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DO WAXY STOMATAL PLUGS IMPACT LEAF GAS EXCHANGE IN A RAIN FOREST GYMNOSPERM *AGATHIS ROBUSTA*?

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Summary. Functions suggested for stomatal plugs (occlusion of the stomatal pore by wax or cutin) include: reduction of water loss, protection 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, we investigated their impact on gas exchange, cuticular water loss and water film formation in the rain forest tree, *Agathis robusta*. The results showed that under saturating PFD, 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 grow, 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 affect the formation of a water film on leaf surfaces of *A. robusta*. This result was again in contrast

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to the results reported for *D. winteri* (Feild et al., 1998). Stomatal plugs increased water use efficiency and decreased water loss from leaves. However, an unavoidable consequence of decreased water loss is that CO_2 assimilation is also limited because of increased resistance to diffusion of CO_2 into leaves. Meanwhile, possessing stomatal plugs might be a disadvantage in hot environments, as our results showed that stomatal plugs can increase leaf temperature through their impact on evaporative cooling of leaves.

***Key words*:** *Agathis robusta*, stomatal plug, water film, transpiration, photosynthesis.

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; Kerstiens, 2006).

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, 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. Firstly, as stomata are the main diffusion pathway for both water and CO_2, it is inevitable that any feature that decreases the rate of water loss will also reduce CO_2 diffusion into leaves, potentially limiting photosynthesis. Secondly, 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 suggest that since CO_2 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 the light of Feild et al.'s (1998) paper and using the same technique to remove plugs from leaves, we investigated the impact of waxy stomatal plugs on leaf gas-exchange (both across the cuticle and through stomata), leaf temperature and the development of water films in another rainforest species, *Agathis robusta*.

MATERIALS AND METHODS

Plant material

All experiments were conducted with 2-year-old and 50-cm tall seedlings of *Agathis robusta* (Araucariaceae). *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. During the study, daily average maximum photon flux density (PFD) was 1450 µmol quanta $m^{-2}s^{-1}$, 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 min every 3 days.

Electron and light microscopy

Micromorphology of leaf surfaces was investigated using a scanning electron microscopy (SEM). Leaves were cut in ~1 cm^2 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 sec and examined using a light microscope at 400X magnification.

Removal of somatal 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 Lendzian, 1981; Prugel et al., 1994) as changing mass over a 55-h period in a dark room. The temperature and humidity in the 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 μmol quanta $m^{-2}s^{-1}$, 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.

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 as well as 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. 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. 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. 1c).

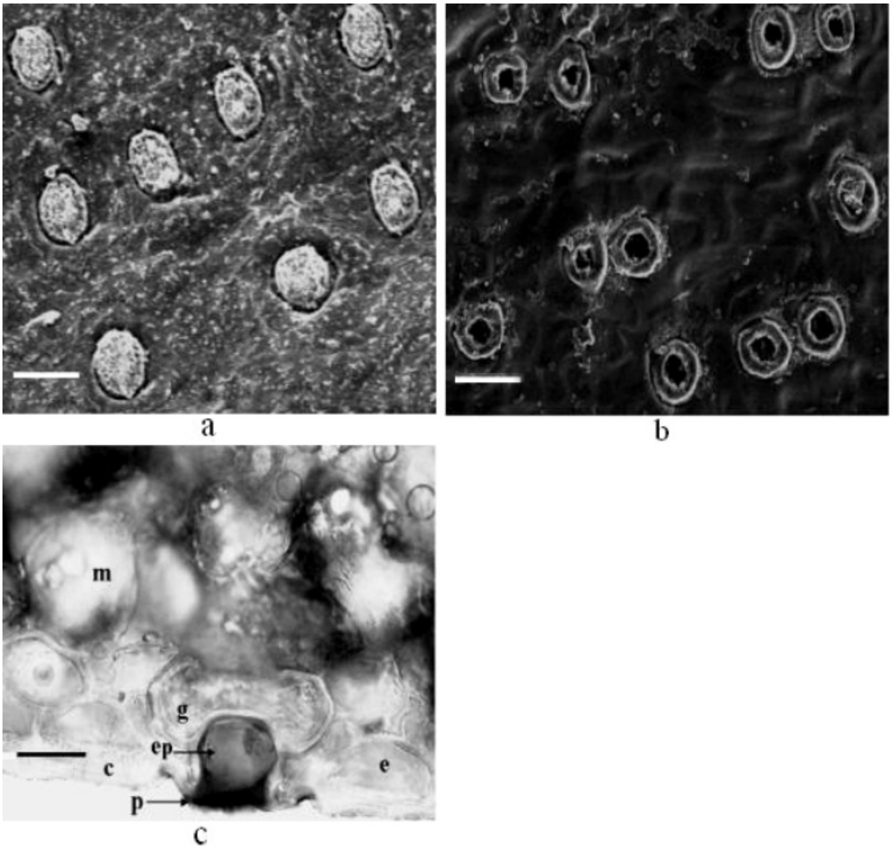

Fig. 1. SEM of the abaxial leaf surface of *Agathis robusta* with stomatal plugs (a) and after removal of stomatal plugs (b). Light micrograph of the cross sectional view of a stoma of *Agathis robusta* (c); mesophyll (m), guard cell (g), epistomatal chamber (ep), stomatal plug (p), epidermal cell (e) 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 period during which measurements were made (Fig. 2; P= 0.0001). Most of this loss would have occurred across the cuticle, as stomata should

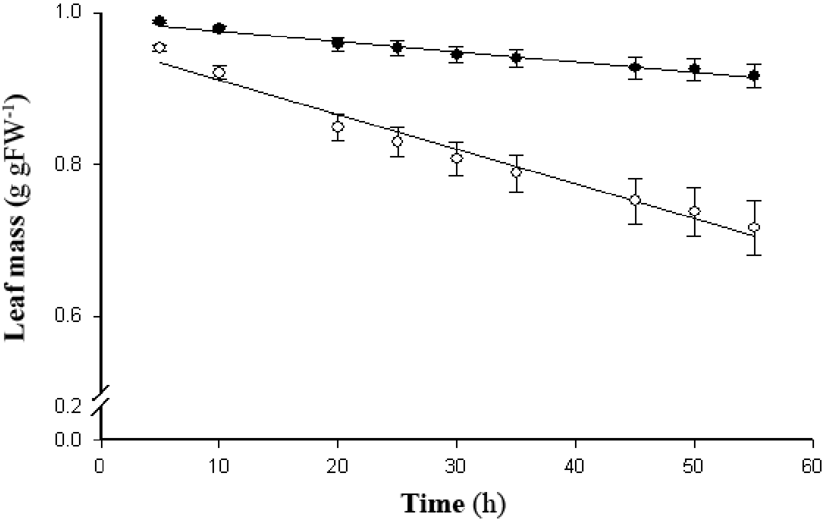

Fig. 2. The rates of water loss of detached leaves of *A. robusta* with (●) and without (○) wax, measured gravimetrically over 55 h in a dark room. Data points are means ± SE (n=10). Linear regressions were calculated using Sigma Plot.

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 µmol quanta m^{-2} s^{-1} and leaf temperature of 26 ºC was significantly higher in leaves without plugs than in control leaves (Fig. 3a; $P<0.001$), confirming that stomatal plugs decrease the rate of water loss from *A. robusta* leaves. Photosynthetic

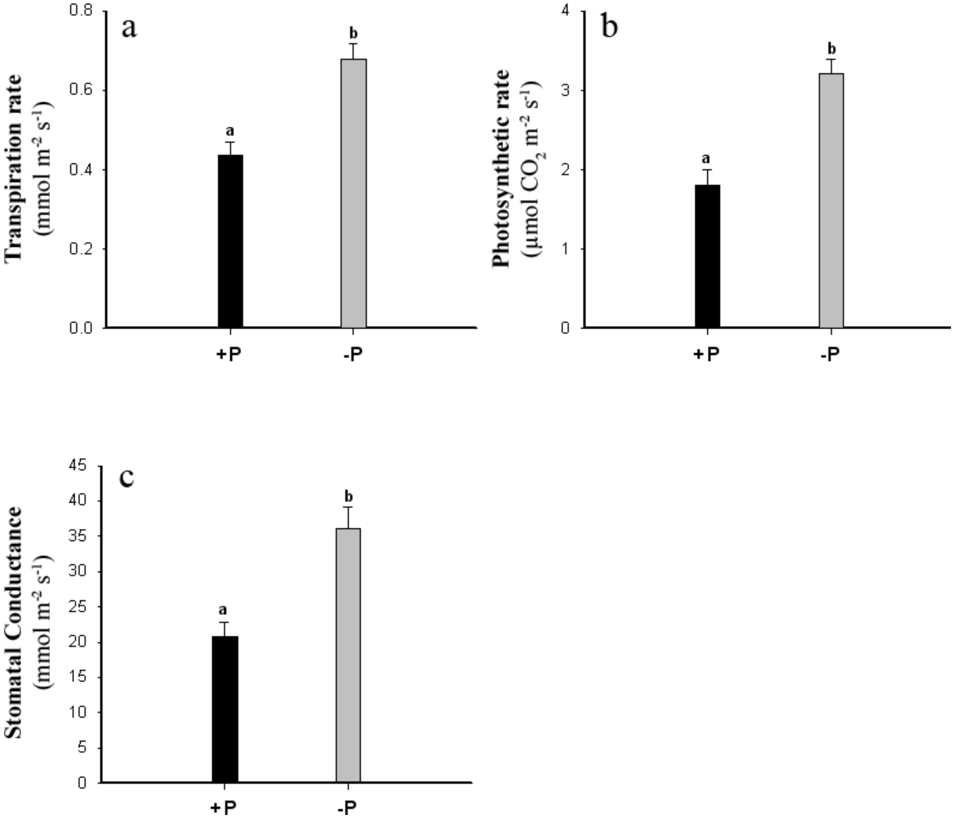

Fig. 3. Transpiration rates (a), photosynthesis (b) and stomatal conductance (c) of the leaves of *A. robusta* at saturating PFD with (■) and without (□) stomatal plugs. Letters a and b above bars indicate that there was significant difference between control and treatments. Data points are means ± SE (n=10).

rate was also significantly higher in leaves from which plugs had been removed compared with control leaves (Fig. 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. 3c; $P<0.001$).

Transpiration rates of leaves without waxy plugs were significantly higher compared with control leaves at all temperatures between 15-40 ^{o}C

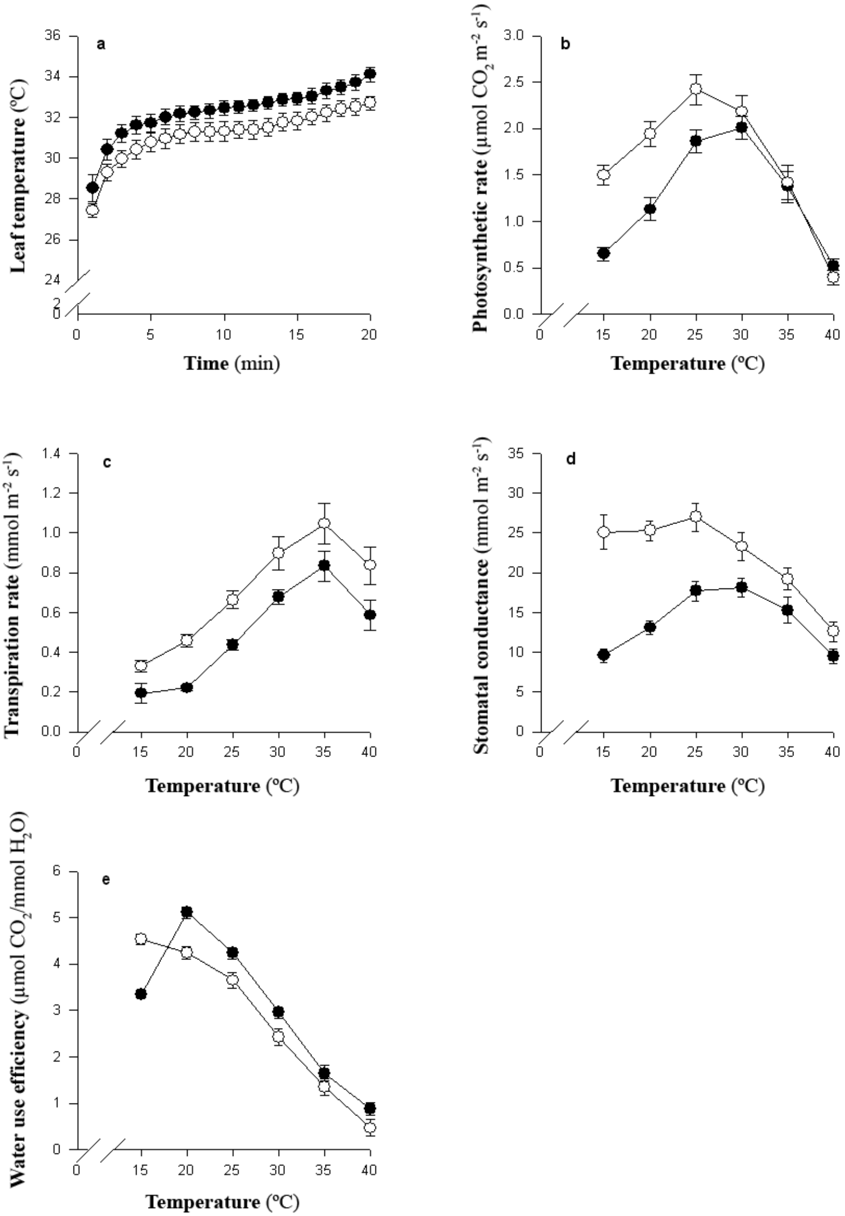

Fig. 4. Temperature (a) of the leaves of *A. robusta* with (●) and without (○) stomatal plugs during 20 min chlorophyll fluorescence measurements. Photosynthetic rates (b), transpiration rates (c), stomatal conductance (d) and water use efficiency (e) of the leaves of *A. robusta* with (●) and without (○) stomatal plugs at leaf temperatures from 15 to 40 °C. Data points are means ± SE, (n=10).

(Fig. 4c; $P<0.001$). However, the pattern of response was similar for both with a transpiration rate increasing between 20-35 °C and then declining at temperatures above 35 °C. Stomatal conductance was higher in the unplugged leaves compared with controls at all temperatures. However, conductance began to decline at a lower temperature (25-30 °C) in unplugged leaves than in control leaves (30-35 °C) (Fig. 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 mmol m^{-2} 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. 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 on either control leaves or leaves from which stomatal plugs had been removed (results not shown). Thus, although Blu-Tack removed more than 90 % of stomatal plugs, leaves were still sufficiently hydrophobic to prevent the formation of a water film.

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, which was consistent with results of earlier studies showing that 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). In addition, this result was in agreement with the result that we found for *Leucadendron lanigerum* (Mansour et al., 2007). 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 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, we 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 that reflectance increased linearly with the amount of epicuticular wax in wheat (*Triticum* spp.). The reflective character of such waxes is probably dependent on their quantity, specific arrangement on the leaf surface and molecular composition.

Our findings are quite different from the results reported by Feild et al. (1998) for *Drimys winteri*. We found that stomatal plugs significantly reduced leaf transpiration rates of *A. robusta* (Fig. 3a). The higher transpiration rates of leaves without plugs were apparently related to increased stomatal conductance compared with control leaves (Fig. 3c). However, we think that these different results may be related to the depressed location of the stomata on leaf surfaces of *D. winteri*. When we applied Blu-Tack with the same amount of pressure as used for removing the stomatal plugs of *A. robusta*, we were 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), our 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. 4a). We also observed lower photosynthetic rates in control leaves in comparison with leaves without plugs (Fig. 3b). Since stomatal conductance is a primary factor which controls photosynthetic rates (Brodribb, 1996), this was most likely due to the lower stomatal conductance of control leaves.

Our 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, 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. Our results broadly supported their findings, although the difference in stomatal conductance between treated 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 et al., 1993), and our results indicated that in *A. robusta*, stomatal conductance was reduced when transpiration rates increased above 0.6 mmol m^{-2} s^{-1}, regardless of whether stomatal plugs were present or not (Fig. 4). This contrasted 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 we observed have revealed some interesting effects of stomatal plugs in *A. robusta*. Firstly, as noted above, plugs do not appear to interfere with the ability of stomata to sense changes in VPD or transpiration rates. Secondly, 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 our 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 our 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 conductance was most likely related to the low stomatal density of *A. robusta* leaves. Similar low conductance values 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 were also very low, averaging 0.8–1.1 µmol m^{-2} s^{-1} (Rijkers et al., 2000; Engelbrecht et al., 2002).

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