylem cavitation (Tyree and Sperry 1988). This is more critical for plants living in dry environments where water is very restricted at times. Therefore, plants must achieve a balance between photosynthetic activity and water loss. A number of leaf modifications may assist plants in achieving this balance.

Trichomes (leaf hairs) that grow on the surfaces of some leaves may help to maintain a boundary layer next to the leaf and as a consequence may reduce transpiration rates (Wuenschel 1970; Seddon 1974; Ehleringer *et al.* 1976; Ehleringer and Mooney 1978; Nobel 1991; Fahn and Cutler 1992; Taiz and Zeiger 1998; Ripley *et al.* 1999). In addition to trichomes, modification of stomatal location and/or morphology might affect resistance in the gas diffusion pathway (Lee and Gates 1964; Brodribb and Hill 1997; Soliman and Khedr 1997; Hill 1998a; Hill 1998b; Roberts 2000). Stomata do not always occur in the same plane as epidermal cells (i.e. superficial stomata). In some leaves, guard cells are recessed beneath subsidiary cells due to a slight depression of the epidermis; these are called sunken stomata. Stomata may also occur in grooves in the epidermis. In

some plant species, guard cells are recessed into deep depressions of the epidermis termed stomatal crypts.

Stomatal crypts are the most extreme form of stomatal protection, especially when there are dense trichomes at the opening of the crypts (Hill 1998a). Such structures may have evolved to reduce water loss (Hadley 1972; Hill 1998a; Taiz and Zeiger 2002). These epidermal depressions, occur in most species of *Banksia* (Proteaceae) a genus endemic to Australia and Papua New Guinea (Hill 1998a; Mast and Givnish 2002). However, they are also found in other taxa e.g. *Nerium oleander* (Apocynaceae) (Gollan *et al.* 1985). Sunken stomata and stomatal crypts have been assumed to be adaptations to reduce transpiration (Seddon 1974; Smith and Nobel 1977; Fahn and Cutler 1992; Carpenter 1994). However, stomatal crypts occur in plants living in both dry and wet environments (Ragonese 1989), and this occurrence in both habitats creates some questions regarding the assumed function of stomatal crypts. Even in some plants living in muddy salt water or brackish waters e.g. *Aegialitis rotundifolia*, a mangrove species from the family Plumbaginaceae, stomata occur in crypts (Das *et al.* 1995); although, the latter habitat could be considered a dry environment because seawater has a very low water potential.

Any molecule diffusing into a leaf faces resistances at different points. For example, a CO₂ molecule entering a leaf is first carried by turbulent air to near the leaf surface, where it enters an immobile boundary layer of air next to the leaf surface. Then it diffuses to a stoma and then into the substomatal cavity. From there it diffuses through the intercellular air spaces of the mesophyll. The CO₂ molecule then dissolves in water next to the cell surface, and then diffuses through

the cell to the stroma of chloroplasts. Finally, it is fixed in the Calvin cycle to produce sugars (Machler *et al.* 1990; Parkhurst 1994; Lal *et al.* 1996).

According to Fick's law of diffusion, the diffusion rate through a tube is proportional to the area of its cross-section but inversely proportional to its length (Campbell 1986). Therefore, it is possible that crypts would increase the pathway of gas diffusion and as a consequence increase the resistance to gas exchange. Also, the crypt should increase the thickness of the immobile boundary layer of air next to the stomata and as a consequence might increase the resistance to gas exchange. Hence, it is reasonable to assume that stomatal crypts decrease gas diffusion from leaves. Surprisingly, despite numerous assumptions about the impact of stomatal crypts on diffusion resistance, the role of stomatal crypts in ensuring the survival of plants in drought conditions has not been studied experimentally.

Research Objectives

The objectives of the present study were:

- 1- To evaluate the impact of leaf surface wax coverage on photoinhibition, cuticular water loss, photosynthesis and transpiration in *Leucadendron lanigerum* (Proteaceae) (Chapter 2).
- 2- To investigate the effect of stomatal plugs on net CO₂ assimilation rates, transpiration rates, stomatal conductance and water use efficiency in a rain forest tree *Agathis robusta* (Araucariaceae) (Chapter 3).
- 3- To examine the initiation process and annual modification of waxy plugs, and also the effect of stomatal plugs on fungal invasion in *Agathis robusta* (Chapter 4).

4- To quantify the impact of stomatal crypts on photosynthesis, transpiration and stomatal conductance in 15 species of *Banksia* (Proteaceae) (Chapter 5).

2. The impact of epicuticular wax on water loss, gas-exchange and photoinhibition in *Leucadendron lanigerum* (Proteaceae)

Introduction

Leaf surface wax has been suggested to be an adaptation to a range of environmental factors including drought and high irradiance (Weiglin and Winter 1991; Jordaan and Kruger 1992; Raveh *et al.* 1998). For example, Shantz (1927) and Jordan *et al.* (1983) reported that the amount of wax per unit area increased on leaves of sorghum under drought conditions. Similar results were obtained with rice (O'Toole *et al.* 1979). In addition, Sanchez *et al.* (2001) found that in most of the 20 cultivars of pea they studied, the epicuticular wax load increased significantly when plants were under drought stress. This kind of acclimatory response has also been reported for peanut and *Theobroma cacao*, such that leaf epicuticular wax increased with decreases in soil moisture (Samdur *et al.* 2003). The latter authors concluded that plants show an acclimatory response to water deficit by increasing epicuticular wax load on leaves to reduce cuticular water loss and thus improve leaf water use efficiency (total dry matter production relative to water used). However, it is not always possible to attribute a precise function to a particular characteristic (Johnson *et al.* 1983).

Epicuticular wax can be exuded from the surface of guard cells, subsidiary cells and epidermal cells in a liquid form. The liquid wax is exuded outwards through micropores in the cuticle, and crystallized into different shapes (Reicosky and Hanover 1978). It has been found that temperature affects the deposition rate of

wax on leaf surfaces. For example, additional wax deposition occurred on the needles of *Picea pungens* in summer relative to other seasons, and from late autumn epicuticular wax started to degrade (Reicosky and Hanover 1978). The higher rates of wax deposition in summer relative to winter suggest some function for epicuticular wax related to temperature, water loss, or possibly photoprotection.

However, seasonal variation in wax deposition can vary with species. For instance, Hauke and Schreiber (1998) found that wax deposition and the chain-length of wax on shade leaves were the same as for sun leaves in *Hedera helix*. Wax thickness also changes significantly with increasing leaf age. For example, in *Hedera helix* the wax deposition of leaf surfaces rapidly increased during the first 30 days, at the period of maximum leaf growth, and during the remaining period of the year the extrusion of wax gradually decreased (Hauke and Schreiber 1998). These authors found that in mature leaves the amount of cutin-bound wax substances was higher than the amount of solvent-extractable wax, and suggested that this could be due to the higher degradation of solvent-extractable wax during the leaf lifetime compared with cutin-bound wax.

It has been suggested that substances like waxes that are exuded onto the cuticle have a considerable influence on light reflectance of leaf surfaces (Jeffree *et al.* 1971; Ehleringer *et al.* 1976; Ehleringer 1981; Robinson *et al.* 1993; Grant *et al.* 1995). Mauseth (1988) reported that epicuticular wax reflected 25% of the incident light in *Echeveria bracteosa*. Reicosky and Hanover (1978) reported that epicuticular waxes of glaucous leaves in *Picea pungens* reflected radiation in the 350 to 800 nm region with the highest reflectance being in the 750 to 800 nm

region. They concluded that this should reduce long wavelengths absorbed by the leaf and result in a reduction in leaf temperature.

A reduction in leaf temperature could be an advantage in dry environments to decrease water loss, but under conditions of low temperatures (winter) a lower leaf temperature may be a disadvantage due to a reduction of net photosynthetic rate of the leaf and possible low temperature damage. Reicosky and Hanover (1978) suggested that under conditions of high temperatures (summer) a lower leaf temperature of glaucous leaves could be an advantage because respiration rates would be lower and temperature should be closer to optimal for photosynthesis. Furthermore, Pierce *et al.* (2001) found that reflectance of infrared wavelengths (IR), was significantly higher (45%) than reflectance of visible light in *Catopsis micrantha*, a rainforest epiphyte. Sanchez (2001) discovered that among 20 cultivars of pea, higher grain yield under conditions of high solar radiation and water deficit was associated with higher deposition of wax on leaf surfaces. They found that cultivars with greater wax loads had significantly lower canopy temperatures than cultivars with less wax.

In high radiation habitats such as semi-arid and arid environments, leaves may develop greater surface reflectance, due to a waxy cuticle, pubescence, or the accumulation of salt on the epidermis, as photoprotective devices when light is excessive (Ehleringer *et al.* 1976; Mooney *et al.* 1977; Demmig and Adams 1992; Robinson *et al.* 1993). Robinson and Osmond (1994) suggested epidermal wax on *Cotyledon orbiculata* is an external mechanism for protection against photo-damage, reflecting up to 60% of the incident light.

By increasing reflectance, epicuticular wax might reduce the risk of over-excitation of photosystem II reaction centres thus preventing photo-oxidative

damage caused by absorption of excess light (Robinson *et al.* 1993). Moreover, the waxy cuticle can also reflect ultra violet (UV) radiation and protect plants from UV damage (Mauseth 1988; Cen and Bornman 1993; Gordon *et al.* 1998). Cen and Bornman (1993) reported that densely arranged epicuticular wax on the adaxial leaf surfaces of UV-treated *Brassica napus* decreased penetration of UV-B radiation by reflectance.

It has also been suggested that the waxy cuticle is relatively impermeable to water and gases and can prevent the formation of water films on leaf surfaces (Mauseth 1988; Grant *et al.* 1995; Gordon *et al.* 1998). The epicuticular waxy layer can make leaves water repellent, so that water droplets roll off rather than remaining on the leaf (Neinhuis and Barthlott 1997). Water proofing can reduce infection by fungal and bacterial pathogens by removing water from the leaf surface and preventing spore germination (Martin and Juniper 1970; Eigenbrode and Espelie 1995).

The deposition of particles on leaf surfaces can considerably affect the physiological activity of leaves including photosynthesis, transpiration, stomatal conductance and leaf temperature. Because particles may enter and block stomatal pores, they can decrease CO₂ assimilation rates and also prevent stomatal closure when it is necessary to decrease transpiration rate. Moreover, particles can absorb long wavelength radiation and increase leaf temperature and also restrict the access of light to leaves for photosynthesis (Hirano *et al.* 1995; Chen 2001). The water proofing ability of wax can facilitate the removal of particles from leaf surfaces, and as a consequence help to maintain the physiological activity of the leaves.

On the other hand, as CO₂ diffuses 10,000 times more slowly through water vapor than air (Weast 1979; Smith and McClean 1989), water repellency might serve to allow a sufficient supply of CO₂ into stomata for photosynthesis by preventing water films that may block stomata (Smith and McClean 1989; Neinhuis and Barthlott 1997). Additionally, this layer might increase the thickness of the boundary layer and further decrease water loss (McKerie and Leshem 1994; Hill 1998a), and confine gas exchange to the stomatal apertures (McKerie and Leshem 1994).

The current project investigated seasonal modification of wax deposition on leaf surfaces, and the impact of epicuticular wax on light reflectance and water loss of leaves of *Leucadendron lanigerum*. The effect of wax on leaf gas exchange and the ability of wax to protect leaves from photodamage were also investigated.

Materials and methods

Plant materials

An investigation of leaf surface wax was made across 120 species from the family Proteaceae growing in the Adelaide, Wittunga and Mount Lofty Botanic Gardens, Australia (Appendix 1). Among these species *Leucadendron lanigerum* had the most epicuticular wax and the most consistent wax coverage relative to other species. All experiments were conducted on 2-year-old seedlings of *L. lanigerum* that were ~ 40 cm in height. The plants were grown in 2 L pots containing premium potting mix (Premium Potting Mix, Australian standard, AS3743) and assigned randomly in a glasshouse at the University of Adelaide, Australia. During the study, daily average maximum photon flux density (PFD) was 1450 $\mu\text{mol quanta m}^{-2}\text{s}^{-1}$, average maximum night and day temperatures in the

glasshouse were 18 and 28°C respectively, and average minimum night and day temperatures were 9 and 12°C respectively. Average humidity during the day was 54% over the course of the study measured with a digital thermohygrometer (Model 37950-10, Cole-Palmer Instruments, Illinois, USA). Plants were watered with tap water automatically by overhead spray for 5 minutes every 3 days. Five grams non-phosphorous slow release fertilizer for Proteaceae (Protea world, Adelaide, Australia) was applied for each pot in spring and autumn.

Electron and Light Microscopy

Leaves were selected randomly, cut in $\sim 1 \text{ cm}^2$ sections, mounted with double-sided adhesive tape and attached to aluminium stubs. The stubs were sputter coated with a thin layer of Gold/Palladium (80%/20%) $\sim 4 \text{ nm}$ thick in a Cressington high-resolution sputter coater (Model 208HR, Cressington, UK). Coated specimens were examined at magnifications from 100 \times to 5000 \times using a Philips XL20 scanning electron microscope (SEM) with an accelerating voltage of 10 kV and a standard tilt of 15° (Philips Electron Optics, Eindhoven, Netherlands). To examine the cross-sectional view of the leaves, hand-sections were prepared using a razor blade, stained with Toluidine Blue for 30 seconds and examined with a light microscope at 400X magnification.

Seasonal modification of wax deposition

Wax deposition on the adaxial and abaxial leaf surfaces of *L. lanigerum* growing in the Wittunga Botanic Garden, Adelaide, Australia was observed throughout the year using SEM. Young fully expanded leaves were used for the survey. Leaves were collected in the middle of summer, autumn, winter and spring in 2004.

Wax removal

To examine the impact of epicuticular wax on water loss and leaf gas-exchange, waxes were removed from the adaxial (upper) and abaxial (lower) leaf surfaces of *L. lanigerum* using Blu-Tack (Bostik, UK). This non-toxic putty was pressed gently against leaf surfaces a number of times enabling the effective removal of wax from leaf surfaces without damage as assessed by SEM.

Cuticular water loss

The effect of the epicuticular waxy coverage on cuticular water loss was assessed with 10 fully expanded, mature darkened leaves of *L. lanigerum*. Leaves were detached and the petiole at the detached end covered with petroleum jelly. Wax was removed from half of the leaves, and water loss was measured gravimetrically as changing mass over 55 hours. Leaves were kept in a photographic dark room throughout the experiment. The temperature and humidity of dark room were 29°C and 41%, respectively.

Light reflectance

Light reflectance of leaves with and without wax coverage was measured in the photosynthetically active radiation range (400-700 nm) using an integrating sphere (Taylor 1920). A projector was used as a light source, and a quantum sensor (LiCor, Li- 190SZ, Lincoln, USA) was used to measure PFD.

Leaf gas exchange

Transpiration, CO₂ assimilation and stomatal conductance of leaves with and without wax coverage were measured in lab using a CIRAS-2 portable infrared gas analyzer (PP Systems, Herts, UK) fitted with an automatic Parkinson leaf cuvette. Light response curves were recorded at various PFDs from 0 to 2000

$\mu\text{mol quanta m}^{-2}\text{s}^{-1}$. CO_2 concentration was $355 \mu\text{mol mol}^{-1}$, VPD was 1.3 kPa and leaf temperature was 25°C . Plants were initially exposed to $300 \mu\text{mol quanta m}^{-2}\text{s}^{-1}$ for 30 minutes. Following this the PFD response was recorded, starting at a PFD of 0 and rising in steps, with 5 minutes interval, to $2000 \mu\text{mol quanta m}^{-2}\text{s}^{-1}$.

Chlorophyll fluorescence

Chlorophyll fluorescence yield (photosystem II efficiency (Genty *et al.* 1989)) of the adaxial surfaces of leaves with and without waxy coverage was examined in lab using a MINI-PAM chlorophyll fluorometer (Walz, D-91090 Effetrich Germany). Fluorescence yield of dark-adapted seedlings was measured half an hour before exposing plants to sunlight. Plants were exposed to sunlight ($1115 \mu\text{mol quanta m}^{-2}\text{s}^{-1}$) for 2 hours, and then transferred to low light ($<10 \mu\text{mol quanta m}^{-2}\text{s}^{-1}$). Recovery of PSII efficiency was measured over 3 hours at half hour intervals, and then again 12 hours later.

Gas exchange and fluorescence yield measurements were made 2 days after removal of wax to decrease the possibility of leaves being disrupted due to wax removal. All measurements were made on attached leaves.

Data analysis

Data were analyzed by repeated measures ANOVA using the statistical package JMPIN, Version 4.03, 2000, SAS institute. Cuticular water loss and quantum yield of the leaves with and without epicuticular wax was analyzed by Analysis of Covariance (ANCOVA), using the statistical program JMPIN. The assumptions of normality and homogeneity of variances were confirmed beforehand, using the Shapiro-Wilk and Levene's tests, respectively, in JMPIN.

Results

Investigations of leaf sections using SEM and light microscopy showed that *L. lanigerum* is amphistomatic, i.e. stomata occur on both the abaxial and adaxial leaf surfaces. There were on average 119 ± 7 stomata mm^{-2} on the adaxial and 116 ± 8 stomata mm^{-2} on the abaxial leaf surfaces. Although waxy leaves look green and not whitish, SEM micrographs of the leaf surfaces showed the presence of wax on both the adaxial and abaxial leaf surfaces of *L. lanigerum* (Fig 2.1a). According to the classification of epicuticular wax by Barthlott *et al.* (1998), the waxy coverage on leaf surfaces of *L. lanigerum* was plate form with distinct edges. SEM analysis of leaf surfaces confirmed the effectiveness of Blu-Tack in removing epicuticular wax (Fig 2.1b). Furthermore, the leaf surface micrographs and the cross sectional view of the leaves indicated that leaves suffered no observable damage from the Blu-Tack treatment, compared with intact leaves. Light microscopy of hand sections showed the presence of Florin rings (raised cuticle) on stomata (Fig 2.1c).

Seasonal modification of epicuticular wax

The deposition of waxes in plate form on juvenile leaves of *L. lanigerum* commenced in spring (Fig 2.2a), and continued during summer (Fig 2.2b). Except for partial transformation of wax from plate form into a flattened form, no visual differences were found between summer and autumn (Fig 2.2c). There was loss of wax, along with partial transformation of wax from plate form into a flattened form, on both the abaxial and adaxial leaf surfaces in winter (Fig 2.2d).

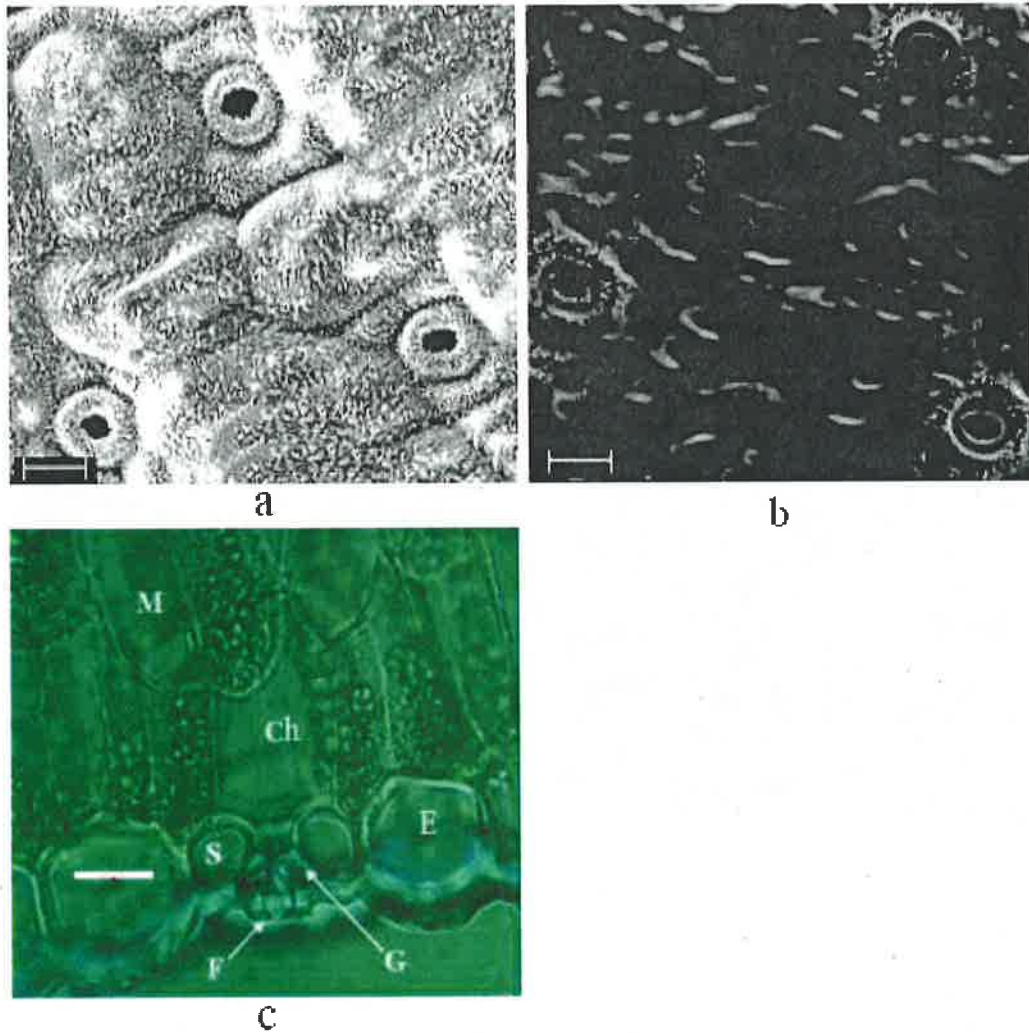


Figure 2.1. SEM of the abaxial leaf surface of *Leucadendron lanigerum* with wax in place (a) and after removal of wax by Blu-Tack (b). Light micrograph of a cross sectional view of a stoma of *L. lanigerum* (c). Mesophyll (M), substomatal chamber (Ch), guard cell (G), subsidiary cell (S), Florin ring (F) epidermal cell (E). Scale bar is the same for all micrographs (20 μm).

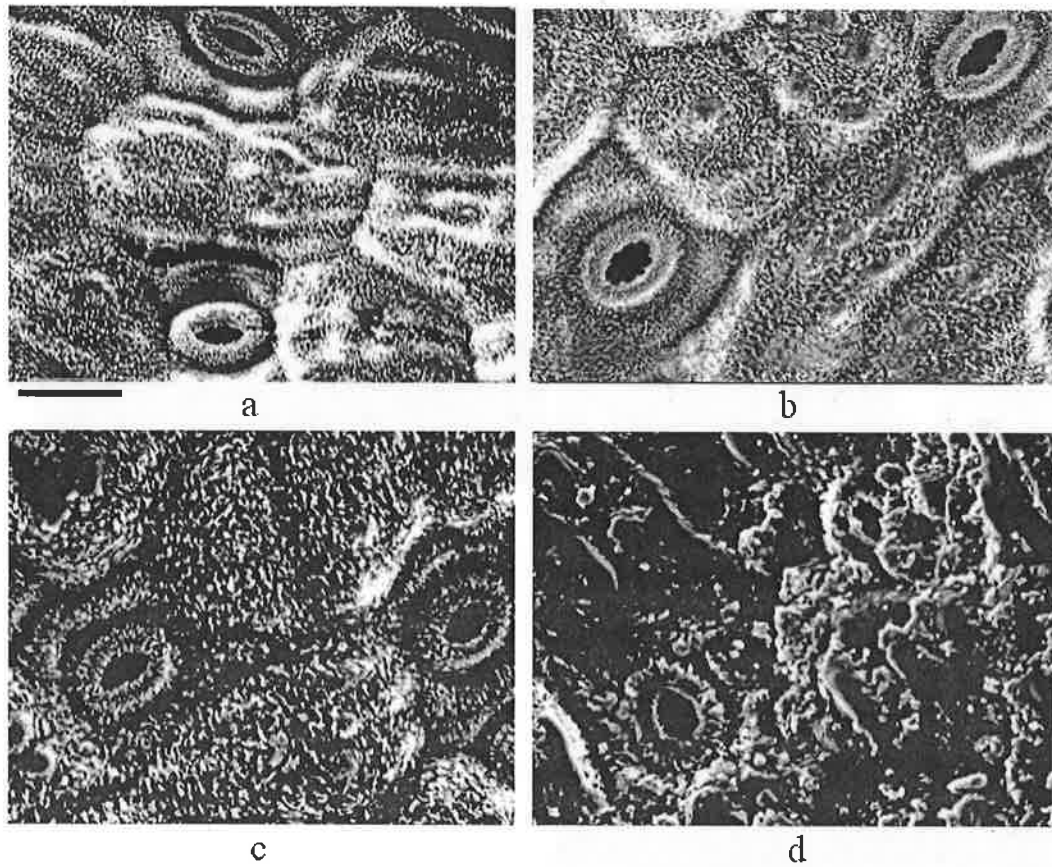


Figure 2.2. SEM of the abaxial leaf surface of *Leucadendron lanigerum* in spring (a), summer (b), autumn (c) and winter (d) showing seasonal modification of leaf surface wax coverage. Scale bar is the same for all micrographs (20 μm).

For those few leaves that were initiated in autumn, the developmental sequence of wax modification was different. These leaves possessed a dense plate form of wax in autumn and little wax erosion was observed in winter relative to the leaves that were initiated in spring. Leaves that were more than 2 years old lacked the intact plate form wax even in summer and autumn.

Cuticular water loss

The rates of water loss from leaves of *L. lanigerum* with and without epicuticular wax were $4.45 \text{ g gfw}^{-1} \text{ h}^{-1}$ and $8.86 \text{ g gfw}^{-1} \text{ h}^{-1}$, respectively over the 55 h during which measurements were made (Fig 2-3; $P= 0.03$, ANCOVA). Most of this loss would have occurred across the cuticle, as stomata should have been closed in the darkened conditions in which the experiment was conducted.

Light reflectance

The reflectance of light from control leaves of *L. lanigerum* was 4% greater than leaves without wax and this was almost significant ($P= 0.07$) (Fig. 2.4).

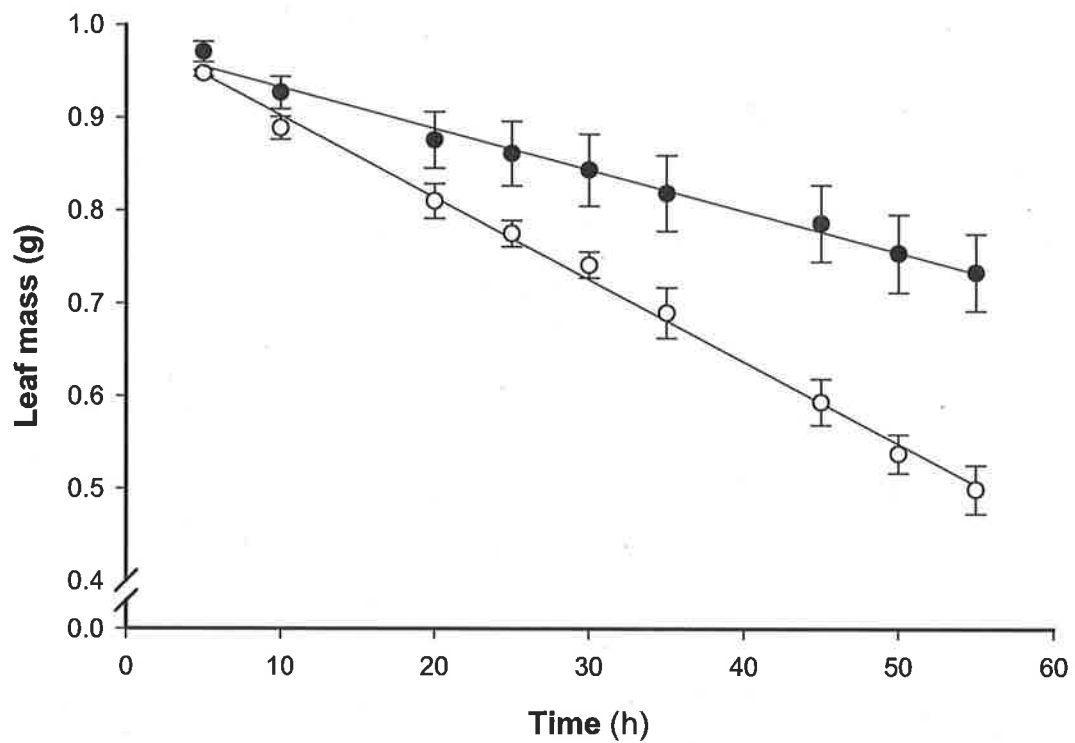


Figure 2.3. Changes in leaf mass with time in detached leaves of *L. lanigerum* with (●) and without (○) wax, measured over 55 h in a dark room. Data points are means \pm s.e., $n=5$. Linear regressions were calculated using Sigma Plot.

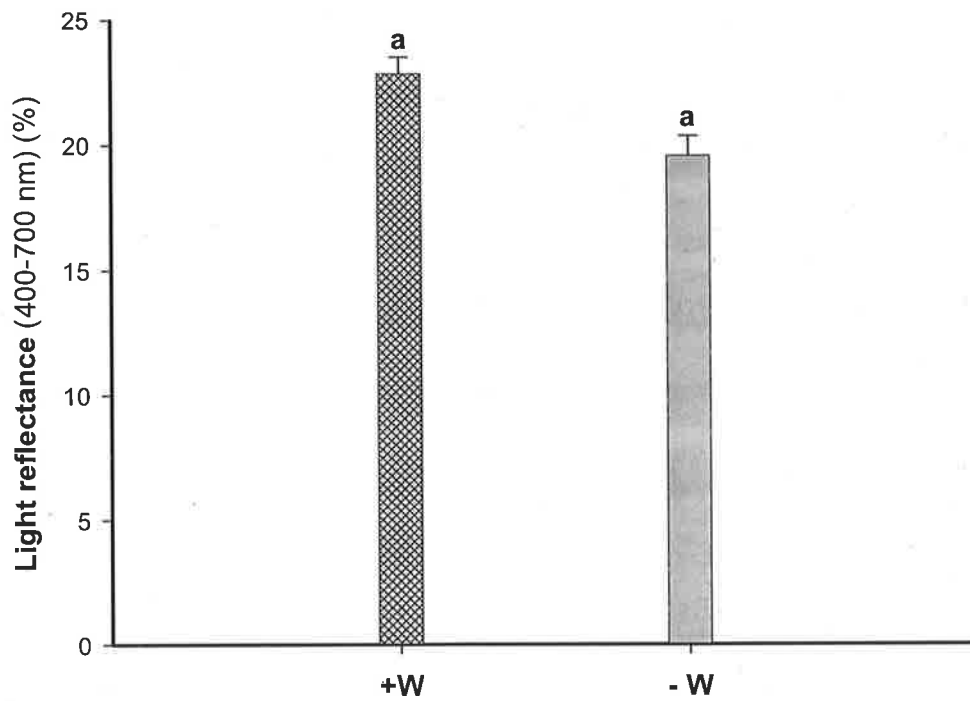


Figure 2.4- Reflectance of the adaxial leaf surfaces of *L. lanigerum* with (+W) and without (-W) waxy coverage. Letters above bars indicate that there was no significant difference between treatments. Data are means \pm s.e., $n=5$.

Gas exchange

Transpiration rates were significantly higher in leaves without wax than in leaves with epicuticular wax across all PFDs (Fig 2.5a; $P < 0.0001$). The photosynthetic rate was also significantly higher in leaves from which wax had been removed compared with control leaves across the PFD range of 0 to 2000 $\mu\text{mol quanta m}^{-2}\text{s}^{-1}$ (Fig 2.5b; $P < 0.0001$). The higher rates of transpiration and photosynthesis observed in leaves without waxes were most likely the result of reduced resistance to gas exchange through the stomata, as indicated by the higher stomatal conductance of the leaves without epicuticular waxes relative to controls (Fig 2.5c; $P < 0.0001$). Additionally, there was a significant difference in temperature of leaves with and without wax. Leaves without epicuticular wax were cooler than wax-covered leaves. The difference was greater when leaves were exposed to PFD higher than 500 $\mu\text{mol quanta m}^{-2}\text{s}^{-1}$ ($P = 0.02$) (Fig 2.5d).

Quantum yield of leaves with wax and without wax, as determined from the initial linear regions of the PFD response curves, were 0.041 and 0.045 $\text{mol CO}_2 \text{ mol photon s}^{-1}$, respectively ($P = 0.24$, ANCOVA).

Chlorophyll fluorescence

Fv/Fm half an hour before exposure to sunlight for leaves with wax was 0.792 ± 0.082 and without wax was 0.787 ± 0.073 , confirming no disruption due to the Blu-Tack treatment. After exposing the leaves to sunlight for 2 hours, there was a significant reduction in yield for leaves both with and without wax relative to pre-exposure Fv/Fm ($P = 0.001$). Yield of the leaves with and without wax recovered to 99% and 93% of pre-exposure values, respectively, after 3 hours in low light. After 12 hours in low light, Fv/Fm of leaves with and without epicuticular wax had recovered to 100% and 96% of pre-exposure Fv/Fm, respectively (Fig 2.6).

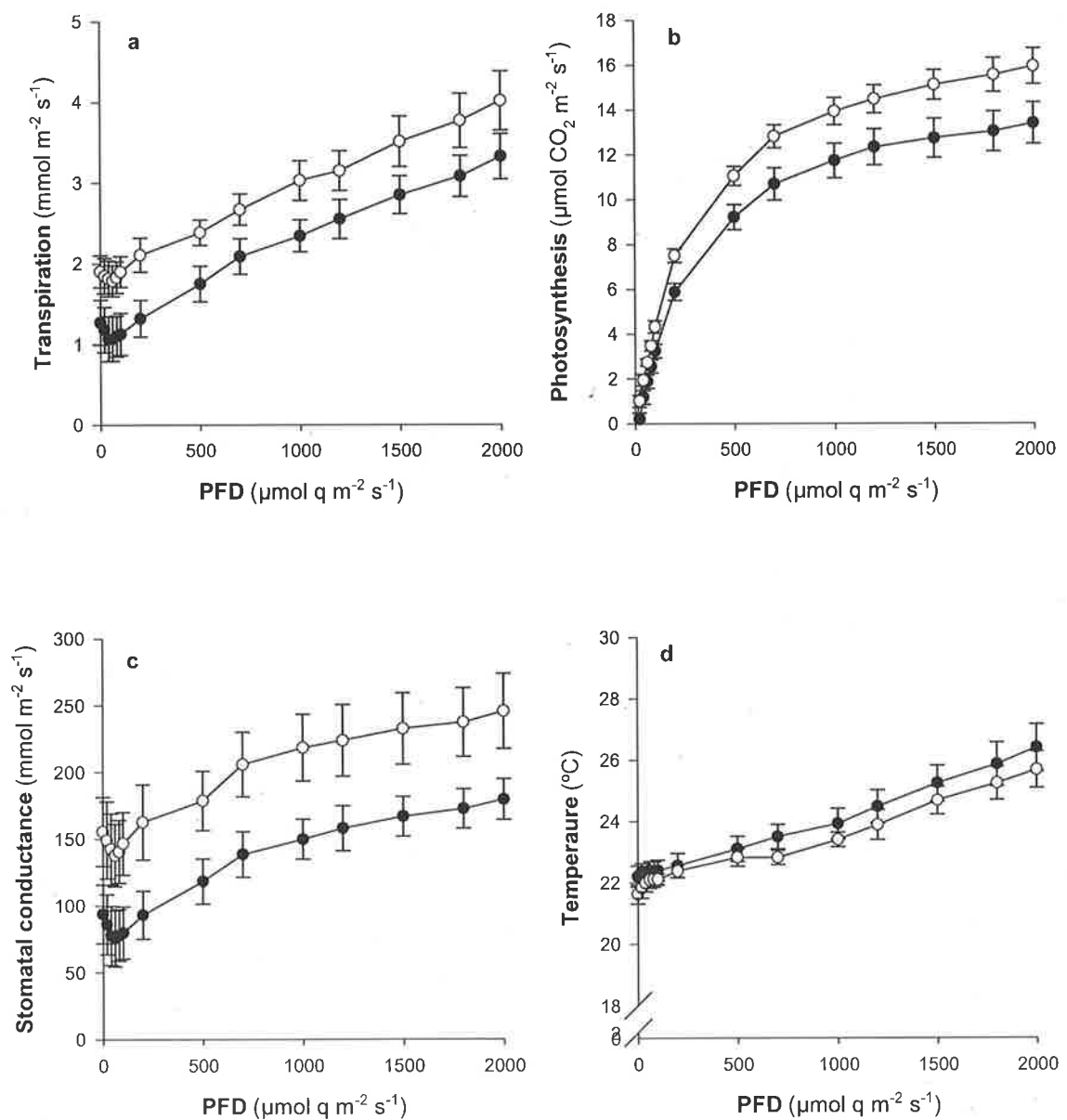


Figure 2.5- Relationship between leaf gas-exchange and PFD for *L. lanigerum* with (●) and without (○) epicuticular wax. Transpiration (a), photosynthesis (b), stomatal conductances (c) and leaf temperature (d). Data points are mean \pm s.e., n= 5.

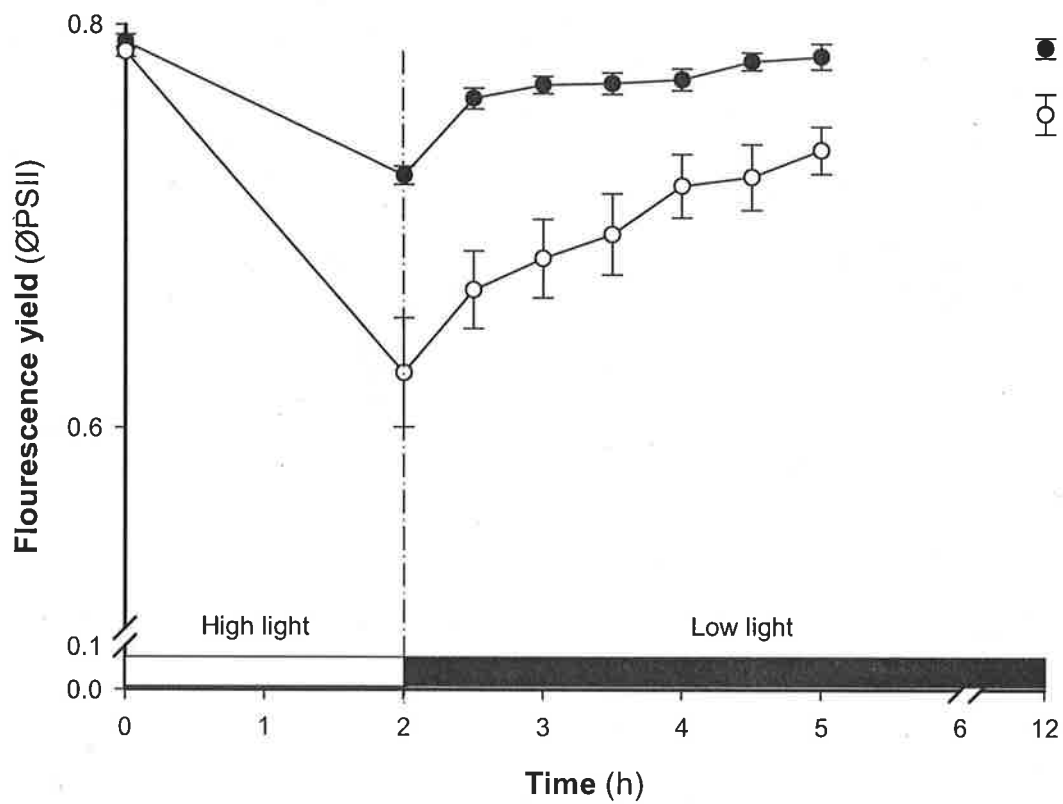


Figure 2.6- Fluorescence yield of the adaxial surface of leaves of *L. lanigerum* with (●) and without (○) waxy coverage prior to and for 12 h after exposure to full sunlight for 2 h. Data points are means \pm s.e., $n=5$.

Discussion

Seasonal modification of epicuticular wax

During the seasonal investigation of leaf surface wax coverage it was found that the form and amount of wax crystals was dependent on both the age of the leaves and the season. Epicuticular wax was produced in spring and crystallized in plate form (Fig 2.2a). This process continued during summer. No visual difference was found between the form of wax crystals in spring and summer (Fig 2.2b). In autumn, the transformation of wax from plate form into a flattened form commenced (Fig 2.2c) and this continued and was accompanied by partial erosion of wax in winter (Fig 2.2d). The degradation of wax from late autumn and into winter has been reported previously in *Picea pungens* (Reicosky and Hanover 1976) and in *Picea abies* and *Pinus cembra* (Anfodillo *et al.* 2002). The latter authors also reported a seasonal transformation of epicuticular wax from tubular form into planar form from spring to summer.

Leaves that were more than two years old, despite producing wax on both leaf surfaces in spring, did not produce the plate form of wax as younger leaves did. This is similar to the results of Hauke and Schreiber (1998) in *Hedera helix* and Prugel *et al.* (1994) and Anfodillo *et al.* (2002) in *Picea abies* and *Pinus cembra* who found wax production decreased with leaf age. The results of the present study indicate that contrary to the results found for leaf trichomes in other species, which were not replaced after shedding (Appendix 2), epicuticular wax is replaced at least for the first two years of the lifetime of leaves in *L. lanigerum*. Regeneration of wax has also been reported previously in 24 plant species (Neinhuis *et al.* 2001). However, the seasonal regeneration of epicuticular wax by *L. lanigerum*, at least for the first two years, is contrary to the findings of Jetter

and Schaffer (2001) who reported that epicuticular waxes of *Prunus laurocerasus* were regenerated in the early stages of leaf development only. This difference might be related to the fact that *P. laurocerasus* is a deciduous plant, while *L. lanigerum* is evergreen and has long-lived leaves. The results of this study are also inconsistent with the results of Neinhuis and Barthlott (1997) who reported that in 200 water-repellent plant species, wax erosion occurred within 4-6 weeks of leaf expansion. Consequently, they concluded that many plants are only water-repellent during leaf expansion.

Cuticular water loss

It has been reported that although cuticular transpiration accounts for only 5 to 10% of total leaf transpiration (Kerstiens 1997; Taiz and Zeiger 1998; Taiz and Zeiger 2002), it can be significant when drought stress is severe (Sanchez *et al.* 2001). Epicuticular wax has been shown to help leaves preserve water by decreasing cuticular transpiration (Jordan *et al.* 1983; Jefferson *et al.* 1989; Premachandra *et al.* 1992). The results for cuticular water loss of *L. lanigerum* with and without waxy coverage support earlier finding on the effect of waxy coverage on cuticular water loss from leaves (Fig. 2.3). Higher rates of cuticular water loss in leaves without wax compared with control leaves is consistent with other studies where epicuticular wax was the main barrier to water loss across the cuticle (e. g. Schönherr 1976; Schönherr and Riederer 1988; Schreiber and Riederer 1996a; Takamatsu *et al.* 2001). Also, my findings are consistent with the conclusion of Clarke and Richards (1988) in wheat and Premachandra *et al.* (1992) and Premachandra *et al.* (1994a) in sorghum. A reduction in transpiration rate because of epicuticular wax has also been reported in *Brassica oleracea* (Denna 1970) and in *Oryza sativa* (O'Toole *et al.* 1979). Furthermore, Samdur *et*

al. (2003) found that the epicuticular wax on leaves of peanut reduced cuticular transpiration and improved leaf water use efficiency.

Light reflectance

The results for light reflectance of *L. lanigerum* are not in agreement with Johnson *et al.* (1983) who found that light reflectance in the PAR range of the abaxial waxy covered leaf surfaces of wheat lines was significantly greater than non-waxy covered leaves. Also, in contrast to their findings, my results indicated that the temperature of leaves without wax was lower than leaves with waxy coverage. This was most likely due to the increased transpiration rates observed for leaves without wax compared with control leaves. Although IR reflectance was not measured, the lower temperatures for leaves from which wax had been removed suggest that evaporative cooling was more effective than IR reflectance for keeping leaves cool.

Gas exchange

According to the results of the current study, wax coverage on leaves of *L. lanigerum* increased the resistance to gas diffusion. The observed increase in photosynthesis and transpiration of leaves without wax was most likely due to an increase in stomatal conductance of leaves after removal of epicuticular wax (Fig. 2.5a-d). The partial occlusion of stomata by wax in some of the Proteaceae has been assumed to prevent the entry of water into the pore (Mickle 1993). However, since the occlusion of stomata by wax decreases the cross-sectional area for diffusion, wax should increase resistance to gas exchange.

Quantum yield of leaves from which wax had been removed was not significantly different from control leaves. This is in agreement with the findings that epicuticular wax had no impact on leaf reflectance in *L. lanigerum*.

Wax and photoinhibition

The results of fluorescence yield measurements in *L. lanigerum* indicated that plants were photoinhibited when they were exposed to sunlight ($1115 \mu\text{mol quanta m}^{-2} \text{ s}^{-1}$) for 2 hours, and that leaves without wax showed significantly greater photoinhibition than control leaves (Fig. 2.6). This result is in agreement with the results of Robinson *et al.* (1993), Robinson and Osmond (1994) and Barker *et al.* (1997) who found that wax is a mechanism for protection against photoinhibition in *Cotyledon orbiculata*. The reflectance of light from the leaf surfaces prevents inactivation and/or damage of the PSII reaction centre and increases the efficiency of photosynthesis (Barker *et al.* 1997; Rohacek and Bartak 1999; Waldhoff *et al.* 2002; Baker and Rosenqvist 2004; Bartak *et al.* 2004). Therefore, although no effect of epicuticular wax on leaf reflectance could be measured, wax did appear to benefit plants by reducing photoinhibition. However, the reduction in yield for leaves without wax was smaller than that reported for leaves of *C. orbiculata* from which wax had been removed (Robinson *et al.* 1993). Thus, the protection afforded by wax in *L. lanigerum* is relatively small compared with some other species.

Conclusions

The results of this study demonstrated that the deposition of epicuticular wax in *L. lanigerum* is dependent on the age of the leaf as well as the season, and generation and regeneration of wax occurs mostly in spring while transformation and also

degeneration of wax crystals occurs in winter. Epicuticular waxes decreased cuticular water loss but had little impact on leaf reflectance. The leaf temperature of leaves without wax was lower than wax-covered leaves, indicating that the rate of transpiration impacted more on leaf temperature than reflectance of light in the PAR or IR range in *L. lanigerum*.

The wax coverage at the entrance of stomata in *L. lanigerum* increased resistance to gas diffusion and as a consequence decreased stomatal conductance, transpiration and photosynthesis. Also, the results indicated that epicuticular waxes do help prevent photodamage in *L. lanigerum*, and so this property could benefit plants living in arid environments with high solar radiation.

3. The influence of waxy stomatal plugs on leaf gas-exchange in a rain forest gymnosperm, *Agathis robusta*

Introduction

Plants exhibit a wide variety of leaf surface modifications that have been suggested to have a range of functions. Examples include the presence of hairs and waxes that may increase leaf reflectance and prevent excessive absorption of radiation (Jeffree *et al.* 1971; Ehleringer 1981; Robinson *et al.* 1993), and stomatal modifications such as crypts, Florin rings and stomatal plugs that have been suggested to reduce water loss (Brodribb and Hill 1997; Hill 1998a; Roberts 2000). Many of these leaf features are likely to affect gas exchange through the stomata and the cuticle, leading to the commonly held view that they may have evolved to reduce water loss in dry environments (Taiz and Zeiger 2002).

Waxy plugs are known to occlude the entrance of stomata, particularly in conifers (Stockey and Ko 1986; Schmitt *et al.* 1987; Barnes *et al.* 1988; Hasemann *et al.* 1990; Mickle 1993; Stockey and Atkinson 1993; Brodribb and Hill 1997; Jimenez *et al.* 2000). Brodribb and Hill (1997) suggested that stomatal plugs affect leaf gas exchange by decreasing the cross-sectional area for diffusion and thus increasing resistance. Based on theoretical calculations of stomatal conductance, they concluded that maximum stomatal conductance in species without plugs should be about twice that of plugged species with similar stomatal densities. However, unlike Jimenez *et al.* (2000), Brodribb and Hill (1997) did not suggest wax plugs as an adaptation to restrict water loss, because the presence of plugged stomata

pre-dates the spread of aridity in Australia during the Tertiary (Hill 1990), and also some conifer species growing in arid areas of Australia lack stomatal plugs.

Two further points can be made about the role of stomatal plugs in reducing water loss. First, as stomata are the main diffusion pathway for both water and CO₂, it is inevitable that any feature that decreases the rate of water loss will also reduce CO₂ diffusion into leaves, potentially limiting photosynthesis. Second, reduced transpiration rates will also affect leaf temperature through their impact on evaporative cooling.

Very few studies have been conducted on the functions of stomatal modifications. However, Feild *et al.* (1998), investigated the function of stomatal plugs in *Drimys winteri*, a species from wet forests of Central and South America. They removed stomatal plugs from leaves and compared transpiration and photosynthetic activities of plugged and unplugged leaves. The authors concluded that stomatal plugs do not protect leaves from water loss but instead serve to prevent the formation of a water film on leaf surfaces in wet environments. They found that under a high evaporative demand, leaves without plugs decreased their conductance to water vapour by 70% while leaves with plugs showed only a 20% decline in conductance. They concluded that stomatal plugs noticeably decreased the capacity of *Drimys winteri* leaves to regulate water loss. The authors suggested that since CO₂ diffuses 10,000 times more slowly through water than air (Weast 1979), and considering the water repellent nature of wax plugs, stomatal plugs in wet environments are more important for promoting photosynthetic activity by preventing the formation of water films on leaf surfaces than protecting the leaf from excessive transpiration.

In light of Feild *et al.*'s (1998) paper, I investigated the impact of waxy stomatal plugs in another rainforest species, *Agathis robusta*. The same technique was used to remove plugs from leaves as was used by Feild *et al.* (1998) and the impact on leaf gas-exchange (both across the cuticle and through stomata), leaf temperature and the development of water films was investigated.

Materials and Methods

Plant material

All experiments were conducted with ~ 2 year old and 50 cm tall seedlings of *Agathis robusta* (Araucariaceae), that were obtained from a commercial nursery (Yuruga Nursery Pty Ltd, Atherton, Qld, Australia). *Agathis robusta* is a southern hemisphere conifer that occurs in tropical and warm temperate regions of lowland rainforest (McGee *et al.* 1999; Brophy *et al.* 2000). The experimental seedlings were grown in 2 L pots containing premium potting mix (Premium Potting Mix, Australian standard, AS3743) in glasshouses at the University of Adelaide, Australia. Glasshouse conditions were as described in Chapter 2. Plants were watered with tap water automatically by overhead spray for 5 minutes every 3 days.

Electron and light microscopy

Micromorphology of leaf surfaces was investigated using scanning electron microscopy (SEM). Leaves were cut in ~1 cm² sections from near the middle of leaves, mounted with double-sided adhesive tape and attached to aluminum stubs. The stubs were sputter coated with a thin layer of Gold/Palladium (80%/20%) to ~ 4 nm thick in a Cressington high-resolution sputter coater (Model 208HR, Cressington, UK). The coated specimens were examined at different

magnifications from 100X to 5000X using a Philips XL20 scanning electron microscope with an accelerator voltage of 10 kV and a standard tilt of 15° (Philips Electron Optics, Eindhoven, Netherlands).

To examine the cross-sectional view of stomatal plugs, hand-sections were prepared using a razor blade. These sections were stained with Toluidine Blue for 30 seconds and examined by light microscope at 400X magnification.

Removal of Stomatal plugs

The waxy plugs occluding stomata were removed using Blu-Tack (Bostik, UK). This non-toxic putty was pressed gently against leaf surfaces a number of times enabling the effective removal of >90% of stomatal plugs from stomatal antechambers, as assessed by SEM. SEM also indicated that leaves suffered no observable damage from the Blu-Tack treatment.

Cuticular water loss

Cuticular water loss was determined on 10 fully expanded, darkened mature detached leaves in which petiole ends had been coated with petroleum jelly. After removing the waxy layer along with waxy plugs from half of the leaves, water loss from leaves was measured gravimetrically (Schoenherr and Lenzian 1981; Prugel *et al.* 1994) as changing mass over a 55 hour period in a dark room. The temperature and humidity of dark room were 25°C and 46%, respectively.

Light reflectance

Light reflectance of leaves with and without stomatal plugs was measured in the photosynthetically active radiation range (PAR, 400 - 700 nm) using an integrating sphere (Taylor 1920). A projector was used as a light source, and a

quantum sensor (LiCor, Li- 190SZ, Lincoln, USA) was used to measure photon flux density (PFD).

Leaf gas exchange

Gas exchange measurements were made on leaves with and without waxy plugs, using a CIRAS-1 portable gas exchange system fitted with an automatic Parkinson Leaf Cuvette (PLC, PP Systems, Hitchin UK). The measurements were made at a PFD of $650 \mu\text{mol quanta m}^{-2}\text{s}^{-1}$, which had previously been determined to be saturating for the experimental plants. CO_2 concentration was 350 ppm and vapor pressure difference (VPD) was 19 mb which is similar to midday conditions in tropical rain forests (Grubb and Whitmore 1966). Temperature response curves were obtained by varying temperature in the leaf Cuvette using the Peltier system on the PLC.

Water film formation

To investigate the effect of waxy plugs on water film formation, leaves with and without waxy plugs were misted with a hand-held water spray, photographed and compared for formation of water films.

Chlorophyll fluorescence

Electron transport rate (ETR) of wet leaves was measured using a MINI-PAM chlorophyll fluorometer (Walz, D-91090 Effetrich Germany). ETR was calculated using the following equation: $\text{ETR} = \Phi\text{PSII} * \text{PFD} * 0.84 * 0.5$. Measurements of external PFD were taken by the microquantum sensor of the MINI-PAM leaf clip. PFD during the experiments was $650 \mu\text{mol quanta m}^{-2} \text{s}^{-1}$. ETR of leaves was first measured in the absence of a water film for 10 minutes. The abaxial surface of

leaves was then misted with water and ETR monitored for a further 10 minutes during which time leaves were frequently misted to maintain a wet surface.

Gas exchange and chlorophyll fluorescence measurements were made 2 days after removal of the plugs to decrease the possibility of the leaves being disrupted due to the Blu-Tack treatment. All measurements were made on attached leaves.

Data analysis

Data for leaves with and without epicuticular wax were compared by repeated measures ANOVA, using the statistical program JMPIN, Version 4.03, 2000, SAS institute. Cuticular water loss of the leaves with and without wax was analyzed by Analysis of Covariance (ANCOVA), using the statistical program JMPIN. The assumptions of normality and homogeneity of variances were confirmed beforehand, using the Shapiro-Wilk and Levene's tests, respectively, in JMPIN.

Results

Investigations of leaf sections using SEM and light microscopy showed that the stomata of *Agathis robusta* occur only on the abaxial surface of leaves. There were, on average, 89 ± 7 stomata mm^{-2} , and all stomata were occluded by waxy plugs (Fig 3-1a). Micrographs also show the raised position of stomata on *A. robusta* leaf surfaces. This feature probably contributed to the ease with which stomatal plugs could be removed by Blu-Tack. SEM analysis of leaf surfaces confirmed the effectiveness of this method, with more than 90% of the stomatal plugs being removed (Fig 3-1b). Furthermore, the micrographs also show that there was no indication of damage to the leaf structure after using Blu-Tack, compared with intact leaves. Light micrographs of the cross sectional view of the stomata of *A. robusta* also confirmed the occlusion of stomata by wax (Fig. 3-1c).

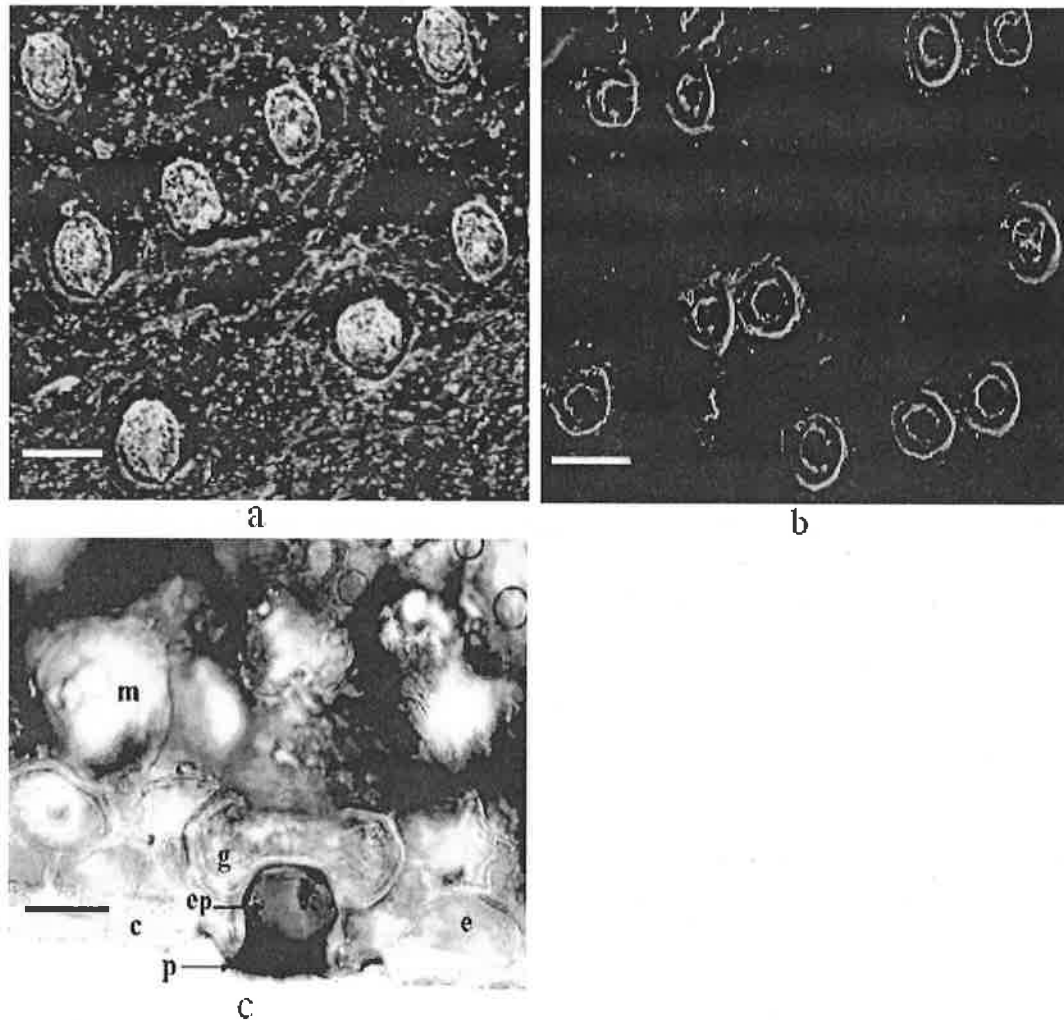


Figure 3.1. SEM of the abaxial leaf surface of *Agathis robusta* with stomatal plugs (a) and after removal of stomatal plugs (b). Light micrograph of the cross sectional view of a stoma of *Agathis robusta* (c); mesophyll (m), guard cell (g), epistomatal chamber (ep), stomatal plug (p), epidermal cell (e) and cuticle (c). The plug can clearly be seen in the epistomatal chamber above the guard cells. Scale bar is 50 μm for all figures.

Cuticular water loss

The rates of water loss from leaves of *A. robusta* with and without wax plugs were 1.37 g gfw⁻¹ h⁻¹ and 4.57 g gfw⁻¹ h⁻¹, respectively over the 55 h during which measurements were made (Fig 3-2; P= 0.0001, ANCOVA). Most of this loss would have occurred across the cuticle, as stomata should have been closed in the darkened conditions in which the experiment was conducted.

Light reflectance

The presence of a waxy coverage did not significantly affect reflectance of *A. robusta* leaf surfaces, across the PAR range, compared with leaves that lacked a waxy coverage (P=0.19). Thus, the waxy surface of *A. robusta* leaves is unlikely to have an impact on photosynthesis or leaf temperature through any affect on light absorption.

Gas exchange

Transpiration rate during 20 minutes at PFD of 650 $\mu\text{mol quanta m}^{-2} \text{s}^{-1}$ and leaf temperature of 26 °C was significantly higher in leaves without plugs than in control leaves (Fig 3-3a; P<0.001), confirming that stomatal plugs decrease the rate of water loss from *A. robusta* leaves. Photosynthetic rate was also significantly higher in leaves from which plugs had been removed compared with control leaves (Fig 3-3b; P<0.001). The higher rates of transpiration and photosynthesis observed in leaves without plugs were most likely the result of reduced resistance to gas exchange through the stomata, as illustrated by the

higher stomatal conductance of the unplugged leaves relative to controls (Fig 3-3c; $P < 0.001$).

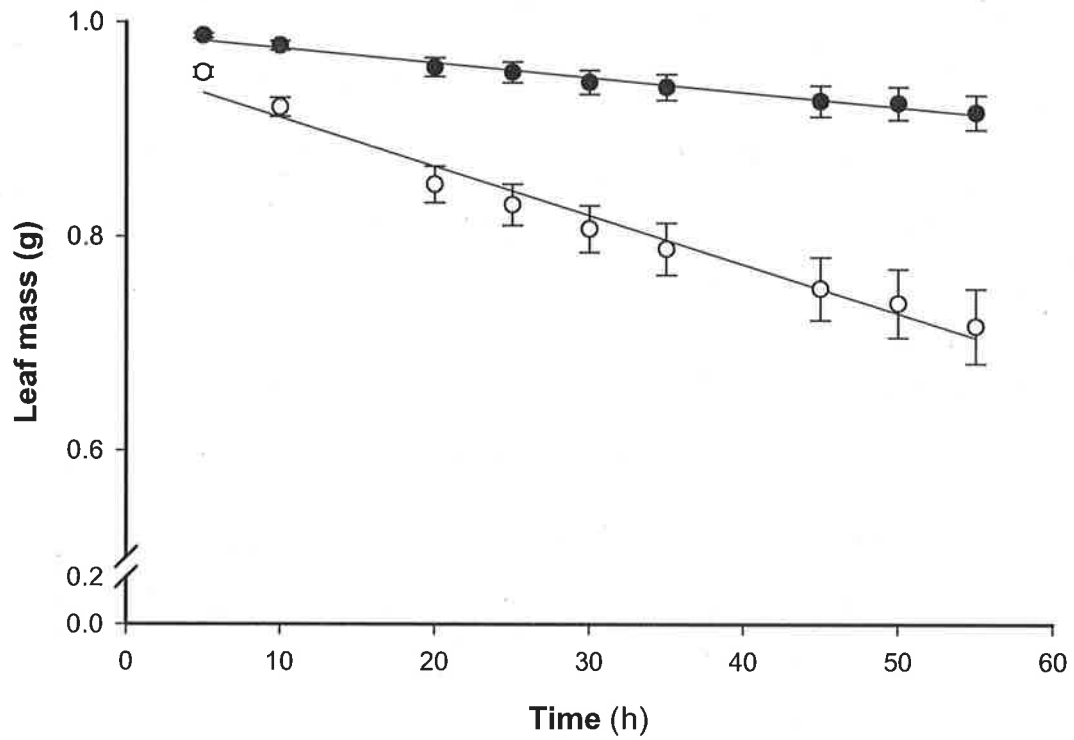


Figure 3.2. Changes in leaf mass of detached leaves of *A. robusta* with (●) and without (○) wax, measured over 55 h in a dark room. Data points are means \pm s.e., $n = 10$. Linear regressions were calculated using Sigma Plot.

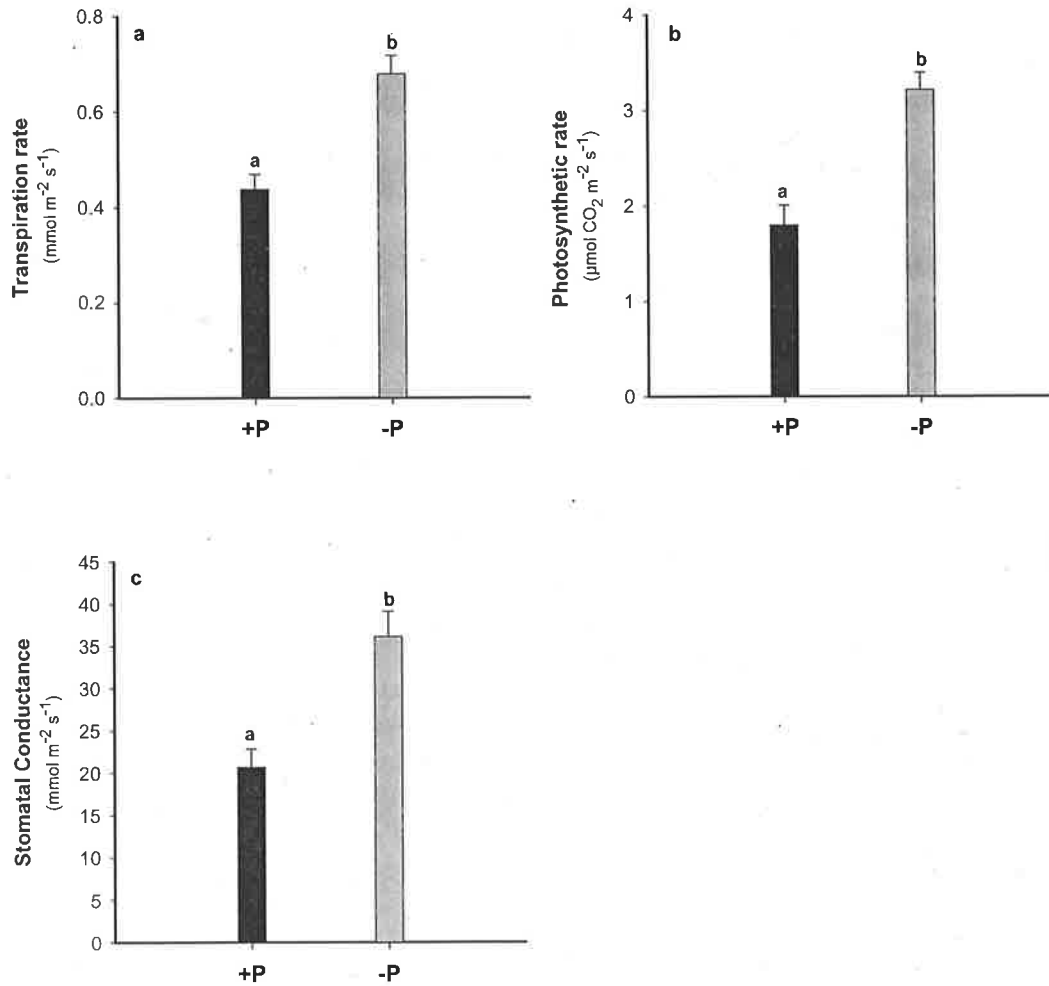


Figure 3.3 Transpiration rates (a), photosynthesis (b) and stomatal conductance (c) of the leaves of *A. robusta* at saturating PFD with (●) and without (○) stomatal plugs. Letters a and b above bars indicate that there was significant difference between control and treatments. Data points are mean \pm s.e., n= 10.

In contrast, there was no significant difference in ETR rates of the unplugged and control leaves when measured using chlorophyll fluorescence ($P=0.85$). However, this was probably the result of the higher leaf temperatures that leaves operated under when using the chlorophyll fluorometer. Since I was unable to control leaf temperature with the chlorophyll fluorometer, as I could with the gas exchange system, both control and treated leaves reached temperatures higher than 30°C (Fig 3-4a). Subsequent measurements indicated that unplugged and control leaves had similar photosynthetic rates at temperatures of 30°C and above (Fig 3-4b). The photosynthetic rate of unplugged leaves was significantly higher at temperatures between 15 and 25°C than that of the control leaves (Fig 3-4b; $P<0.001$). Maximum photosynthesis was reached at 25°C for unplugged leaves and at 30°C for control leaves, after that photosynthetic rate decreased in both. At temperatures above 30°C there was no significant difference in photosynthesis between unplugged and control leaves ($P=0.63$).

Transpiration rates of leaves without waxy plugs were significantly greater than for control leaves at all temperatures between 15 - 40°C (Fig 3-4c; $P<0.001$). However the pattern of response was similar for both with transpiration rate increasing between 20 - 35°C and then declining at temperatures above 35°C . Stomatal conductance was higher in the unplugged than the control leaves at all temperatures. However, conductance began to decline at a lower temperature (25 - 30°C) in unplugged leaves than in control leaves (30 - 35°) (Fig 3-4d; $P= 0.009$). Interestingly, the temperatures at which stomatal conductance began to decline

corresponded to the temperatures at which transpiration rates were similar in both sets of leaves ($0.6 \text{ mmol m}^{-2} \text{ s}^{-1}$). This suggests that the presence or absence of plugs did not affect the ability of leaves to sense when transpiration had reached a critical level.

The relative responses of photosynthesis and transpiration to temperature in unplugged and control leaves resulted in different instantaneous water use efficiencies (WUE) for both sets of leaves when exposed to different temperatures (Fig 3-4e). Control leaves had higher WUE than unplugged leaves at all temperatures except the lowest (15°C), and the difference at temperatures above 15°C was statistically significant ($P= 0.005$).

Water film formation

No water film was observed to form on either control leaves or leaves from which stomatal plugs had been removed (Fig 3-5). Thus, although Blu-Tack removed more than 90% of stomatal plugs, leaves were still sufficiently hydrophobic to prevent the formation of a water film.

Photosynthetic response to leaf surface wetness

Electron transport rates (ETR) of the leaves with and without waxy plugs showed no significant difference during 20 minutes of chlorophyll fluorescence measurements. Misting had no effect on ETR of either control or unplugged leaves ($P= 0.29$) (Fig. 3-6).

In all of the experiments, the results for young fully expanded leaves were the same as mature leaves. However, the differences for young leaves were greater than those observed in mature leaves (results are not shown).

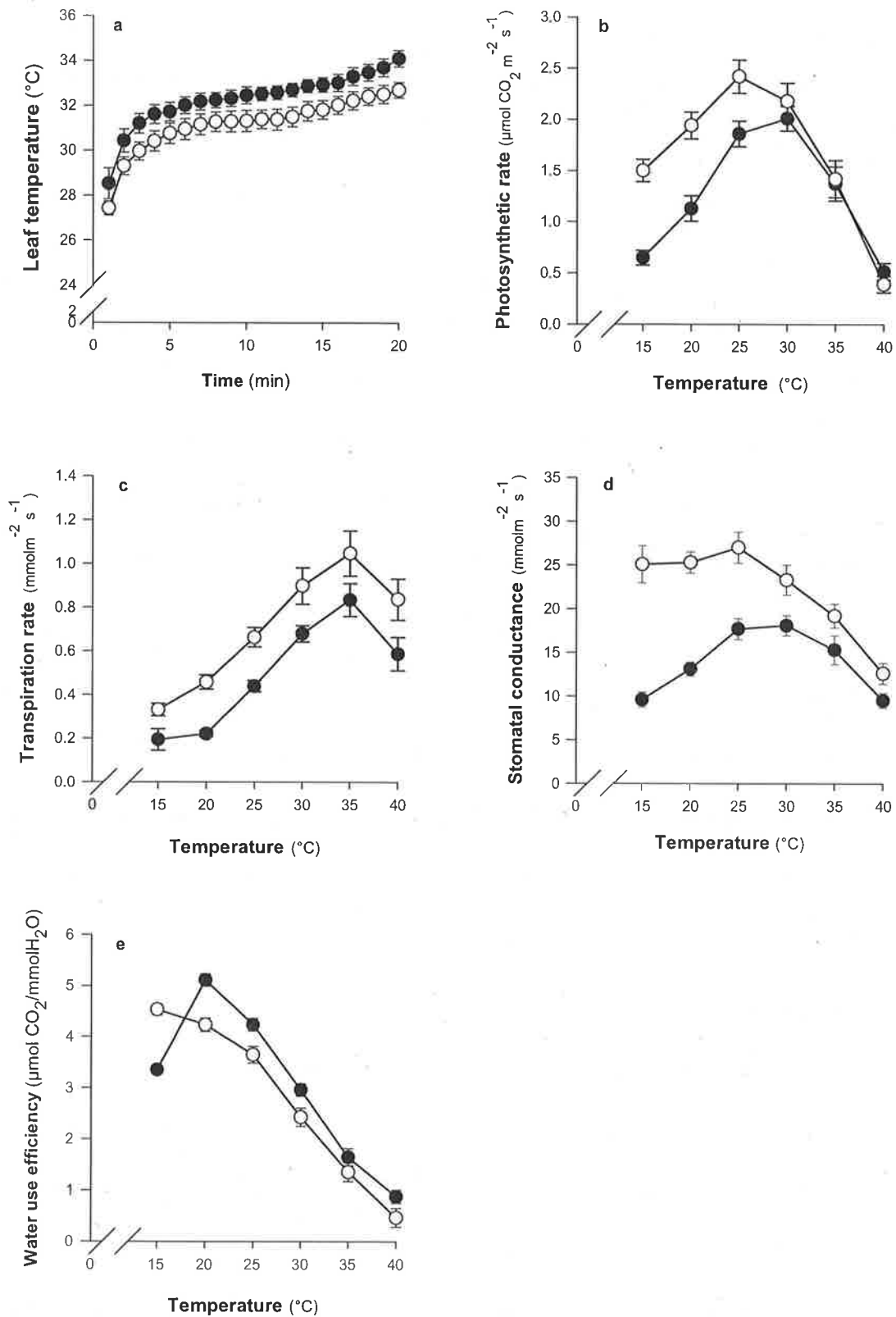


Figure 3.4 Temperature (a) of the leaves of *A. robusta* with (●) and without (○) stomatal plugs during 20 minutes chlorophyll fluorescence measurements. Photosynthetic rates (b), transpiration rates (c), stomatal conductances (d) and water use efficiency (e) of the leaves of *A. robusta* with (●) and without (○) stomatal plugs at leaf temperatures from 15 to 40°C. Data points are mean \pm s.e., $n=10$.

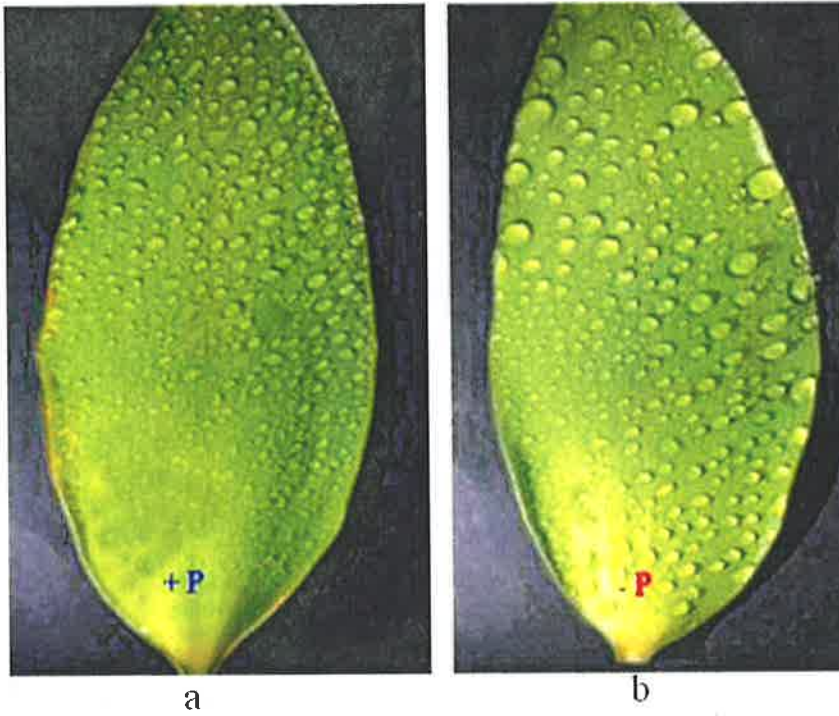


Figure 3.5. Misted leaves of *Agathis robusta* with (a) and without (b) waxy plugs.

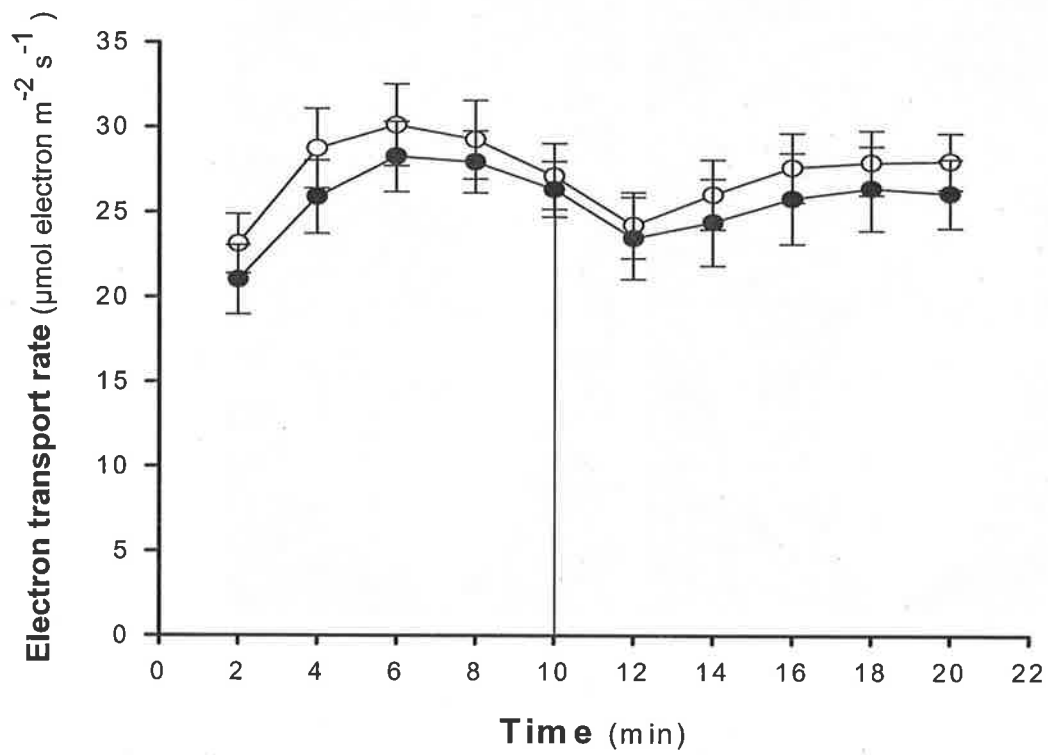


Figure 3.6. Effect of misting on electron transport rate of leaves of *Agathis robusta* with (●) and without (○) stomatal plugs. Data points are mean \pm s.e., $n=10$. ETR was measured for 10 minutes prior to misting (as indicated by the vertical line).

Discussion

The results of this study indicated that removing the waxy surface of *A. robusta* leaves did affect resistance across the cuticle. Leaf cuticular water loss was significantly greater in Blu-Tack treated leaves than control leaves, consistent with results of earlier studies that showed removal of epicuticular wax increased water loss across the cuticle in *Cryptomeria japonica* (Takamatsu *et al.* 2001) and *Sorghum bicolor* (Jordan *et al.* 1983; Riederer and Schreiber 2001). Also, this result was in agreement with the result that I found for *Leucadendron lanigerum* reported in Chapter 2. Therefore, epicuticular waxes appear to contribute to the resistance of the cuticle as a barrier to water loss from leaves (DeLucia and Berlyn 1984). Generally, cuticular transpiration accounts for 5 to 10% of the total leaf transpiration depending on the magnitude of the leaf to air vapour pressure difference (VPD) (Kerstiens 1997). Thus, it may become a significant site of water loss and an important feature affecting the ability of plants to survive severe water deficits (Muchow and Sinclair 1989; Hauke and Schreiber 1998). The effectiveness of epicuticular waxes as barriers to water loss varies with species. For instance, Ristic and Jenks (2002) found that in maize lines, the amount of epicuticular wax did not affect epidermal water loss. However, it is not only the amount of wax, but also the physical arrangement and the chemical composition of wax crystals that determine its ability to decrease water permeability of the cuticle (Rao and Reddy 1980; Hadley 1981; Johnson *et al.* 1983; Riederer and Schneider 1990; Reynhardt and Riederer 1994).

Epicuticular wax can also increase reflectance of light from the leaf surface, consequently reducing light absorption (Ehleringer *et al.* 1976). However, I found that the relatively small amount of epicuticular wax on *A. robusta* leaves did not

have a significant impact on leaf reflectance, supporting the result of Johnson *et al.* (1983) who found reflectance increased linearly with the amount of epicuticular wax in wheat (*Triticum* spp.). The reflective character of such waxes is also probably dependent on quantity, their specific arrangement on the leaf surface and their molecular composition.

Gas Exchange

My findings are quite different from the results reported by Feild *et al.* (1998) for *Drimys winteri*. I found that stomatal plugs significantly reduced leaf transpiration rates of *A. robusta* (Fig. 3-3a). The higher transpiration rates of leaves without plugs were apparently related to increased stomatal conductance compared with control leaves (Fig. 3-3c). However, I think that these different results may be related to the depressed location of the stomata on leaf surfaces of *D. winteri*. When I applied Blu-Tack with the same amount of pressure as used for removing the stomatal plugs of *A. robusta*, I was unable to successfully remove plugs from *D. winteri* leaves. The recessed position of stomata on the leaf surface of *D. winteri* means that greater pressure needs to be used when applying Blu-Tack to remove waxy plugs. Hence, there is more possibility of damaging stomata, and perhaps interfering with their function.

While it is possible that wax could reduce transpiration by increasing leaf reflectance and keeping leaves cooler (Barthlott 1990), my results indicated that the waxy coverage of *A. robusta* did not impact on reflectance. Furthermore, when leaf temperature was not controlled, leaves with waxy plugs had higher temperatures than leaves without plugs when exposed to the same PFD (Fig 3-4a). I also observed lower photosynthetic rates in control leaves in comparison with leaves without plugs (Fig. 3-3b). Since stomatal conductance is a primary factor

which controls photosynthetic rates (Brodribb 1996), this was most likely due to the lower stomatal conductances of control leaves.

My results support the suggestion of Brodribb and Hill (1997) and Jimenez *et al.* (2000) that stomatal plugs affect leaf gas exchange rate possibly through decreasing the cross-sectional area for diffusion and thus increasing resistance. Brodribb and Hill (1997) calculated that the increase in stomatal conductance of leaves without stomatal plugs should be about twice that of leaves with plugs. My results broadly supported their findings, although the difference in stomatal conductance between treatment and control leaves varied with temperature. Plants have been shown to respond to increasing VPD and transpiration rates by closing stomata (Mott and Parkhurst 1991; Tinoco Ojanguren and Pearcy 1993), and my results indicated that in *A. robusta*, stomatal conductance was reduced when transpiration rates increased above $0.6 \text{ mmol m}^{-2} \text{ s}^{-1}$, regardless of whether stomatal plugs were present or not (Fig 3-4). This contrasts strongly with the results for *D. winteri* in which stomatal conductance of leaves with plugs appeared to be insensitive to VPD (Feild *et al.* 1998).

The interactions between leaf temperature and leaf gas exchange characteristics that I observed have revealed some interesting effects of stomatal plugs in *A. robusta*. First, as noted above, plugs do not appear to interfere with the ability of stomata to sense changes in VPD or transpiration rates. Second, the fixed resistance presented by stomatal plugs provides leaves with an advantage in terms of water loss, as leaves with plugs had higher instantaneous WUE at all temperatures other than the lowest used in my study. These results suggest that stomatal plugs can benefit *A. robusta* by reducing water loss across a range of

temperatures (and VPD) and only present a disadvantage, in terms of carbon gain, at temperatures above ~30 °C.

Photochemical efficiency of PSII measured in dark adapted leaves with and without plugs was 0.78 and 0.76, respectively (data not shown), indicating that my experimental plants were healthy and not under stress (Bjorkman and Demmig 1987; Hall *et al.* 1993; Long *et al.* 1993). Rates of stomatal conductance were low in *A. robusta* compared with values reported for crop species. However, as there is a positive relationship between stomatal density and stomatal conductance (Muchow and Sinclair 1989; Awada *et al.* 2002), the low stomatal conductances were most likely related to the low stomatal density of *A. robusta* leaves. Similar low conductances have been reported for other conifers (Roberts 2000). Low stomatal conductance has also been reported for other rain forest species. For example, stomatal conductance was 26 mmol m⁻² s⁻¹ for *Tetragastris panamensis* and 11–13 mmol m⁻² s⁻¹ for *Trichilia tuberculata* and *Quararibea asterolepis* and photosynthetic rates also were very low, averaging 0.8–1.1 μmol m⁻² s⁻¹ (Rijkers *et al.* 2000; Engelbrecht *et al.* 2002).

Do plugs enhance photosynthesis of wet leaves?

While removing wax plugs did affect resistance across the leaf, it did not affect leaf wettability. In *A. robusta*, removing stomatal plugs had no effect on water film formation compared with leaves with plugs, suggesting that waxy plugs do not affect the hydrophobic properties of the leaf surface (Fig. 3-5). However, it is possible that the presence of plugs may still have helped prevent water from entering stomata and impeding diffusion of CO₂ into leaves. ETR, as a proxy of photosynthetic rate (Genty *et al.* 1989; Edwards and Baker 1993; Oberhuber *et al.* 1993; Valentini *et al.* 1995; Fryer *et al.* 1998; Rascher *et al.* 2000), was used to

assess the impact of surface water on rates of photosynthesis in leaves with and without plugs. The results showed no difference in ETR between wet and dry leaves with and without stomatal plugs, suggesting that in *A. robusta*, waxy stomatal plugs do not serve to maintain photosynthetic rates of wet leaves by preventing water from entering the stomata at least for the short term of misting used in this study.

Conclusions

The results of the present study showed that waxy plugs significantly decreased stomatal conductance, transpiration and photosynthesis in *A. robusta* in contrast to the results of Feild *et al.* (1998) for *D. winteri*. Although most species in dry environments lack stomatal plugs, in alpine regions where conifers commonly occur, seasonal soil-water deficit occurs when soil is frozen, and the leaves can still be exposed to dry air and high sunlight (Roberts 2000). This might explain the presence of stomatal plugs in some of conifer species.

The present results also showed that stomatal plugs did not impact on the formation of a water film on leaf surfaces of *A. robusta*. This result was again in contrast to the results reported for *D. winteri* (Feild *et al.* 1998).

According to the results, stomatal plugs increased water use efficiency and decreased water loss from leaves. However, an unavoidable consequence of decreased water loss is that CO₂ assimilation is also limited because of increased resistance to diffusion of CO₂ into leaves. Meanwhile, possessing stomatal plugs might be a disadvantage in hot environments, as my results showed that stomatal plugs can increase leaf temperature through their impact on evaporative cooling of leaves.

Thus, although I have clearly demonstrated that, at least in *A. robusta*, waxy plugs do reduce water loss, this may not be their primary adaptive function.

4. Stomatal plugs and their impact on fungal invasion in

Agathis robusta

Introduction

Plants exhibit a wide variety of leaf surface characteristics that have been suggested to provide different benefits in different habitats. Wax is one of the leaf characteristics that some plant species possess in both arid and wet environments. In general, wax is exuded outwards from the surface of guard cells, subsidiary cells and epidermal cells in a liquid form and then crystallized into various shapes depending on plant species (Reicosky and Hanover 1978). Leaf surface wax is a feature that has been assumed to be an adaptation to drought stress (Shantz 1927; Jordan *et al.* 1983). For example, it has been shown that epicuticular wax deposition on leaves of peanut increases with moisture deficit stress (Samdur *et al.* 2003). The authors reported that water deficit increases the epicuticular wax load on leaves, possibly reducing transpiration and thus improving leaf water use efficiency.

In addition, it has been suggested that epicuticular wax increases reflection of light from leaf surfaces, and so reduces light absorption protecting plants from photodamage and reducing leaf temperature relative to leaves without wax (Jeffree *et al.* 1971; Ehleringer *et al.* 1976; Ehleringer 1981; Robinson *et al.* 1993). The water proofing ability of waxy layers can also reduce fungal infection by removing water from the leaf surface and consequently preventing fungal germination (Martin and Juniper 1970). However, the function of wax coverage in different environments and for different plants is likely to vary.

Wax plugs are a leaf characteristic that occlude the entrance of stomata, particularly in conifers (Stockey and Ko 1986; Schmitt *et al.* 1987; Mickle 1993; Stockey and Atkinson 1993; Brodribb and Hill 1997). Feild *et al.* (1998) investigated the function of stomatal plugs in *Drimys winteri*, a non-coniferous species, and concluded that stomatal plugs do not decrease water loss but instead prevent the formation of a water film on leaf surfaces, which is more important in wet environments. In contrast, I found (chapter 3) that wax plugs reduce gas diffusion through stomatal pores in *Agathis robusta*, probably by decreasing the cross sectional area for diffusion (Brodribb and Hill 1997). However, the presence of stomatal plugs in environments like rain forests, where plants are less likely to face water restriction, and thus adaptations to reduce water loss would be of little benefit, suggests other functions for wax plugs. As yet, no study has been conducted into the initiation process of stomatal plugs and also the impact of stomatal plugs on fungal invasion in plants.

The diversity of fungi on plant leaves is remarkable. For example, thirty-six species of fungi were identified on only one leaf of a rain forest plant in North Queensland, Australia (Parungao *et al.* 2002). Furthermore, McKenzie *et al.* (2002) recorded 264 species of fungi on rainforest trees in New Zealand, mostly on *Agathis australis*. Although most fungi in rain forests are found on fallen leaves, wood and litter, 40 species of fungi found in the rainforest area of New Zealand were found on living leaves (Gadgil 1995; McKenzie *et al.* 2002). Therefore there is a great defensive need for plants living in rain forests to prevent fungal invasion especially through the stomata that are often open and thus prone to fungal penetration.

Generally, fungal penetration, in addition to the mostly fatal effect on leaves in the long term, can have a short term negative effect through blocking stomata and thus reducing stomatal conductance and CO₂ assimilation rates (Manter *et al.* 2000). Fungal penetration usually occurs when the fungal mycelium breaches the physical and chemical barriers of host plants to establish a parasitic relationship (Gallardo and Merino 1993; Canhoto and Graca 1999). Penetration by hyphae might occur in various ways including through stomatal pores, wounds on plant surfaces or direct penetration of the cuticle and cell walls. It has been suggested that the most important route is through stomatal pores (Lucas 1998; Canhoto and Graca 1999). However, hyphae have been shown to penetrate the leaf structure of different plant species in other ways as well.

For example, Hoehl *et al.* (1990) and Angelini *et al.* (1993) found that the mycelium of *Ascochyta rabiei* penetrated the stem of chickpea through the cuticle, and Ilarslan and Dolar (2002) found that penetration occurred through both the cuticle and stomata of chickpea leaves. Furthermore, Heath and Wood (1969) reported that penetration by *Ascochyta pisi* in pea occurs through both the stomata and the cuticle. However, the hyphae of *Phyllactinia corylea* in mulberry leaf enter only through stomatal apertures (Kumar *et al.* 1998), as do the hyphae of *Stemphylium floridanum* in *Solanum gilo* (Wu and Hanlin 1992), and the hyphae of *Pseudoperonospora cubensis* in cucumber (Cohen and Eyal 1980).

As shown in chapter 3, wax plugs significantly decrease transpiration and CO₂ assimilation rates in *A. robusta*. But *A. robusta*, and many other species with wax plugs, occur in moist environments. Thus, Hill (1998b) suggested that stomatal plugs might benefit plants in very wet environments by protecting stomata from

entry of fungal hyphae or entry of the proboscis of leaf feeding insects. However, there has been no empirical investigation of these hypotheses.

The present study investigated the initiation and seasonal modification of stomatal plugs, and also the impact of wax plugs on fungal invasion in *Agathis robusta*.

Materials and methods

Plant material

More than 50-year-old trees of *Agathis robusta* (Araucariaceae) growing in the Adelaide Botanic Garden, Australia, were chosen for this study. Average annual maximum temperatures for Adelaide are 18-21°C and average annual minimum temperatures range between 9-12°C. Rainfall is 600 mm per annum, falling mainly during the winter months, June-August (Commonwealth Bureau of Meteorology, Melbourne, Australia). Plants in the Adelaide Botanic Garden also receive additional water through irrigation.

Electron and light microscopy

Detached leaves of *A. robusta* were cut in ~1 cm² sections, mounted with double-sided adhesive tape and attached to aluminum stubs. The stubs were sputter coated with a thin layer of Gold/Palladium (80%/20%) about 4 nm thick in a Cressington high-resolution sputter coater (Model 208HR, Cressington, UK). The coated specimens were examined at different magnifications from 100× to 5000× using a Philips XL20 scanning electron microscope (SEM) with an accelerating voltage of 10 kV and a standard tilt of 15° (Philips Electron Optics, Eindhoven, Netherlands). Cross-sections of leaves were prepared by hand using a razor blade. The sections were then stained with Toluidine Blue for 30 seconds and examined with a light microscope at 400X magnification.

Freeze-fracture

The position of stomatal plugs in the stomatal antechamber of fresh mature leaves of *A. robusta* was assessed by freeze-fracture SEM. Leaf samples 8 mm² were attached to a clamping holder using Tissue-Tek OCT compound mixed with carbon dag, plunge frozen in liquid nitrogen slush, and transferred under vacuum to the prechamber of an Oxford CT 1500 HF cryotransfer system, maintained at approximately -130 °C, where they were fractured with the end of a scalpel blade. After fracturing, samples were sublimed, coated with platinum (approximately 2nm), transferred to the cold stage of a Philips XL30 Field Emission Gun SEM (FEGSEM) and examined at -190 °C.

Somatal plug replacement

The wax plugs occluding stomata were removed using Blu-Tack (Bostik, UK). This non-toxic putty was pressed gently against leaf surfaces a number of times enabling the effective removal of > 90% of stomatal plugs from stomatal antechambers, as assessed by scanning electron microscopy (SEM). SEM also indicated that leaves suffered no observable damage from the Blu-Tack treatment. After removing wax plugs from one side of the mid-vein of 100 attached leaves of *A. robusta*, wax plug replacement was investigated for 10 weeks from 20th of January to 29th of March 2003. Sampling occurred at weekly intervals, and each time 10 leaves were used. Leaves were examined using SEM as described above.

Seasonal modification of wax deposition

Modification of wax deposition on the adaxial (upper) and abaxial (lower) leaf surfaces of *A. robusta* and also the position of wax plugs at the entrance of stomata were monitored throughout the year in the middle of each season using

SEM. Since most mature leaves did not have complete wax coverage in spring, young fully expanded leaves were chosen for the survey.

Fungal infection

Experiment 1:

To investigate the impact of stomatal plugs on leaf fungal invasion, two species of fungi, *Botrytis cinerea* and *Alternaria solani* were used. Separate solutions of *B. cinerea* and *A. solani* spores in sterile distilled water were prepared. Fungi had been raised on Potato Dextrose Agar (PDA). Two droplets of the solution were placed onto a haemocytometer by sterile pipette and the number of spores was counted. Forty-eight leaves were detached from *A. robusta* trees, and wax plugs removed from 24 of the leaves. The petiole was covered by Vaseline to decrease water loss during the experiments. Leaves were placed on moist filter paper in sterile petri dishes and then inoculated with fungal spores. Spore suspensions of *B. cinerea* (44000 spores mL⁻¹) and *A. solani* (6000 spores mL⁻¹) were sprayed onto 6 leaves with and 6 without stomatal plugs in separate sterile petri dishes at 26 ± 2°C ambient temperature. Forty-eight hours after leaf inoculation, leaf samples were cut into 1 cm² sections and examined for fungal penetration.

Experiment 2:

As *B. cinerea* and *A. solani* mostly infect horticultural plants, to investigate the impact of the fungi that are naturally present on *A. robusta* leaves, 10 leaves were detached at random from a mature *A. robusta* tree, at approximately 2 meters height. To remove the saprotrophic spores, leaves were placed in 10% commercial bleach for 20 seconds, washed twice in sterile distilled water, cut into segments of about 2 cm² and placed on PDA in a petri dish. After one week, 3 distinctive types of fungi (white, black and grey) were isolated and transferred to new petri dishes

containing PDA. This transfer was repeated three times to isolate the fungi. After growing the fungi in new petri dishes, spores from different parts of each petri dish were collected and examined using light microscopy to confirm that a single species of fungus occurred in each petri dish.

After separating each species of fungus, a solution of the spores produced was made for each using sterile distilled water. Two droplets of each solution were sterile pipetted onto a clean haemocytometer and the number of spores was counted. Spore suspensions of white (15000 spores mL⁻¹), black (24000 spores mL⁻¹) and grey fungi (28000 spores mL⁻¹) were sprayed onto 6 leaves with stomatal plugs and 6 without stomatal plugs in separate sterile petri dishes at 26 ± 2°C ambient temperature. After 3 days, 1 cm² samples were cut to examine spore germination using light microscopy. Investigations using light, SEM and Laser Scanning Confocal microscopy (LSCM) were repeated after 4, 7 and 10 days of leaf inoculation.

Confocal visualisation

Infected leaf samples were stained overnight in a 0.01% solution of acid fuchsin (Brundrett *et al.* 1994 [NOTE: cited in ; Dickson and Kolesik 1999]). Stained leaf samples were cut in 1 cm² pieces, mounted in 75% glycerol on glass cover slips and observed with a 40X water-immersion objective lens with a numerical aperture of 1.15 and working distance of 210µm. A Bio-Rad MRC-1000 laser scanning confocal microscope (LSCM) in combination with a Kr/Ar laser and a Nikon Diaphot 300 inverted microscope was used to visualise the samples. A series of optical xy-slices from above the leaf toward the inside of the leaf, each with a 1.5 µm interval on the z-axis, were collected. For observing the acid

fuchsin stained hyphae, red fluorescence at 568nm excitation and 605/32nm emission was used. Leaf surfaces were visualized using green fluorescence at 488nm excitation and 522/35nm emission, and wax plugs were visualized using reflectance mode. Each image was averaged over 4 scans using a Kalman filtering process and saved as a digital file of 765 x 512 pixels. Amira software was used to produce 3D photographs of infected leaves.

Results

Initiation and seasonal modification of stomatal plugs

Investigations using SEM showed that *A. robusta* is hypostomatic, i.e. stomata occur only on the abaxial surface of the leaf. As previously found (chapter 3), there were on average, 89 stomata $\text{mm}^{-2} \pm 7$ on the abaxial surface. Stomata occurred in discontinuous rows, the average distance between rows was $58.8 \pm 3 \mu\text{m}$ and the average distance between stomata on each row was $40.5 \pm 3 \mu\text{m}$.

SEM micrographs of young leaf surfaces of *A. robusta* in the middle of spring showed that the extrusion of wax outwards occurs from the epidermal cells on the leaf surface. Extrusion of wax from guard and subsidiary cells at the entrance of stomata forms the stomatal plugs.

The wax plugs of one-month-old leaves in spring appeared fuzzy because of the rodlet shape of wax plugs (Fig. 4-1a). In summer, when the leaves were mature, rodlet waxes in the stomatal antechamber were almost solidified into a crystal form (Fig. 4-1b). In autumn the solidification of stomatal plugs was complete (Fig. 4-1c). After solidification of wax plugs, the waxy coverage on the leaf surface of *A. robusta* remained in rodlet form, according to the classification of leaf epicuticular wax by Barthlott *et al.* (1998).

There was wax erosion on both abaxial and adaxial leaf surfaces in winter. Wax plug breakdown had begun in the stomatal antechamber by the middle of winter, and most wax plugs had some cracks. By the following spring, old wax plugs had started to detach from stomata, and as the separation of degraded wax plugs started, guard and subsidiary cells began to extrude wax to establish new wax plugs (Fig. 4-1d). In contrast, leaves that were more than 2 years old lacked intact wax plugs throughout the year.

Freeze-fracture

Anatomical investigations using FEGSEM showed that wax plugs do not entirely fill the stomatal antechamber of *A. robusta*, but only occur at the entrance of the antechamber. The wax plugs, therefore, act like a lid located in the upper parts of the stomatal antechamber and very little wax exists in the chamber (Fig. 4-2 a). SEM micrographs showing the structure of detached wax plugs confirmed the FEGSEM results (Fig. 4-2b), and it was clear in hand sections of the leaf as well.

Stomatal plug replacement

No new plugs were produced on leaves from which plugs had been removed during the 10 week investigation of wax plug replacement in summer. There was little wax extrusion on the cuticular layer of leaf surfaces and very little wax accumulated at the entrance of stomata.

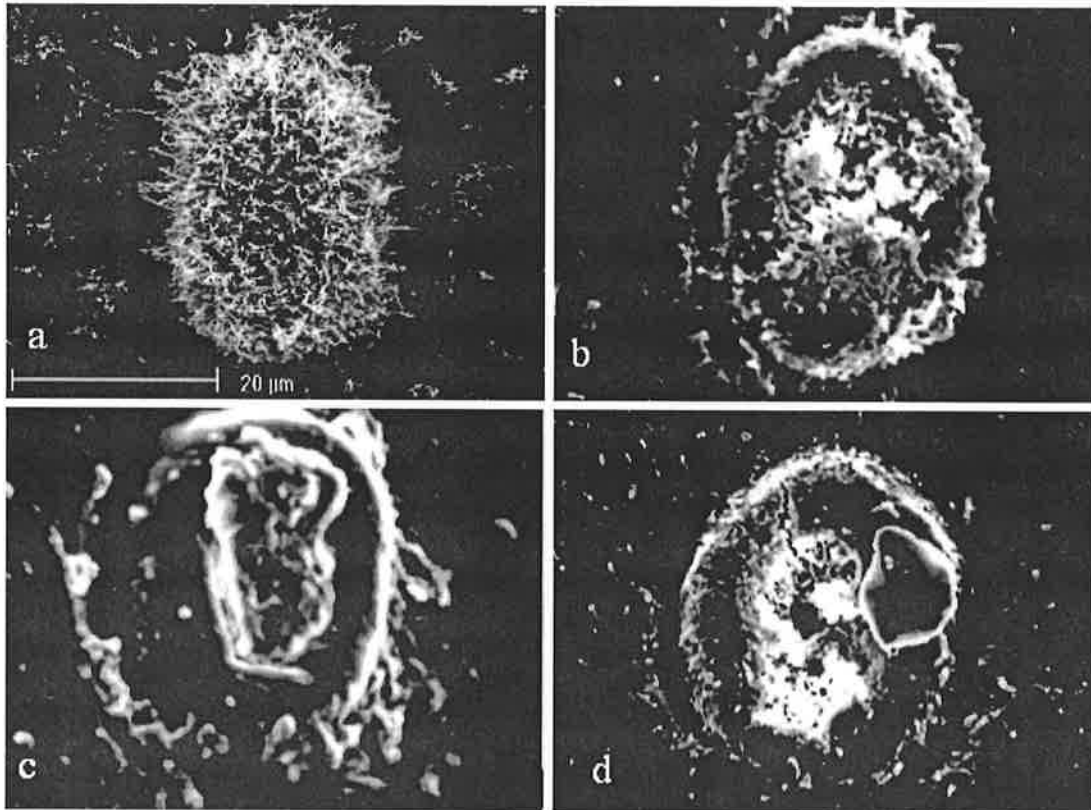


Figure 4.1. SEMs showing seasonal modification of stomatal plug in *A. robusta*. (a) Wax plug of a young one-month-old leaf in spring. (b) Wax plug in summer when the leaves were mature, showing the transformation of rodlet form waxes into a solid crystal. (c) Wax plug in autumn blocking the entrance of a stoma. (d) Wax plug degradation and also the extrusion of rodlet wax to establish a new wax plug from the middle of winter. Scale bar for all micrographs is 20µm.

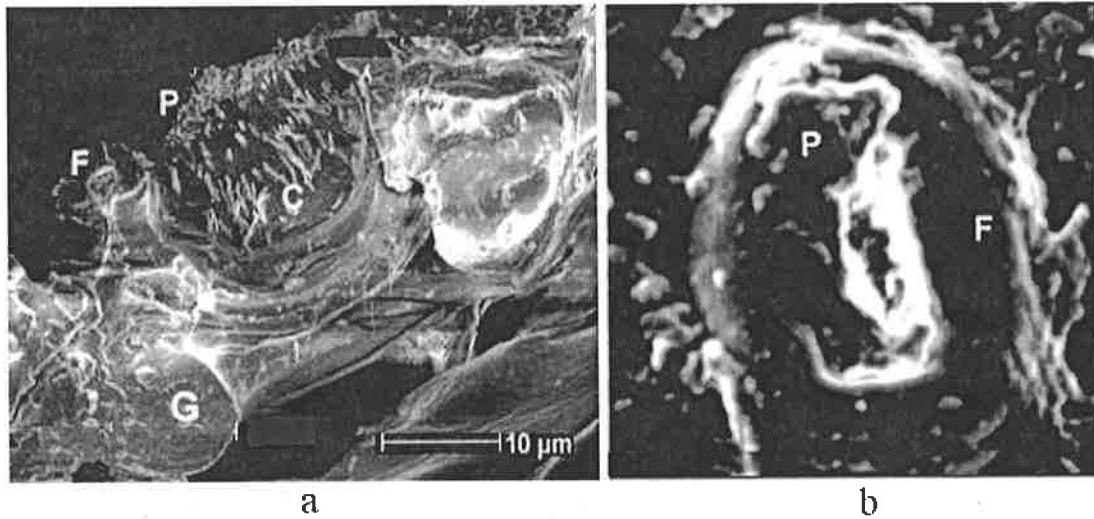


Figure 4.2. (a) FEGSEM micrograph of a stoma of *A. robusta*, showing guard cells (G), Florin rings (F), stomatal antechamber (C) and stomatal plug (P). (b) SEM micrograph of a stoma of *A. robusta*, showing the separation of the plug from the top of the stomatal antechamber; Florin rings (F) and stomatal plug (P). Scale bar for both micrographs is 10µm.

Fungal infection

Germination of spores of *B. cinerea* and *A. solani* on detached leaves of *A. robusta* was observed 3 days after inoculation. However, most spores of *A. solani* germinated later (from day 4) than *B. cinerea*, and hyphal penetration was observed 7 days after inoculation in both fungi. The hyphal density of *B. cinerea* on the infected sites 7 days after inoculation was greater than that of *A. solani*. However, there was no noticeable difference in frequency of hyphal penetration through stomata or the cuticle between *B. cinerea* and *A. solani* 10 days after leaf infection.

Two of the 3 fungi isolated from *A. robusta*, living in the Adelaide Botanic Gardens, were most likely species of *Fusarium* (white) and *Alternaria* (grey) but, the third genus, which had dark brown spores, could not be identified, *Sadasivania* has a similar form of spores Barnett (1960). The spores of *Alternaria* are ovoid with multiple cells and average length of $27 \pm 2 \mu\text{m}$ (Fig. 4-3a). *Fusarium* spores are crescent shaped, mostly 4 celled with an average length of $40 \pm 3 \mu\text{m}$ (Fig. 4-3b), and the unidentified dark brown spores are spherical in shape with average diameter of $22.5 \pm 2 \mu\text{m}$ (Fig. 4-3c).

SEM and confocal visualisation

SEM investigations of the abaxial leaf surface of *A. robusta* infected by *B. cinerea* and *A. solani* showed that fungal hyphae failed to penetrate through stomata that were blocked completely with wax plugs. Hyphae extended past stomata that were blocked with wax plugs and penetrated through stomata with incomplete or damaged wax plugs (Fig. 4-4).

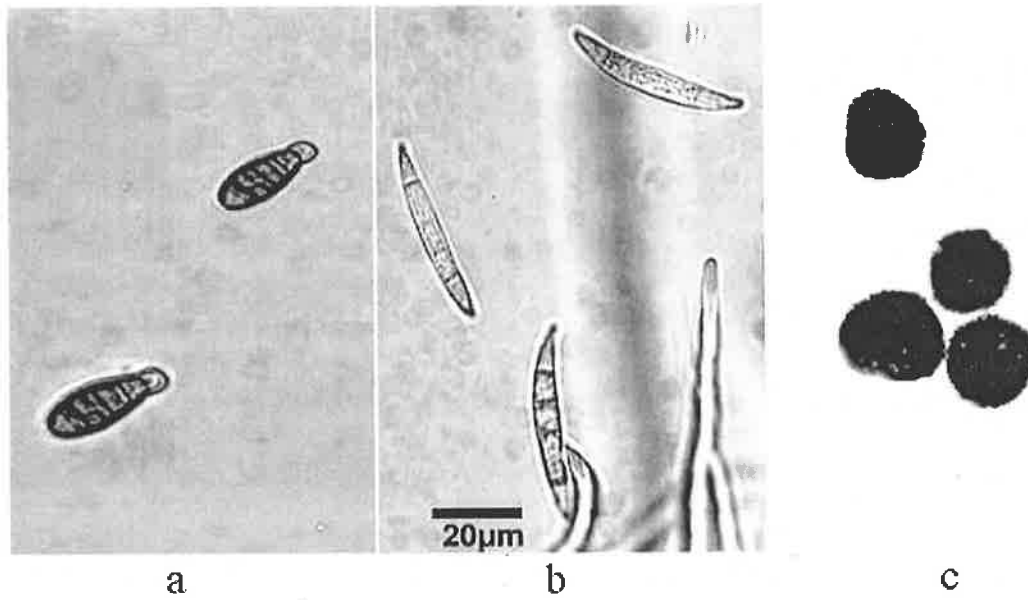


Figure 4.3. Micrographs of spores of isolated fungi from leaves of *A. robusta*: (a) *Alternaria*, (b) *Fusarium* and (c) possibly *Sadasivania*. Scale bar for all 3 micrographs is 20 µm.

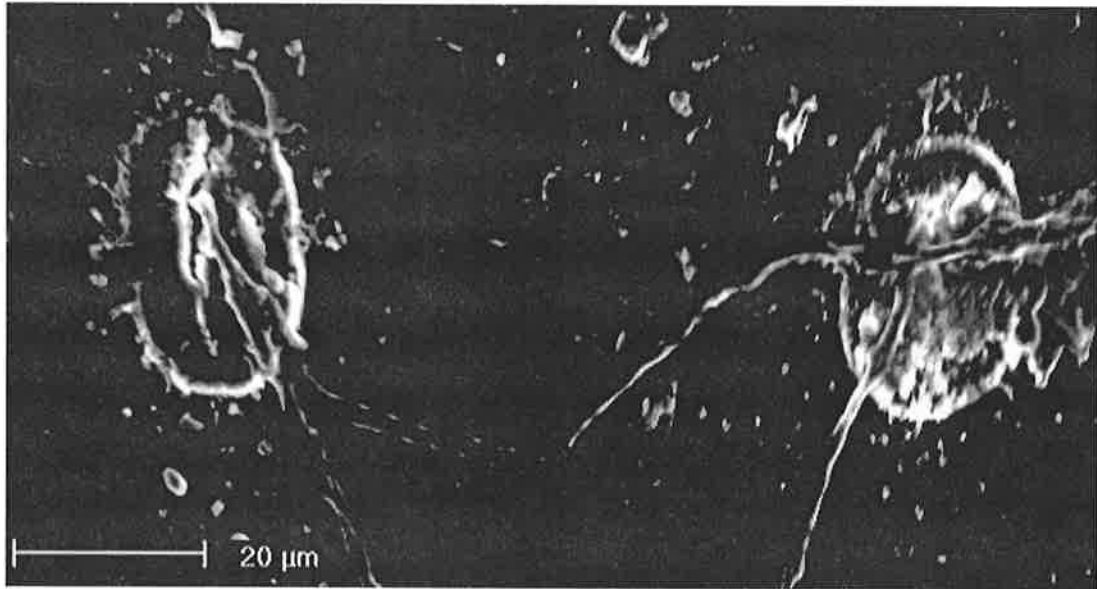


Figure 4.4. SEM of the leaf surface of *A. robusta* infected by *A. solani*. The hypha can be seen passing over the stoma blocked with a wax plug (right) and penetrating the stoma with an incomplete wax plug (left).

Hyphae never penetrated stomatal pores for all the samples blocked with wax plugs, while hyphae were successful in penetrating all stomata in leaves from which plugs had been removed. The results also indicated that the protruding Florin rings on the leaf surface of *A. robusta* changed the direction of growth of hyphae and decreased the chance of hyphae approaching stomatal pores (Fig. 4-5). For LSCM, hyphae were observed as red in colour, stomatal plugs were blue and the host tissues were green. Hyphae of both species were seen to penetrate through stomata that lacked wax plugs. However, in some cases hyphae penetrated directly through the cuticle as well (Fig. 4-6a, b).

Similar results were found for fungi that occurred naturally on *A. robusta*. That is, on no occasion did fungi penetrate the leaves through stomata when they were blocked by wax plugs. However, as was found for *B. cinerea* and *A. solani*, these fungi also penetrated the leaves either directly through the cuticle (Fig. 4-7a) or through unplugged stomata (Fig. 4-7b).

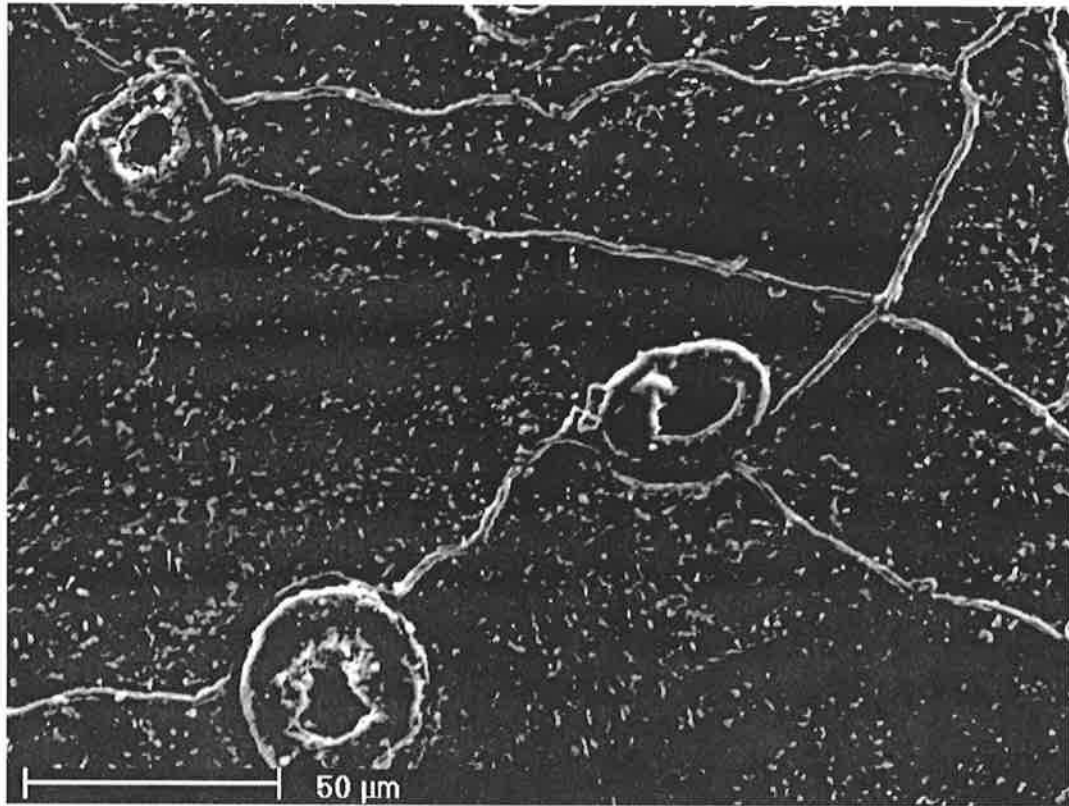


Figure 4.5. SEM of the leaf surface of *A. robusta* infected by *A. solani*. Florin rings encircling antestomatal chambers changed the direction of hyphal growth.



Figure 4.6. Three-dimensional image of the leaf surface of *A. robusta* using LSCM. The leaf was infected by *B. cinerea*, showing the blockage of hyphae by wax plugs of the two left side stomata (a1 and a2), and the penetration of the hypha through the cuticle at the right side (a3). Image b showing just the hyphae of *B. cinerea* and wax plugs of *A. robusta* of image a, after omitting the leaf surface to make more clear the blockage of the hyphae at point 1 and 2 and penetration of the hypha through the cuticle at point 3. Leaf surface (green), hyphae (red) and wax plug (blue). Scale bar is the same for both images (20 μ m).

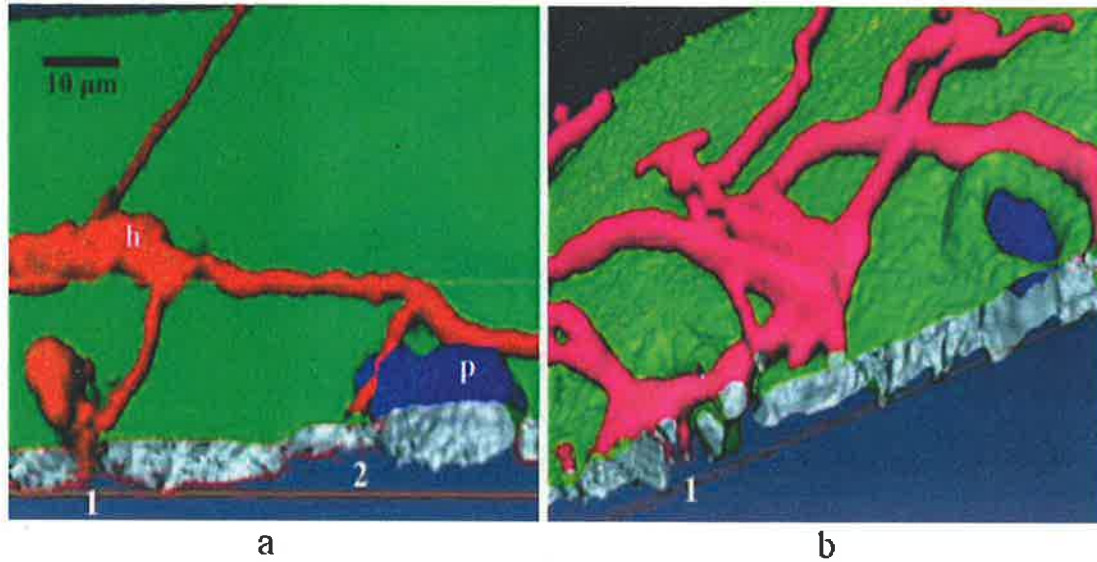


Figure 4.7. Three-dimensional images of the leaf of *A. robusta* as viewed with LSCM. The leaf infected by *Alternaria species* (a) and *Fusarium species* (b) isolated from leaves of *A. robusta*, showing the direct penetration of the hypha through the cuticle at the left side (a1), and the blockage of hypha by wax plug at the right side (a2), and the penetration of the hypha through a stoma that lack of wax plug (b1). Hyphae (h), stomatal plug (p). Leaf surface (green), hyphae (red) and wax plug (blue). Scale bar is the same for both images (10 μ m).

Discussion

Anatomy of wax plugs in A. robusta

Wax plugs are created by extrusion of wax outwards from guard cells and subsidiary cells in the stomatal antechamber (Jeffree *et al.* 1971). Jeffree *et al.* (1971) reported that stomatal plugs in *Sitka spruce* extend to the bottom of the stomatal antechamber and occupy about half the cross-sectional area at the mouth of the chamber. My anatomical observations, using FEGSEM, SEM and light microscopy, showed that wax plugs in *A. robusta* do not extend to the bottom of stomatal antechamber, rather, they occur as an elliptical plug at the entrance of the stomatal antechamber. Thus, wax plugs in *A. robusta* are like a lid covering the stomatal antechamber and very little wax extends into the chamber itself (Fig. 4-2a). Also, the anatomical observations showed that wax plugs in *A. robusta* occupy more than half of the cross-sectional area of the stomatal antechamber, so that very little space can be seen at the side of the stomatal plugs. These results contrast with reports that suggest stomatal plugs extend to the bottom of the stomatal antechamber. Further investigations with *A. australis* showed similar plug morphology to *A. robusta* (data are not shown).

The seasonal investigation of wax plugs in *A. robusta* indicated that stomatal plugs renewed annually, with new plugs being initiated in spring. Most leaves form in spring and their plugs are completed by autumn, followed by winter degradation of wax (Fig. 4-1a-d). Leaves formed in other seasons followed the same developmental sequence of wax plug formation and degradation, but in different seasons. Nevertheless, since most new leaves form in spring, we can say that generally the extrusion, solidification and degradation of wax plugs are seasonal and occur in spring, summer-autumn and winter, respectively. Therefore,

it is likely that *A. robusta* leaves are more vulnerable to fungal invasion in winter rather than during other seasons. The degradation of wax from late autumn, especially in winter, has also been reported in *Picea pungens* (Reicosky and Hanover 1978) and in *Picea abies* and *Pinus cembra* (Anfodillo *et al.* 2002). Also, in beech (Prasad and Guelz 1990) and in *Tilia tomentosa* (Guelz *et al.* 1991) the biosynthesis and exudation of wax outwards occurs during leaf development and growth in spring.

Leaves that were more than two-year-old, despite extruding wax outwards on both leaf surfaces and at the entrance area of stomata in spring, did not produce complete wax plugs at the entrance of stomata and lacked the regular shape of stomatal plugs found in younger leaves. These incomplete wax plugs may make the older leaves of *A. robusta* more susceptible to fungal attack. This result is in agreement with those of Hauke and Schreiber (1998) for *Hedera helix* and Prugel *et al.* (1994) and Anfodillo *et al.* (2002) for *Picea abies* and *Pinus cembra*, who found wax extrusion decreased as leaf age increased. Also, Wirthensohn and Sedgley (1996) found that wax regeneration depended on the age of the leaves in eighteen species of *Eucalyptus*.

In addition to a reduction of wax extrusion as leaves aged, there was also a transformation of epicuticular wax form on leaf surfaces from rodlet to planar. This meant that the waxes on the two-year old leaves were mostly flattened rather than in rodlet form, similar to the seasonal epicuticular wax transformation reported by Anfodillo *et al.* (2002) for *Picea abies* and *Pinus cembra*. The annual regeneration of stomatal plugs in *A. robusta*, at least in leaves younger than 2 years, was contrary to the results found for trichomes in *Banksia* species, which were not replaced after shedding (Appendix 2).

Regeneration of wax has been reported previously for 18 *Eucalyptus* species (Wirthensohn and Sedgley 1996) and 24 water-repellent plant species (Neinhuis *et al.* 2001). However, the annual regeneration of epicuticular wax as well as wax plugs in *A. robusta*, at least for the first 2 years of the leaf lifetime, was contrary to the findings of Jetter and Schaffer (2001) for *Prunus laurocerasus*, in which epicuticular waxes were regenerated only in the early stages of leaf development. However, *P. laurocerasus* is a deciduous plant and *A. robusta* is an evergreen plant with leaves that live for a number of years. My findings are also different from those of Neinhuis and Barthlott (1997) who reported that in 200 water-repellent plant species, wax extrusion occurred only during leaf development and the erosion of wax occurred after leaf expansion was complete.

Wax plugs and fungal infection

The occlusion of stomata in *A. robusta* by wax plugs led to the hypothesis that wax plugs might prevent penetration by fungal hyphae. Investigation of infection strategies by fungal pathogens has included mechanisms of spore attachment to leaf surfaces and fungal penetration of leaves (Mendgen and Deising 1993; Mendgen *et al.* 1996; Dean 1997; Tucker and Talbot 2001). The current study investigated the impact of stomatal plugs on hyphal penetration in *A. robusta*.

Although stomata are sunken in *A. robusta*, the presence of Florin rings creates a protrusion of the cuticle around stomatal antechamber. This may increase the chance of infection, because it facilitates the landing and germination of fungal spores compared with leaves that have flat surfaces. It has been shown that surface topography can act as an inductive signal stimulating fungal infection, and even growth orientation of germ tubes and hyphae (Wynn 1976; Terhune and Hoch 1993; Read *et al.* 1997; Patto and Niks 2001). However, the results of this

study indicated that although the protrusion created by Florin rings might increase the chance of landing and germination of fungal spores on leaf surfaces, they also influenced the direction of the hyphal growth by diverting hyphae away from stomatal pores (Fig. 4-5).

Hoch *et al.* (1987) suggested that topographical features of the stomatal complex can provide signals for the differentiation of appressoria (flattened hyphae of a parasitic fungus that penetrate host tissues) over stomata in the fungal pathogen *Uromyces appendiculatus*. It has been shown that hydrophilic surfaces are an important stimulus to leaf infection and appressorium development in a large number of fungal species (Hamer *et al.* 1988; Lee and Dean 1993; Jelitto *et al.* 1994; Wosten *et al.* 1994; Temple and Horgen 2000). In some fungal species, like *Magnaporthe grisea*, hydration alone seems to be sufficient to induce spore germination (Tucker and Talbot 2001). In contrast, the hydrophobic surface of plants such as *A. robusta* can decrease water availability on leaf surfaces (Beattie and Marcell 2002), and as a consequence restrict the germination of fungal spores. However, Rubiales and Niks (1996) suggested that the wax layer itself plays a role in the growth of the germ tube of rust fungi across the leaf towards stomata. They also suggested that wax covering stomata in *Hordeum chilense* might have evolved as a defense mechanism against penetration by pathogens. In contrast, Patto and Niks (2001) found that the leaf wax layer did not contribute to, or impede, the orientation of the germ tube of rust fungi in *H. chilense*.

The results of the present study indicated that the hyphae of *A. solani*, *B. cinerea* and the fungi isolated from *A. robusta* leaves penetrated through both the cuticle and stomatal pores when the stomata lacked wax plugs. However, in the early stages (i.e. for the first 7 days) fungal penetration only occurred through stomata

that lacked wax plugs (Fig. 4-4). Both SEM and FEGSEM visualization showed that the hyphae of the fungi appeared to grow randomly across leaf surfaces, and where they encountered unprotected stomatal antechambers, they penetrated leaves, supporting the findings of Patto and Niks (2001) in *H. chilense*.

After 7 days, some hyphae penetrated directly through the cuticle, especially in areas where there were depressions in the epidermis such as between the prominent Florin rings and adjacent epidermal cells. The characteristic leaf surface topography of *A. robusta* might also be a signal for appressorium differentiation and thus stimulate direct penetration of hyphae through the cuticle as well. These results support the findings of previous researchers in different species (Wynn 1976; Hoch *et al.* 1987; Terhune and Hoch 1993; Patto and Niks 2001).

Although the hyphae of *A. solani*, *B. cinerea* and the other fungi isolated from *A. robusta* leaves did not specifically target stomata, on those occasions that hyphae attempted to penetrate the leaves through stomata, they were blocked by stomatal plugs. These results support the suggestion of Brodribb and Hill (1997) and Hill (1998a) that stomatal plugs act as physical barriers to fungal invasion. The impact of Florin rings on the direction of hyphal growth and also the ability of wax plugs to prevent fungal penetration through stomata has not been previously reported.

In addition to providing physical barrier to fungal penetration, the hydrophobic property of wax plugs may also inhibit germination of the spores landing on stomata (Tucker and Talbot 2001). Thus, the small number of fungal species found on the attached leaves, compared with the huge number of fungal species found on fallen leaves and wood in rainforests (McKenzie *et al.* 2002), might reflect the impact of the waxy leaves of *Agathis* trees on spore germination.

Conclusions

The generation and degeneration of stomatal wax plugs occurred annually at the entrance of Florin rings in spring and winter, respectively. However, the annual process of change in wax plugs is dependent on both the age of leaves and the season in which leaves develop. Stomatal plug initiation is more dependent on the age of leaves than season, as leaves more than 2 years old did not produce complete wax plugs.

With regard to the impact of wax plugs on fungal invasion, the present study showed that wax plugs could significantly block the penetration of fungal species that lack the ability to penetrate directly through the cuticle. Moreover, even when direct penetration through the cuticle occurs, by blocking penetration through stomata, the leaves of *A. robusta* reduce the overall rate of penetration by fungal hyphae relative to leaves without wax plugs. Thus, wax plugs, either through facilitating the removal of fungal spores from the entrance of stomata or by blocking penetration of hyphae through stomata, could be very important for plants, and especially for those that live in rain forest where there are numerous fungal species and warm, moist conditions that favour fungal growth.

5. The impact of stomatal crypts on gas-exchange in *Banksia* species

Introduction

Gas exchange across leaves is regulated primarily by stomata (Jarvis and Davies 1998; Jones 1998), with more than 95% of the water lost by plants, and almost all of the carbon dioxide gained, passing through them (Jones *et al.* 1993). Thus, there is great interest in factors that may affect the function of stomata and consequently photosynthesis and water loss. There is considerable selective pressure on organisms in arid environments to conserve water. It is likely that these selective pressures have led to evolutionary modifications in morphology and physiology (xeromorphy) that reduce water loss (Dudley 1996; Hill 1998a). This may include modifications in stomatal morphology that could aid in reducing water loss from leaves.

To survive in drought conditions plants need to maintain their water content so that physiological activity can continue. Traits that may facilitate this include: small leaf area, small intercellular air spaces, thick cuticle and waxy layer, abundant trichomes, low stomatal density and hidden stomata (Carpenter 1994; Hawksworth 1996; Brodribb and Hill 1997; Hill 1998b; Villar-de-Seoane 2001; Balok and St Hilaire 2002), and fewer veins (Groom *et al.* 1994). However, some of these features, e.g. small leaves and a thick cuticle, could also be related to low phosphorous availability (Hill 1998a). Features such as a thick cuticle, waxy layer and numerous trichomes also occur in wet environments (Brewer *et al.* 1991; Brewer and Smith 1997; Neinhuis and Barthlott 1997). Thus, there is uncertainty

as to whether such so called xeromorphic features actually evolved as adaptations to reduce water loss.

Stomatal crypts, which are leaf epidermal depressions containing stomata and trichomes, have been assumed to decrease transpiration rates by increasing the diffusion path for water and also the boundary layer thickness above stomata (Lee and Gates 1964; Brodribb and Hill 1997; Hill 1998a; Hill 1998b; Roberts 2000). However, these features are also likely to affect diffusion of CO₂ into leaves as well as water out of leaves (Wilkinson 1979; Hill 1998b). Hill (1998a) suggested that such modifications might decrease water loss in dry environments and restrict the entry of water into stomatal pores in wet environments. However, although the evolution of stomatal crypts in *Banksia* species in southern Australia coincided with the onset of aridity in the Oligocene and Miocene (leading to the conclusion that crypts are xeromorphic structures (Hill 1998a)), the fact that stomatal crypts are not just limited to arid regions may indicate that they provide adaptive benefit for multiple purposes (Gutschick 1999). For example, crypts increase the leaf surface area which may enhance gas exchange.

In accordance with Fick's law of diffusion (Campbell 1986), if we assume the structure of crypts to be a tube, as the depth of crypts increases, the resistance for diffusion of gas should increase as well. Consequently, at constant cross sectional area, leaves with deeper crypts should have lower rates of water loss. Crypts might also affect the thickness of the boundary layer above stomata, and as a consequence affect transpiration and assimilation rates. Vesala (1998) stated that an increase in the thickness of the boundary layer results in a decrease in water vapour loss from the stomatal pore, and also a net decrease in the number of CO₂ molecules that enter the stomata per unit time. For example, Pachepsky *et al.*

(1999) reported that transpiration rate was inversely proportional to boundary layer thickness in *Arachis hypogaea*. Hence, the effect of stomatal crypts on the thickness of the boundary layer above stomata could be an important means, especially in arid zones, for decreasing water loss. However, increasing the thickness of the boundary layer can also cause an increase in leaf temperature, which may affect leaf function in other ways.

Despite assumptions about the function of stomatal crypts, there are surprisingly no published studies on the physiological effects of crypts. This study evaluated the micromorphology of stomatal modifications in a range of *Banksia* species and the impact of stomatal crypts on leaf gas exchange. I hypothesised that as crypt depth increased transpiration and photosynthesis should decrease for a given VPD. If this were the case, this would support the idea that crypts are an adaptation to reduce water loss in arid environments.

Materials and methods

Plant materials

Leaf cross-sections and micrographs of over 110 species of the Proteaceae family were examined (Appendix 1). Among these species stomatal crypts were found only in *Banksia* species. Fourteen species of *Banksia* as well as *Dryandra praemorsa* were selected for this study. Recent phylogenetic studies have indicated that *D. praemorsa* should be grouped within the genus *Banksia*, thus it was included in the study (Mast and Givnish 2002). Two-year-old seedlings of the 15 species were obtained from Protea World, Adelaide, Australia, and grown for one year in 2 L pots containing premium potting mix (Premium Potting Mix, Australian standard, AS3743) in a glasshouse at the University of Adelaide,

Australia. Glasshouse conditions were as described in Chapter 2. Plants were watered with tap water automatically by overhead spray for 5 minutes every 3 days. Five grams non-phosphorous slow release fertilizer for Proteaceae (Protea Word, Adelaide, Australia) was applied in spring and autumn.

Electron and Light Microscopy

For SEM and light microscopy, one-year old leaves from each species were cut in $\sim 1 \text{ cm}^2$ sections, mounted with double-sided adhesive tape and attached to aluminum stubs. The stubs were sputter coated with a thin layer of Gold/Palladium (80%/20%) about 4 nm thick in a Cressington high-resolution sputter coater (Model 208HR, Cressington, UK). The coated specimens were examined at different magnifications from 100 \times to 5000 \times using a Philips XL20 scanning electron microscope with an accelerating voltage of 10 kV and a standard tilt of 15 $^\circ$ (Philips Electron Optics, Eindhoven, Netherlands).

Crypt dimensions were obtained by taking very thin cross-sections through leaves with a razorblade. Sections were placed in a 25% solution of commercial bleach and water until bleached. The bleached leaf pieces were then placed in fresh water with a few drops of 2% ammonia to help remove air bubbles trapped in the crypts. Once the leaf sections were waterlogged, they were again rinsed in fresh water and stained with Toluidine Blue for 30 seconds. These sections were then examined with a light microscope at different magnifications and measurements of crypt depth and entrance width were made.

Stomatal densities of each species were assessed using light microscopy. Leaves were cut into sections approximately 3 \times 3 mm. These were placed in 2.5 mL vials containing a solution of 1:1 80% ethanol and 100% hydrogen peroxide. The vials

were suspended in a water bath at 60°C until the cuticle began to separate from the leaf (about 48 hr). Approximately one quarter of this solution was decanted and replaced with fresh 100% hydrogen peroxide daily. The leaf pieces were then rinsed in water before their abaxial cuticles were carefully removed with forceps. Stomatal crypts occur only on the abaxial leaf surfaces of the species used in this study. This layer was then gently cleaned from the inside with a paintbrush to remove any adhering cellular material. Finally the squares of leaf abaxial layers were stained with Toluidine Blue for 30 seconds and mounted on microscope slides with the internal surface facing up. Stomata per crypt were counted using a light microscope at 400× magnification. Crypt densities were obtained by counting the number of crypts in 2.2 mm² sections of prepared cuticle. Finally, the mean number of stomata per crypt was multiplied by the mean number of crypts per mm² to get stomatal density.

Images of the prepared cuticle were taken at 100X magnification. Images were altered using Corel photo paint (Corel Draw Version 10, Corel Corp. USA), so that the crypts were black and the surrounding cuticle white. The areas of the crypts were then calculated in μm² using Scion Image beta version 4.0.2 (Scion Corp. Maryland, USA).

Cuticular water loss

The impact of stomatal crypts on cuticular water loss was assessed using detached, darkened leaves from 8 of the 15 species. These 8 species represented a range of crypt depths and widths. After detaching two-year-old leaves, the end of the petiole was coated with petroleum jelly to eliminate water loss from cut ends. Water loss from leaves was measured gravimetrically (Schoenherr and Lenzian 1981; Prugel *et al.* 1994) as changing mass over a 65 hour period in a dark room.

Leaves had been in the dark room for 40 minutes before the measurements began. Leaves were obtained from 5 plants (1 leaf each) of each of the 8 species. Temperature and relative humidity in the dark room were 19.5°C and 45% respectively, measured with a digital thermohygrometer (Model 37950-10, Cole-Palmer Instruments, Illinois, USA), and were stable over the course of the measurements.

Gas exchange

Transpiration, CO₂ assimilation and stomatal conductance of the 15 species were measured using a CIRAS-2 portable infrared gas analyzer (PP Systems, Herts, UK) fitted with an automatic Parkinson Leaf Cuvette. During measurements, vapour pressure deficit (VPD) was altered by changing the vapour pressure of the reference gas flowing into the leaf chamber. CO₂ concentration was 350 ppm, PFD was 650 $\mu\text{mol quanta m}^{-2}\text{s}^{-1}$ (which had previously been shown to be saturating for all species) and leaf temperature was 25°C. Transpiration, stomatal conductance and photosynthetic rates of two-year-old leaves were measured after 20 minutes at each VPD. Photosynthetic induction was complete in all plants prior to the start of each experiment. All measurements were made on attached leaves.

Data analysis

Relationships between stomatal conductance, transpiration and photosynthesis to VPD were analyzed by repeated measures ANOVA using the statistical package JMPIN, Version 4.03, 2000, SAS institute. Data for other relationships were analyzed by Analysis of Covariance (ANCOVA), using the statistical program JMPIN. The assumptions of normality and homogeneity of variances were

confirmed beforehand, using the Shapiro-Wilk and Levene's tests, respectively, in JMPIN.

Results

Leaf characteristics

The leaf surface characteristics of the 15 different species were examined on both the adaxial (upper) and abaxial (lower) surfaces. In some species, like *B. marginata*, dense trichomes covered the abaxial leaf surface and the inside of crypts (Fig. 5-1 a; Table 5-1), while in other species, e.g. *B. baxteri*, trichomes occurred only the inside of crypts, and mostly at the entrance of crypts on the abaxial surface (Fig. 5-1 b; Table 5-1). All species had sparse trichomes on the adaxial surface.

Maximum depth of crypts varied among the 15 species, ranging from 100 μm in *B. marginata* to 425 μm in *B. blechnifolia* (Fig. 5-1 c; Table 5-1). Maximum width of the entrance of crypts also varied among the 15 species, ranging from 110 μm in *B. repens* to 395 μm in *B. ashbyi* (Table 5-1). Two species, *B. spinulosa* and *D. praemorsa* lacked crypts. Stomatal density also varied among the 15 species, ranging from 144 stomata mm^{-2} in *B. caleyi* to 388 stomata per mm^{-2} in *B. speciosa* (Fig. 5-1 d; Table 5-1). Leaf thickness varied from $200 \pm 11 \mu\text{m}$ in *B. spinulosa* to $730 \pm 24 \mu\text{m}$ in *B. blechnifolia* (Table 5-1).

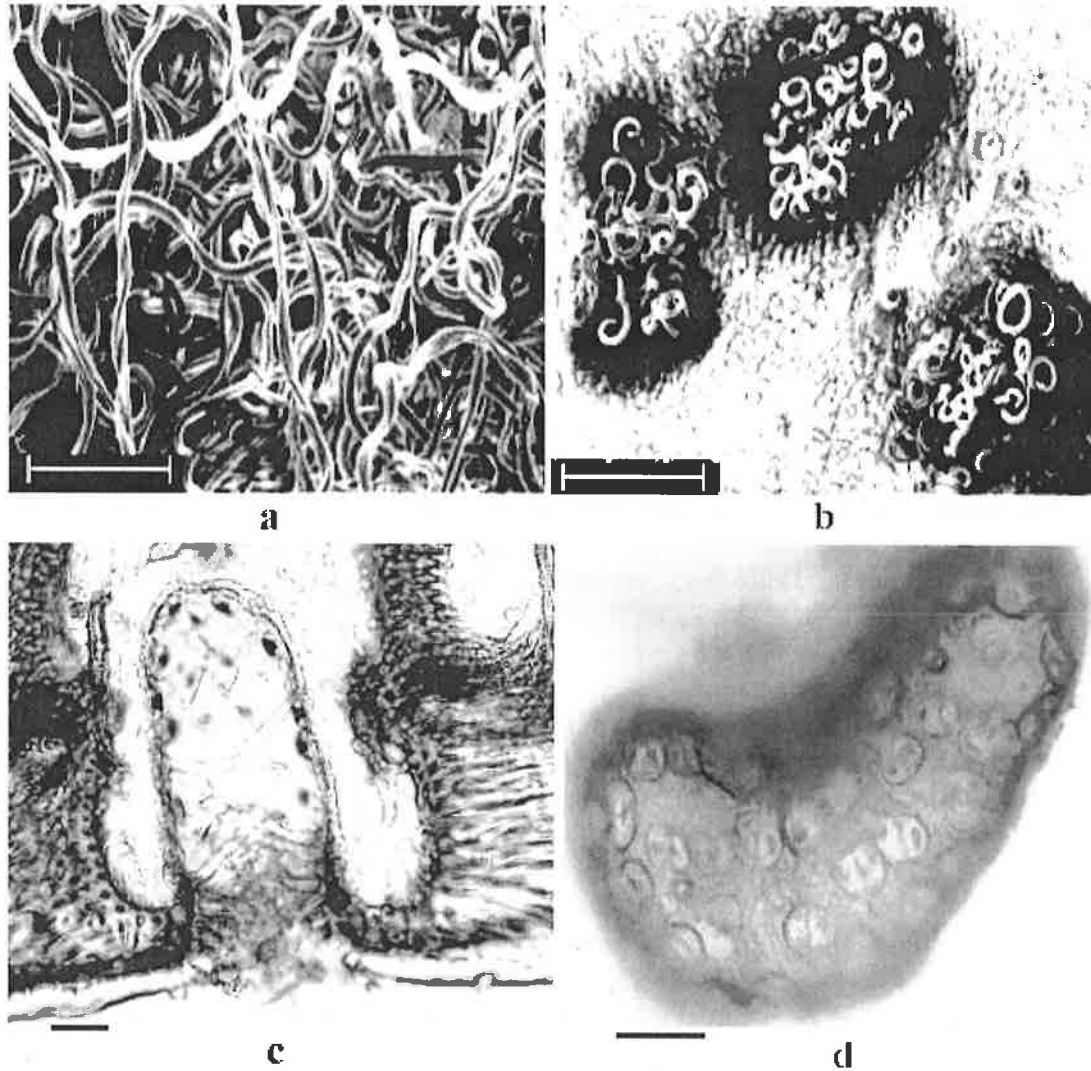


Figure 5.1. SEM micrographs of the abaxial (lower) leaf surface of *B. marginata* (a) and *B. baxteri* (b). The abaxial surface of *B. marginata* was covered with dense trichomes, while in *B. baxteri* trichomes occurred only inside and mostly at the entrance of the stomatal crypts. Cross-sectional view of a leaf of *B. blechnifolia* showing the depth and width of a crypt, the position of stomata inside the crypts and the occurrence of numerous trichomes inside and mostly at the entrance of stomatal crypts (c). Stomata inside a crypt of *B. blechnifolia* after removing the abaxial cuticle (d). Scale bar for figures (a) and (b) is 100 μm and for (c) and (d) is 50 μm.

Table 5.1. Depth and width of crypts, leaf thickness, stomatal density and trichome coverage of 14 *Banksia* species and *Dryandra praemorsa*. Species are ranked according to crypt depth in decreasing order i.e. the species with the deepest crypts is ranked 1. Data are means \pm s.e., n= 15, (3 leaves from 5 separate plants).

Rank	Species	Crypt Depth (μm)	Crypt Width (μm)	Leaf Thickness (μm)	Stomatal Density (mm^{-2})	Trichomes
1	<i>Banksia blechnifolia</i>	425 (± 12)	144 (± 7)	730 (± 24)	187 (± 8)	Crypt
2	<i>B. repens</i>	355 (± 12)	110 (± 6)	600 (± 19)	219 (± 9)	Crypt
3	<i>B. menziesii</i>	230 (± 9)	183 (± 7)	475 (± 12)	237 (± 10)	Surface & crypt
4	<i>B. prionotes</i>	225 (± 12)	175 (± 9)	400 (± 15)	243 (± 13)	Crypt
5	<i>B. caleyi</i>	215 (± 11)	154 (± 8)	505 (± 18)	144 (± 8)	Crypt
6	<i>B. baxteri</i>	175 (± 10)	148 (± 8)	510 (± 16)	147 (± 6)	Crypt
7	<i>B. media</i>	155 (± 7)	160 (± 8)	445 (± 15)	232 (± 10)	Crypt
8	<i>B. ashbyi</i>	150 (± 8)	395 (± 15)	375 (± 15)	283 (± 10)	Surface & crypt
9	<i>B. praemorsa</i>	150 (± 7)	173 (± 10)	405 (± 15)	205 (± 10)	Crypt
10	<i>B. robur</i>	125 (± 9)	273 (± 10)	325 (± 12)	251 (± 13)	Surface & crypt
11	<i>B. speciosa</i>	125 (± 8)	320 (± 13)	375 (± 14)	388 (± 14)	Surface & crypt
12	<i>B. grandis</i>	100 (± 6)	370 (± 13)	345 (± 13)	296 (± 11)	Surface & crypt
13	<i>B. marginata</i>	100 (± 8)	381 (± 15)	305 (± 16)	315 (± 14)	Surface & crypt
14	<i>B. spinulosa</i>	-	-	200 (± 11)	371 (± 15)	Surface
15	<i>Dryandra praemorsa</i>	-	-	355 (± 15)	340 (± 12)	Surface

There was a significant, positive relationship between the depth of crypts and leaf thickness ($r^2 = 0.87$, $P < 0.0001$; Fig. 5-2a). In contrast, there was a significant, negative relationship between stomatal density and leaf thickness ($r^2 = 0.44$, $P = 0.007$; Fig. 5-2b). There was also a significant, negative relationship between crypt depth and width ($r^2 = 0.45$, $P = 0.01$) (Data not shown).

Cuticular water loss

The rate of water loss from detached, darkened leaves of 8 *Banksia* species with different crypt depths was negatively correlated with crypt depth, over the 65 h for which measurements were made ($r^2 = 0.29$, $P = 0.009$; Fig. 5-3, slope 1). When *B. marginata*, which had very high rates of water loss, was excluded, the relationship was stronger ($r^2 = 0.52$, $P = 0.005$; Fig. 5-3, slope 2). Most of this loss would have occurred across the cuticle, as stomata should have been closed in the darkened conditions in which the experiment was conducted.

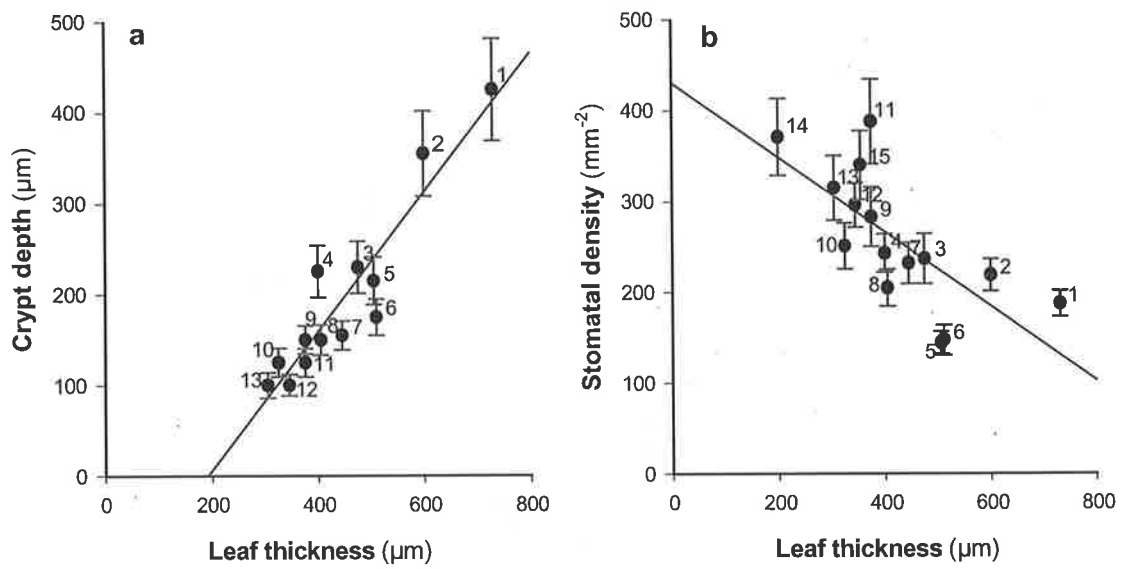


Figure 5.2. The relationship between leaf thickness (μm) and crypt depth (μm) in 13 *Banksia* species (a) and the relationship between leaf thickness (μm) and stomatal density (mm^{-2}) in 14 *Banksia* species and *Dryandra praemorsa* (b). Species are numbered as in Table 5.1. Data points are means \pm s.e., $n=15$, from 5 plants.

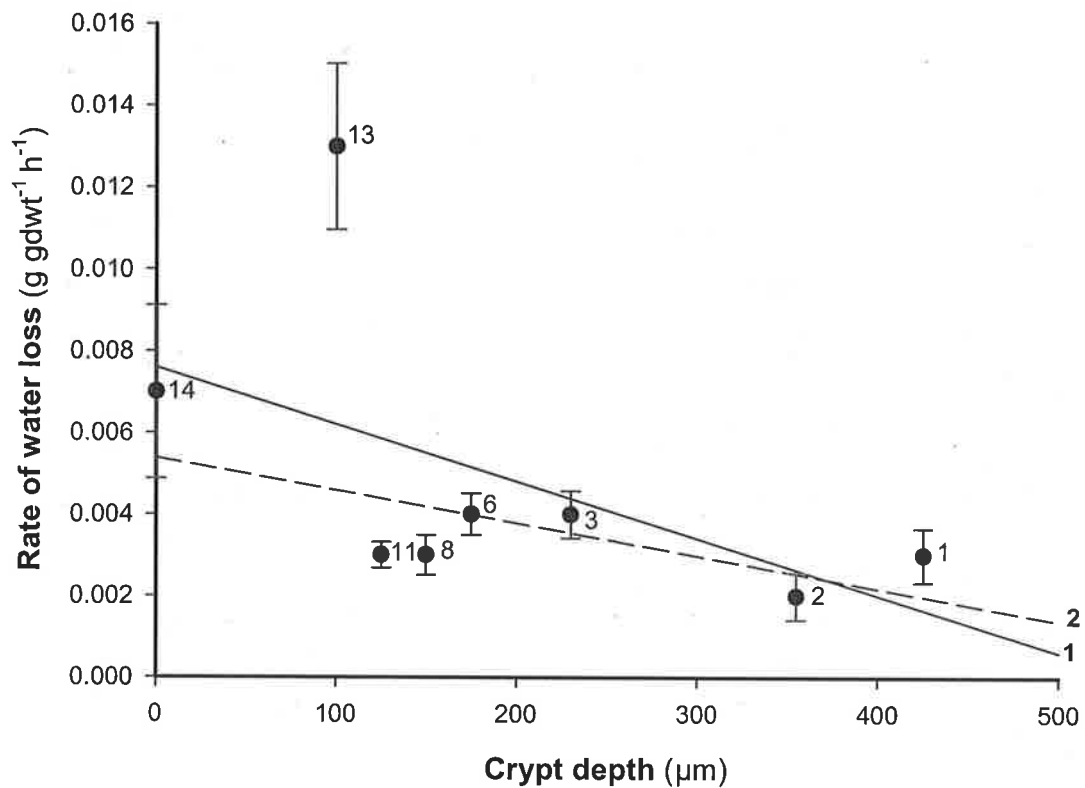


Figure 5.3. Cuticular water loss from detached, darkened leaves of 8 (slope 1) and 7 (slope 2) *Banksia* species with different crypt depths, including *B. blechnifolia* (1), *B. repens* (2), *B. menziesii* (3), *B. baxteri* (6), *B. praemorsa* (8), *B. speciosa* (11), *B. marginata* (13), and *B. spinulosa* (14). For slope 2, *B. marginata*, that had very high rates of water loss, was excluded. Data points are means \pm s.e., for each species $n=5$.

Gas exchange

Stomatal conductance increased in all species as VPD increased up to 14 mb. At higher VPDs stomatal conductance declined slightly in all species except *B. baxteri* and *B. repens*, (Fig. 5-4a). However, in none of the species were the reductions in stomatal conductance statistically significant. Transpiration increased with increasing VPD in all 15 species. However, after VPD approached approximately 17 mb, transpiration slowed and then plateaued for all species. *Banksia spinulosa* was the only species that showed a decline in transpiration when VPD reached about 19 mb, however, the reduction was not statistically significant ($P=0.69$; Fig. 5-4b). There was also a slight reduction in photosynthesis for all species after VPD approached 17 mb. However, these reductions were not statistically significant for any species (Fig. 5-4c).

There was no relationship between stomatal density and either maximum transpiration rate ($r^2=0.005$, $P=0.80$; Fig. 5-5a), or maximum stomatal conductance ($r^2=0.003$, $P=0.85$; Fig. 5-5b).

The relationship between crypt depth and transpiration at a VPD of 14 mb, where most of the species had their maximum stomatal conductance, was not statistically significant ($r^2=0.036$, $P=0.49$; Fig. 5-6a). There was also no significant relationship between VPD at which stomata began to close and crypt depth ($r^2=0.107$, $P=0.27$; Fig. 5-6b).

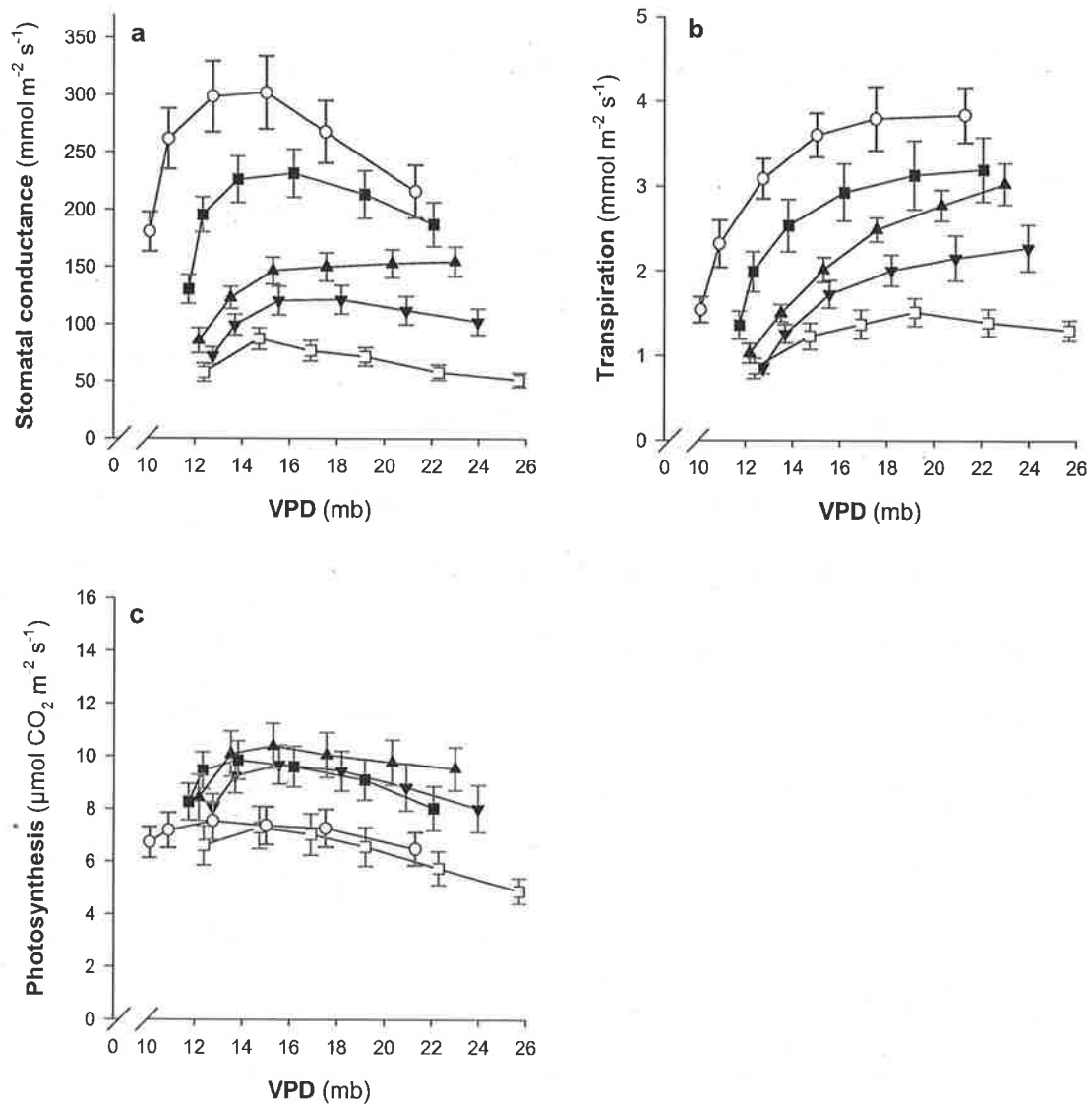


Figure 5.4. Representative responses of stomatal conductance (a), transpiration (b) and photosynthesis (c) to VPD (mb) in 5 of 15 study species. *Dryandra praemorsa* (↓), *B. marginata* (*), *B. repens* (⬛), *B. blechnifolia* (⤴) and *B. spinulosa* (♠). All 15 species were measured but only 5 are shown for clarity. These 5 species represent the full range of responses observed across the 15 experimental species. Data points are means \pm s.e., $n=5$.

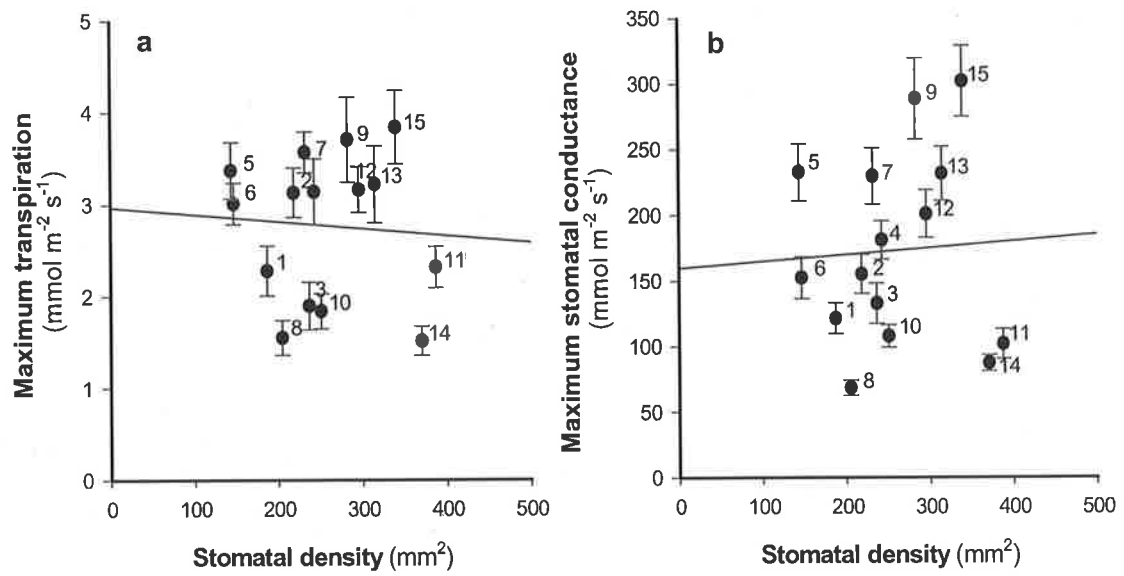


Figure 5.5. The relationship between stomatal density and maximum transpiration (a) and maximum stomatal conductance (b) of the 14 *Banksia* species and *Dryandra praemorsa*. Species are numbered as in Table 5.1. Data points are means \pm s.e., $n=5$.

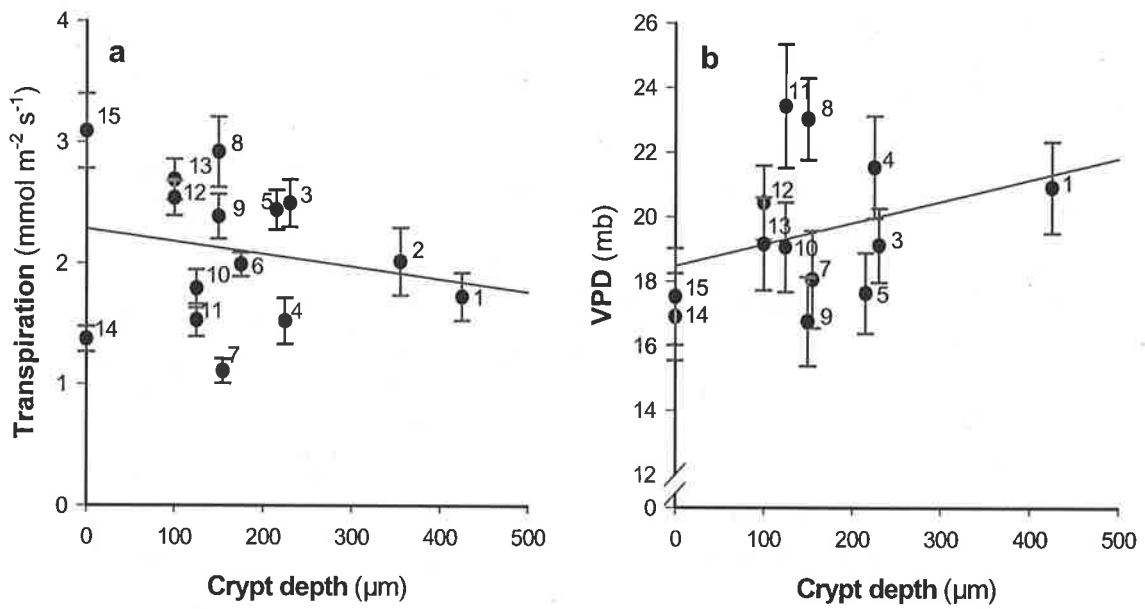


Figure 5.6. Relationships between crypt depth and transpiration rate at a VPD of 14 mb (a) and the VPD at which stomata began to close (b). Two species, *B. blechnifolia* and *D. praemorsa*, did not close their stomata at all in the face of increasing VPD and are not included in Fig. 5.6b. Species are numbered as in Table 5.1. Data points are means \pm s.e., $n=5$.

Discussion

Leaf morphology

The results of the present study showed that among 110 species of Proteaceae investigated, stomatal crypts occurred only in the genus *Banksia* (Appendix 1). The presence of stomatal crypts has been reported in a few other families e.g. Apocynaceae (*Nerium oleander*), Rhizophoraceae (mangrove taxa) (Das 2002) and Compositae (*Eupatorium bupleurifolium*) (Ragonese 1989). To my knowledge no study has been conducted to quantify the range of crypt depth across different plant species. However, Ragonese (1989) investigated the leaf anatomy of *Eupatorium bupleurifolium* and surprisingly found no differences in the crypt characteristics of the specimens collected from humid or in dry environments. The author came to the conclusion that crypts might not present a protective function for the stomata.

The negative relationship between leaf thickness and stomatal density found in this study for a range of *Banksia* species has also been reported in other species. Beerling and Kelly (1996) analysed data collected by Koerner *et al.* (1989) and found that there was a negative relationship between leaf thickness and abaxial stomatal density of 30 species from high altitude (3,000 m) in the Central Alps of Europe. The authors suggested that high light at these altitudes may be responsible for the thick leaves observed and may also affect distribution and density of stomata.

Cuticular Water loss

According to the literature, although cuticular transpiration accounts for 5 to 10% of total leaf transpiration (Kerstiens 1997; Taiz and Zeiger 2002), it can be

significant when drought stress is severe (Sanchez *et al.* 2001). Therefore, it is possible that stomatal crypts may help in reducing water loss even when stomata are closed. The results of this study support this hypothesis, because there was a negative relationship between the depth of crypts and the rate of water loss (Fig. 5-3 slope 1 and 2). Although the relationship was not very strong, detached leaves with deeper crypts such as *B. repens* had significantly lower cuticular water loss than leaves without crypts like *B. spinulosa* or leaves with shallower crypts such as *B. marginata*. Cross sectional views of stomatal crypts (Fig. 5-1c) showed that the epidermis surrounding stomata in crypts is much thinner than the epidermis outside crypts. Therefore, it is likely that leaves facing very high VPD and severe water deficit could decrease the rate of water loss from closed stomata by localizing stomata in crypts and also from the thinner epidermis inside the crypts. The unexpectedly higher cuticular water loss of *B. marginata*, which has shallow crypts, compared with *B. spinulosa* which lacks crypts might be related to the different anatomical characteristics of the leaves of these two species. They both have almost the same stomatal density, but trichome coverage on the abaxial leaf surfaces of *B. spinulosa* is denser than in *B. marginata*. Also, in *B. spinulosa* mesophyll cells are more densely packed and have more sclereids and smaller intercellular air spaces than in *B. marginata* (Appendix 3).

Gas exchange

It was expected that species with shallow crypts, or lacking crypts altogether, would have higher transpiration rates at a given VPD than species with deep crypts. It was also expected that stomata in species with shallow or no crypts would be more sensitive to increasing VPD than those in species with deep crypts. For example, I expected that at a given VPD increased, transpiration rates in *B.*

spinulosa and *Dryandra praemorsa*, which lack crypts, should be higher than other species which possess crypts. However, the results did not support my hypothesis. As VPD increased, all species showed almost the same pattern of response in transpiration. *B. blechnifolia* with the deepest crypts and longest diffusion pathway was expected to be less sensitive to increasing VPD, and indeed it did not close its stomata as VPD increased. However, *Dryandra praemorsa* did not close its stomata either, even though it lacked crypts and was expected to be more sensitive to increasing VPD. Thus, crypts appear to have little or no impact on water loss from open stomata in the 15 species studied.

There was no relationship between stomatal density and maximum transpiration rate or maximum stomatal conductance. This is in contrast to previous work that has shown a positive relationship between stomatal density and stomatal conductance (Muchow and Sinclair 1989; Awada *et al.* 2002). However, my findings support the results of Schurr *et al.* (2000) who found that assimilation and transpiration rates were not correlated with stomatal density in *Ricinus communis*.

Unexpectedly, despite the fact that both *B. spinulosa* and *Dryandra praemorsa* lacked stomatal crypts and had almost the same stomatal density and similar patterns of trichome coverage, they had significantly different maximum stomatal conductance and transpiration rates. *Banksia spinulosa* had the 2nd lowest stomatal conductance and the lowest maximum transpiration rate, while *Dryandra praemorsa* had the highest maximum stomatal conductance and transpiration rate of the 15 species studied.

Therefore, contrary to the assumption that stomatal crypts are an example of an adaptation that reduces water loss (Curtis and Barnes 1989; Campbell *et al.* 1999;

Taiz and Zeiger 2002), the results of this study showed that there was no evidence to support this assumption in the 15 species studied.

My results are consistent with the findings of Matthews (2003) who modeled the impact of crypts on gas exchange in three species, *Banksia media*, *B. baxteri* and *B. menziesii*, and concluded that crypts have a very small effect on reducing transpiration compared with the resistance of stomatal pores and the leaf boundary layer.

It has been reported that stomata respond directly to the rate of transpiration and not to the relative or absolute humidity (Mott and Parkhurst 1991). Thus, if stomatal crypts did reduce water loss at low ambient humidity, they might allow plants to keep their stomata open and maintain photosynthesis even in dry environments. However, any effect of stomatal crypts on water loss would also affect rates of CO₂ diffusion into leaves, probably canceling out any advantage in terms of photosynthesis. Besides, the results of this study do not support the idea that stomatal crypts can reduce transpiration rates. My results support the idea that the resistance created by stomata is the most important factor limiting water loss in dry environments (Gollan *et al.* 1985; Ogle and Reynolds 2002). Neither did the results of the present study suggest that in *Banksia* species increased boundary layer thickness in crypts acts to decrease transpiration or net photosynthesis.

What are crypts for?

The leaves of *Banksias* are characterised by thick cuticle and epidermis and tightly packed mesophyll, all of which probably increase resistances for CO₂ influx into leaves. In addition, as leaves become thicker, mesophyll resistance will increase further. The significant, positive relationship between the thickness of the leaves and the depth of crypts found in this study (Fig. 5-2a), suggests that

stomatal crypts might act as a pathway to deliver carbon dioxide into the interior of thick leaves. Thus, for very thick leaves stomatal crypts may help to overcome the significant mesophyll resistances to CO₂ diffusion, and as a consequence increase the availability of CO₂ to photosynthetic tissues.

On the other hand, it has been found that dust is capable of increasing transpiration through mechanically holding open the stomatal pore, thereby preventing it from closing to regulate water loss (Beasley 1942; Ricks and Williams 1974; Hirano *et al.* 1995). These results in an increased rate of transpiration, and in a plant already suffering from water stress, may lead to death. Trichomes have been shown to prevent dust from entering the pores of stomata in mangroves (Paling *et al.* 2001) and in *Dryandra praemorsa* and 4 *Banksia* species (Matthews 2003). However, the leaves of *Banksia* species can live up to 13 years (Witkowski *et al.* 1992), and even with trichome coverage there is a high probability of dust entering stomatal pores during the long lifetime of the leaves.

Conclusions

The current study demonstrated that crypts occurred in the epidermis of the *Banksia* species examined at different depths and widths but did not impact on gas diffusion through stomata. Contrary to my hypothesis, leaves with crypts had no significant extra resistance to gas diffusion compared with leaves that lacked crypts. However, the present results showed that deeper stomatal crypts did have significant impact on cuticular water loss compared with leaves that had shallower crypts or no crypts. This might benefit plants in arid environments facing very high VPD and severe water deficit by decreasing water loss when stomata are closed.

The positive relationship between leaf thickness and depth of crypts and the negative relationship between leaf thickness and stomatal density in *Banksia* species might suggest that stomatal crypts possibly act as a means of overcoming mesophyll resistance to CO₂ diffusion. Further studies are required to investigate this possibility.

6. Summary and conclusions

Epicuticular wax and gas exchange

The results of this study indicated that the position of wax on the leaf surface and the shape of wax crystals in *Leucadendron lanigerum*, a species from the Proteaceae family, are dependent on both the age of the leaves and the season. Generation and regeneration of epicuticular wax occurs mostly in spring on leaves that are less than 2 years old, while transformation of leaf surface wax crystals from the plate form into the flattened form, accompanied with more or less erosion of wax, occurred in winter. Epicuticular waxes decreased cuticular water loss but did not significantly increase leaf reflectance. Temperature of leaves with wax removed was lower than control leaves. Thus, epicuticular wax in *L. lanigerum* is more important for reducing water loss than for decreasing reflectance of light and keeping leaves cool.

In addition, the wax coverage at the entrance of Florin rings in *L. lanigerum*, increased the resistance to gas diffusion, resulting in lower stomatal conductance, transpiration and photosynthesis than for leaves without wax. Removal of wax, prior to exposure to high light, resulted in increased photoinhibition relative to leaves with wax. The reduction of photoinhibition provided by the epicuticular wax layer may be an advantage for these plants in high light environments.

Therefore, the results of the current study indicated that epicuticular waxes in *L. lanigerum* reduce cuticular water loss and leaf transpiration rates, consequently increasing water use efficiency. Despite being unable to detect an effect of wax on reflectance, there was evidence that wax can reduce photoinhibition in *L. lanigerum*,

and so could benefit plants living in arid environments with high solar radiation. This is supported by the findings that seasonal accumulation of wax coincided with spring and summer in *L. lanigerum* when radiation is highest.

Stomatal plugs and gas exchange

Functions suggested for stomatal plugs (occlusion of the stomatal antechamber by wax or cutin) include: reducing water loss, protecting against insects and fungi, preventing entry of water into stomatal pores and preventing the formation of a water film on leaves. By removing plugs experimentally, I was able to investigate their impact on gas-exchange, cuticular water loss and water film formation in the rain forest tree, *Agathis robusta*.

The results indicated that under saturating PFD, leaves with plugs had significantly lower transpiration rates, stomatal conductance and photosynthetic rates, but higher leaf temperatures than unplugged leaves. Maximum photosynthetic rates occurred at 30°C for plugged leaves and 25°C for leaves without plugs; possibly because the higher transpiration rate of the unplugged leaves induced early stomatal closure. At temperatures above 30°C, rates of photosynthesis were the same for plugged and unplugged leaves, but transpiration rates were lower for the former, resulting in higher instantaneous water use efficiency.

Cuticular water loss, measured gravimetrically over 55 h in a dark room, was significantly greater in unplugged than plugged leaves. In contrast, plugs had no impact on water film formation and wet leaves of plugged and unplugged leaves had similar electron transport rates, as measured by chlorophyll fluorescence. However,

it is possible that leaves exposed to precipitation or misting for periods longer than 10 minutes, could be affected by water entering unprotected stomata.

Stomatal plugs and fungal invasion

Protecting stomata against fungal invasion is another function that has been attributed to stomatal plugs. The results of this study indicated that the establishment of plugs occurs annually, and unlike trichomes, stomatal plugs can be replaced at least for the first two years of a leaf's life. Leaves that are more than 2 years old, failed to produce a complete wax plug.

The investigation of leaves infected by fungi showed that when hyphae did attempt to penetrate the leaf tissue through stomata, waxy plugs always blocked them. Hyphae penetrated the leaf tissue either through stomata that lacked waxy plugs or, at later stages in infection, directly through the cuticle. My results suggest that stomatal plugs in *A. robusta* do present a significant barrier against fungal penetration through stomata at least in leaves less than 2 years old, and so prevent the infection of leaves by fungi. Therefore, this function of stomatal plugs could be critical for the trees living in rainforest environments when the chance of fungal invasion is high.

Moreover, the water proofing ability of waxy plugs could ease the removal of fungal spores from the entrance of stomata when exposed to wind or precipitation. Hence, I believe that waxy plugs in addition to blocking hyphal penetration through stomata, can also create a water proofing situation over the stomata disrupting primary attachment and displacing fungal spores or other particles.

According to the results, stomatal plugs in *Agathis robusta* do present a significant barrier to water loss. Therefore, even though stomatal plugs mostly occur in plants living in rainforest environments that rarely face a restriction of water availability, I believe that this property could have multiple functions. The functions could include decreasing water loss, preventing penetration of water into stomatal pores in rainforest habitats and also reducing fungal penetration through stomatal pores in rainforest trees with abundant opportunity for fungal invasion. In regard to the oily property of wax plugs, removing or displacing of particles from the entrance of stomata could be another function of stomatal plugs that plants could face in many environments.

Stomatal crypts and gas exchange

Stomatal crypts are among the most frequently cited examples of an adaptation that reduces water loss. Numerous assumptions exist in the literature suggesting that the structure of crypts increases the diffusion path length and as a consequence the resistance to diffusion of gases.

The results of this study indicated that maximum stomatal conductance, transpiration and photosynthesis were not related to either stomatal density or the width or depth of crypts in the *Banksia* species used. However, stomatal crypts did impact on cuticular water loss of *Banksia* species when stomata were closed. This can be important for plants living in arid environments facing severe water deficit to decrease the loss of water from the epidermis when stomata are closed. Contrary to my hypothesis, the data indicated that stomatal crypts do not significantly increase resistance to gas

diffusion. Instead, crypts may facilitate transfer of CO₂ to the photosynthetic tissues of thick leaves. This idea is supported by the significant positive relationship that was found between crypt depth and leaf thickness in the *Banksia* species used in this study. An alternative function could be to prevent particles into stomatal pores that could affect the physiological activity of leaves, by preventing pores from closing or by increasing resistance to diffusion. Plants in arid zones, where there is an abundance of dust, might particularly benefit from such a filtering function.

In conclusion, the exact functions of crypts are not clear yet, and more research needs to be done to illuminate their function in different environments and species.

7. References

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Appendices

1. Plant materials

To investigate different morphological characteristics of leaves, 126 species from the families Proteaceae, Araucariaceae, Atherospermataceae, Crassulaceae, Winteraceae and Podocarpaceae grown in the Adelaide, Wittunga and Mount Lofty Botanic Gardens, Australia were examined and detailed. Leaf surfaces were examined using Scanning Electron Microscopy (SEM). Internal leaf structure and stomatal morphology were also assessed from cross sections using light microscopy.

The results were used to select suitable species for testing the hypotheses outlined in this thesis. Stomatal plugs were observed in the families Araucariaceae, Winteraceae and Podocarpaceae; Florin rings in the families of Araucariaceae, Atherospermataceae, Podocarpaceae and Proteaceae, and stomatal crypts just in the family Proteaceae (*Banksia* species). Trichomes occurred only on the leaf surfaces of species from the families Atherospermataceae and Proteaceae (Table 1).

Table 1. Results of a morphological survey of leaf surface characteristics of 126 species from 7 families.

	Species	Family	Abaxial Stomata	Adaxial Stomata	Abaxial Trichome	Adaxial Trichome	Stomatal Plugs	Stomatal Crypts	Florin Rings
1	<i>Adenanthos obovatus</i>	Proteaceae	+	+	-	-	-	-	-
2	<i>Agathis atropurpurea</i>	Araucariaceae	+	-	-	-	+	-	+
3	<i>A. australis</i>	"	+	-	-	-	+	-	+
4	<i>A. robusta</i>	"	+	-	-	-	+	-	+
6	<i>Araucaria araucana</i>	"	+	-	-	-	+	-	-
7	<i>A. bidwillii</i>	"	+	-	-	-	+	-	-
8	<i>Atherosperma moschatum</i>	Atherospermataceae	+	-	+	+	-	-	-
9	<i>Aulax cancellata</i>	Proteaceae	+	+	-	-	-	-	+
10	<i>Banksia ashbyi</i>	"	+	-	+	+	-	+	-
11	<i>B. baxteri</i>	"	+	-	+	+	-	+	-
12	<i>B. blechnifolia</i>	"	+	-	+	+	-	+	-
13	<i>B. caleyi</i>	"	+	-	+	+	-	+	-
14	<i>B. coccinea</i>	"	+	-	+	+	-	+	-
15	<i>B. gardneri</i>	"	+	-	+	+	-	+	-
16	<i>B. grandis</i>	"	+	-	+	+	-	+	-
17	<i>B. integrifolia</i>	"	+	-	+	+	-	-	-
18	<i>B. marginata</i>	"	+	-	+	+	-	+	-
19	<i>B. media</i>	"	+	-	+	+	-	+	-
20	<i>B. menziesii</i>	"	+	-	+	+	-	+	-
21	<i>B. occidentalis</i>	"	+	-	+	+	-	-	-

	Species	Family	Abaxial Stomata	Adaxial Stomata	Abaxial Trichome	Adaxial Trichome	Stomatal Plugs	Stomatal Crypts	Florin Rings
22	<i>B. ornata</i>	"	+	-	+	+	-	+	-
23	<i>B. paludosa</i>	"	+	-	+	+	-	+	-
24	<i>B. pilostylis</i>	"	+	-	+	+	-	+	-
25	<i>B. plagiocarpa</i>	"	+	-	+	+	-	+	-
26	<i>B. praemorsa</i>	"	+	-	+	+	-	+	-
27	<i>B. Prionotes</i>	"	+	-	+	+	-	+	-
28	<i>B. repens</i>	"	+	-	+	+	-	+	-
29	<i>B. robur</i>	"	+	-	+	+	-	+	-
30	<i>B. saxicola</i>	"	+	-	+	+	-	+	-
31	<i>B. serrata</i>	"	+	-	+	+	-	+	-
32	<i>B. spinulosa</i>	"	+	-	+	+	-	-	-
33	<i>B. speciosa</i>	"	+	-	+	+	-	+	-
34	<i>Bellendenia montana</i>	"	+	+	-	-	-	-	+
35	<i>Buckinghamia celsissima</i>	"	+	-	+	+	-	-	+
36	<i>Cenarrhenes nitida</i>	"	+	-	-	-	-	-	+
37	<i>Cotyledon orbiculata</i>	Crassulaceae	+	+	-	-	-	-	-
38	<i>Drimys lanceolata</i>	Winteraceae	+	-	-	-	+	-	-
39	<i>D. winteri</i>	"	+	-	-	-	+	-	-
40	<i>Embothrium coccineum</i>	Proteaceae	+	-	-	-	-	-	-
41	<i>Grevillea apricot glow</i>	"	+	-	+	+	-	-	-
42	<i>G. aquifolium</i>	"	+	-	+	+	-	-	-

	Species	Family	Abaxial Stomata	Adaxial Stomata	Abaxial Trichome	Adaxial Trichome	Stomatal Plugs	Stomatal Crypts	Florin Rings
43	<i>G. arenaria</i>	"	+	-	+	+	-	-	-
44	<i>G. brachystylis</i>	"	+	-	+	+	-	-	-
45	<i>G. bronz rambler</i>	"	+	-	+	+	-	-	-
46	<i>G. coconut ice</i>	"	+	-	+	+	-	-	-
47	<i>G. hilliana</i>	"	+	-	+	+	-	-	-
48	<i>G. ilicifolia</i>	"	+	-	+	-	-	-	-
49	<i>G. lady</i>	"	+	-	+	+	-	-	-
50	<i>G. merylesii</i>	"	+	+	+	+	-	-	-
51	<i>G. nedkelly</i>	"	+	-	+	+	-	-	-
52	<i>G. poorinda</i>	"	+	-	+	+	-	-	-
53	<i>G. robin gordon</i>	"	+	-	+	+	-	-	-
54	<i>G. robusta</i>	"	+	-	+	+	-	-	-
55	<i>G. shiressii</i>	"	+	-	-	-	-	-	-
56	<i>G. speciosa</i>	"	+	-	+	+	-	-	-
57	<i>G. splendour</i>	"	+	-	+	-	-	-	-
58	<i>G. thelemanniana</i>	"	+	+	+	-	-	-	-
59	<i>G. vestita</i>	"	+	-	+	+	-	-	-
60	<i>G. victoria</i>	"	+	-	+	+	-	-	-
61	<i>Hakea ambigua</i>	"	+	+	-	-	-	-	+
62	<i>H. burrendong beauty</i>	"	+	+	+	-	-	-	+
63	<i>H. carinata</i>	"	+	+	+	+	-	-	+

	Species	Family	Abaxial Stomata	Adaxial Stomata	Abaxial Trichome	Adaxial Trichome	Stomatal Plugs	Stomatal Crypts	Florin Rings
64	<i>H. cristata</i>	"	+	+	-	-	-	-	+
65	<i>H. cucullata</i>	"	+	+	+	+	-	-	+
66	<i>H. dactyloides</i>	"	+	+	+	+	-	-	-
67	<i>H. drupacea</i>	"	+	+	-	-	-	-	-
68	<i>H. elliptica</i>	"	+	+	+	+	-	-	-
69	<i>H. eriantha</i>	"	+	+	+	-	-	-	-
70	<i>H. flabellifolia</i>	"	+	+	+	-	-	-	+
71	<i>H. florida</i>	"	+	+	+	+	-	-	+
72	<i>H. francisiana</i>	"	+	+	+	+	-	-	-
73	<i>H. grammatophylla</i>	"	+	+	+	+	-	-	-
74	<i>H. laurina</i>	"	+	+	+	+	-	-	-
75	<i>H. marginata</i>	"	+	+	-	+	-	-	+
76	<i>H. multilineata</i>	"	+	+	-	-	-	-	-
77	<i>H. myrtoides</i>	"	+	+	+	+	-	-	+
78	<i>H. nitida</i>	"	+	+	-	-	-	-	+
79	<i>H. pandanicarpa</i>	"	+	+	+	+	-	-	-
80	<i>H. petiolaris</i>	"	+	+	+	+	-	-	+
81	<i>H. Salicifolia</i>	"	+	+	-	-	-	-	-
82	<i>H. victoria</i>	"	+	+	-	-	-	-	+
83	<i>Hicksbeachia pinnatifolia</i>	"	+	-	+	+	-	-	+
84	<i>Lambertia inermis</i>	"	+	-	+	+	-	-	-

	Species	Family	Abaxial Stomata	Adaxial Stomata	Abaxial Trichome	Adaxial Trichome	Stomatal Plugs	Stomatal Crypts	Florin Rings
85	<i>Laurelia sempervirens</i>	Atherospermataceae	+	-	-	-	-	-	+
86	<i>Leucadendron argenteum</i>	Proteaceae	+	+	+	+	-	-	+
87	<i>L. cordifolium</i>	"	+	+	+	+	-	-	+
88	<i>L. lanigerum</i>	"	+	+	-	-	-	-	+
89	<i>L. linifolium</i>	"	+	+	+	+	-	-	+
90	<i>L. macowanii</i>	"	+	+	-	-	-	-	+
91	<i>L. microcephalum</i>	"	+	+	+	+	-	-	+
92	<i>L. muirii</i>	"	+	+	-	-	-	-	+
93	<i>L. praecox</i>	"	+	+	+	+	-	-	+
94	<i>L. salignum</i>	"	+	+	-	-	-	-	+
95	<i>L. safari sunset</i>	"	+	+	-	-	-	-	+
96	<i>Lomatia arborescens</i>	"	+	-	+	+	-	-	+
97	<i>L. dentata</i>	"	+	-	+	+	-	-	+
98	<i>L. fraseri</i>	"	+	-	+	+	-	-	+
99	<i>L. frruginea</i>	"	+	-	+	-	-	-	+
100	<i>L. polymorpha</i>	"	+	-	+	-	-	-	+
101	<i>Macadamia tetraphyla</i>	"	+	-	-	-	-	-	-
102	<i>Orites excelsa</i>	"	+	-	+	-	-	-	-
103	<i>O. lancifolia</i>	"	+	-	-	-	-	-	-
104	<i>Persoonia attenuata</i>	"	+	-	-	-	-	-	+
105	<i>P. gunnii</i>	"	+	+	-	-	-	-	-

	Species	Family	Abaxial Stomata	Adaxial Stomata	Abaxial Trichome	Adaxial Trichome	Stomatal Plugs	Stomatal Crypts	Florin Rings
106	<i>Phyllocladus aspleniifolius</i>	Podocarpaceae	+	-	-	-	+	-	-
107	<i>Ph. trichomanoides</i>	"	+	-	-	-	+	-	-
108	<i>Podocarpus chinensis</i>	"	+	-	-	-	+	-	+
109	<i>P. elatus</i>	"	+	-	-	-	+	-	+
118	<i>Prumnopitys ladei</i>	"	+	+	-	-	+	-	+
110	<i>Protea aurea</i>	Proteaceae	+	+	-	-	-	-	+
111	<i>P. cynaroides</i>	"	+	+	-	-	-	-	+
112	<i>P. mundii</i>	"	+	+	-	-	-	-	+
113	<i>P. nerifolia</i>	"	+	+	-	-	-	-	+
114	<i>P. obtusifolia</i>	"	+	+	+	+	-	-	+
115	<i>P. Pink Ice</i>	"	+	+	-	-	-	-	+
116	<i>P. repens</i>	"	+	+	-	-	-	-	+
117	<i>P. susannea</i>	"	+	+	+	+	-	-	+
119	<i>Stenocarpus salignus</i>	"	+	-	+	-	-	-	+
120	<i>S. sinuatus</i>	"	+	-	-	-	-	-	-
121	<i>Tasmania lanceolata</i>	Winteraceae	+	-	-	-	+	-	-
122	<i>Telopea mongaensis</i>	Proteaceae	+	-	-	-	-	-	+
123	<i>T. oreades</i>	"	+	-	-	-	-	-	+
124	<i>T. speciosissima</i>	"	+	-	+	-	-	-	+
125	<i>T. truncate</i>	"	+	-	-	-	-	-	+
126	<i>Triunia youngiana</i>	"	+	-	+	-	-	-	+

2. Seasonal modification of leaf trichome density

I hypothesized that for plants to optimize their photosynthetic activity and decrease transpiration rate, they may increase the number of trichomes in summer and decrease them in winter, assuming that trichomes affect leaf reflectance. To test this hypothesis, 19 species from the families of Atherospermataceae and Proteaceae growing in the Adelaide, Wittunga and Mount Lofty Botanic Gardens, Australia were examined. Trichome density of the adaxial (Ad) and abaxial (Ab) leaf surfaces was quantified in the middle of each season has been on micrographs using SEM. Five, fully expanded, north facing leaves were used for each species. The results of the seasonal modification of the leaf trichome densities failed to support my hypothesis. In only 5 species (*Atherosperma moschatum*, *Grevillea vestita*, *Hakea eriantha*, *H. myrtoides* and *H. pandanicarpa*) an increase in trichome frequency observed on the adaxial and abaxial leaf surfaces from spring to summer. However, the increases were not significant. On the other hand, 10 species decreased trichome density on the adaxial and abaxial leaf surfaces from spring to summer. No species significantly increased the number of trichomes on the adaxial and abaxial leaf surfaces from winter to summer (Table 2).

Therefore, the present study showed that none of these species tested increased the density of trichomes in summer and decreased them in winter. It seems that trichomes are produced during leaf expansion, which occurs mostly in spring, and after that no more trichomes are formed. Neither did any of the species significantly lose trichomes from spring to winter. Even when leaves formed in summer, this survey found that the trichome density of these leaves was not significantly different from leaves in other seasons. My results are contrary to

those of Ehleringer *et al.* [, 1976 #835] who reported that *Encelia farinosa* responds to an increase in temperature and aridity during the growing season by producing new leaves which are more pubescent than earlier ones. However, since Ehleringer [, 1978 #887] did not count the number of trichomes on the leaf surfaces of *E. farinosa*, there is some doubt about the conclusion. Because of the lack of quantitative assessment they could not determine whether there was an increase in hair density or whether the hairs were just much longer and gave the appearance of an increase in the leaf trichome frequency.

Table 2. Seasonal trichome frequency of 19 species from the families of Atherospermataceae and Proteaceae.

Species		Autumn (mm ⁻²)	Winter (mm ⁻²)	Spring (mm ⁻²)	Summer (mm ⁻²)	
1	<i>Atherosperma moschatum</i>	Ad	10	2	6	7
		Ab	118	66	101	104
2	<i>Buckinghamia celsissima</i>	Ad	5	0	9	1
		Ab	217	189	285	234
3	<i>Grevillea aquifolium</i>	Ad	38	0	7	5
		Ab	56	39	56	54
4	<i>G. vestita</i>	Ad	51	59	49	51
		Ab	57	53	55	57
5	<i>G. victoria</i>	Ad	1	1	0	1
		Ab	34	34	35	24
6	<i>Hakea eriantha</i>	Ad	2	1	0	1
		Ab	4	2	2	4
7	<i>H. francisiana</i>	Ad	11	9	19	5
		Ab	11	7	17	5
8	<i>H. myrtoides</i>	Ad	8	1	1	3
		Ab	9	4	1	7
9	<i>H. pandanycarpa</i>	Ad	2	1	1	3
		Ab	1	1	3	4
10	<i>H. petiolaris</i>	Ad	11	9	9	3
		Ab	16	8	33	6
11	<i>Hicksbeachia pinnatifolia</i>	Ad	0	1	3	1
		Ab	8	11	9	6
12	<i>Leucadendron cordifolium</i>	Ad	9	4	2	6
		Ab	44	45	49	36

Species		Autumn (mm ²)	Winter (mm ²)	Spring (mm ²)	Summer (mm ²)
13	<i>L. linifolium</i>	Ad	2	1	1
		Ab	35	16	24
14	<i>L. microcephalum</i>	Ad	7	4	18
		Ab	9	7	27
15	<i>Lomatia arborescens</i>	Ad	5	7	8
		Ab	3	3	3
16	<i>L. dentata</i>	Ad	7	4	7
		Ab	13	12	11
17	<i>L. fraseri</i>	Ad	4	1	2
		Ab	152	171	246
18	<i>Orites excelsa</i>	Ad	0	0	0
		Ab	127	150	189
19	<i>Stenocarpus salignus</i>	Ad	0	0	0
		Ab	1	1	3

3. Leaf structure of two *Banksia* species

The unexpectedly higher cuticular water loss of *B. marginata* that had shallow crypts (100 μm) compared with *B. spinulosa* that lacked crypts might be related to differences in anatomical characteristics of the leaves of these two species. Although they both have almost the same stomatal density, trichome coverage on the abaxial leaf surfaces of *B. spinulosa* is denser than on *B. marginata*. Also, in *B. spinulosa* mesophyll cells are more densely packed and have more sclereid cells and less intercellular air spaces than in *B. marginata* (Fig. 1, 2). The high cuticular water loss of *B. marginata* compared with *B. spinulosa* could also be related to differences in the vasculature of their leaves. However, I did not find any quantitative difference in the vascular bundles between these leaves. I did not investigate qualitative differences that might reflect the capacity of their xylem cells to transfer water throughout the leaves.

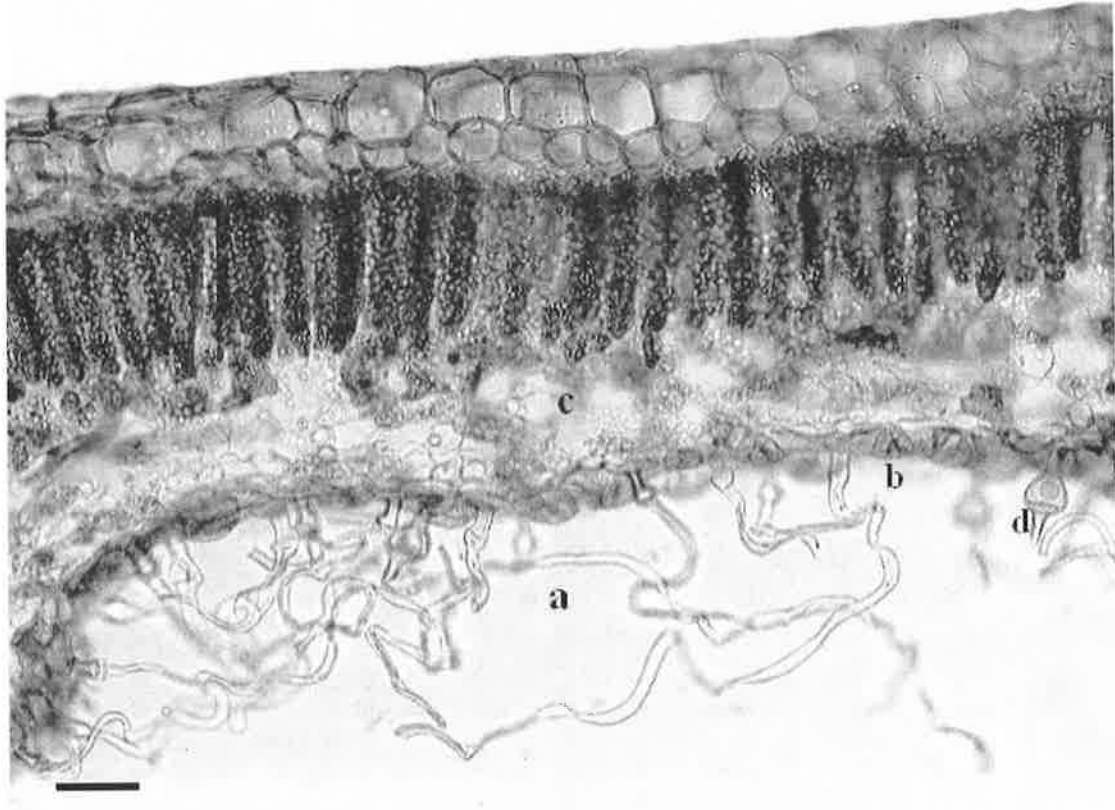


Fig. 1. Cross-sectional view of a leaf of *B. marginata* (showing a shallow crypt (a) including stomata (b), intercellular airspaces (c) and trichome (d). Scale bar is 50 μ m.

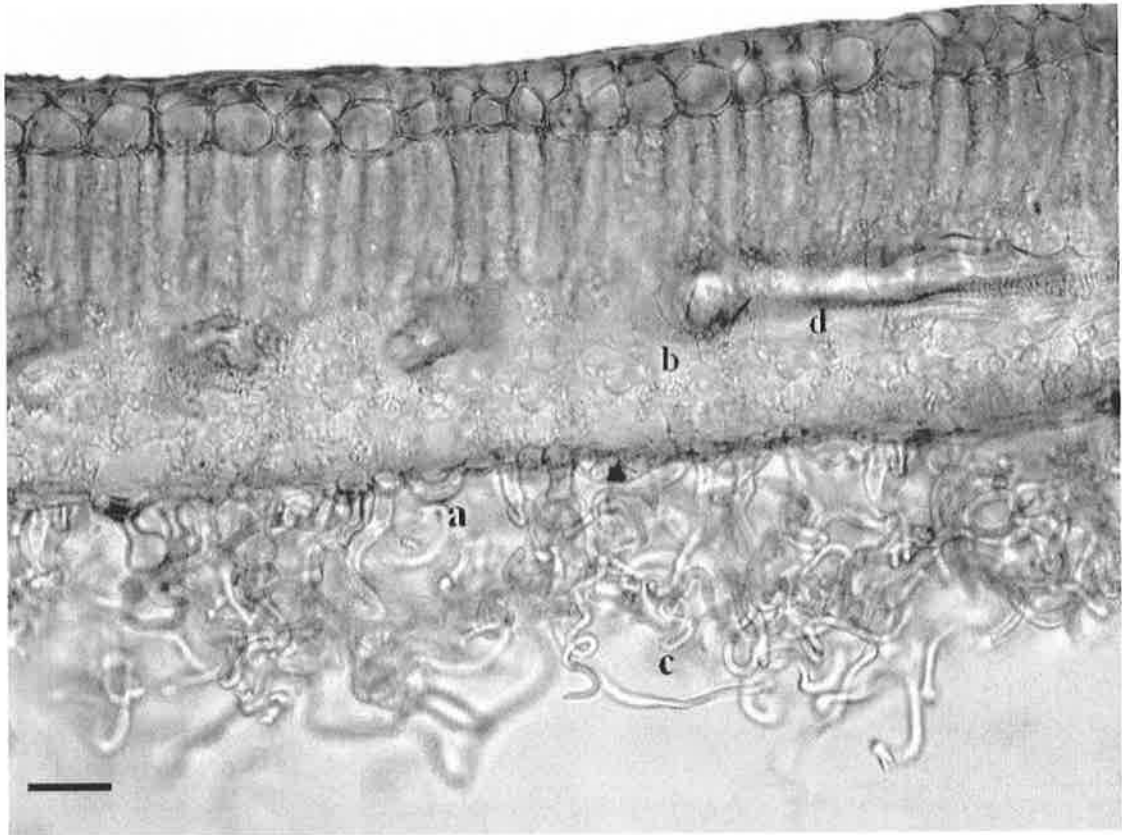


Fig. 2. Cross-sectional view of a leaf of *B. spinulosa* (showing stomata (a), intercellular airspaces (b), trichome (c) and sclereid (d). Scale bar is 50 μ m.