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Research Article

The stomatal crypts in some *Banksia* speacies does not reflect their assumed functions

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Abstract

Stomatal crypts, the depressions of the epidermis containing stomata, are among the most frequently cited as examples of an adaptation that reduces water loss. The effect of stomatal crypts, despite the considerable assumption regarding its possible impact on gas diffusion, has never actually been quantified. In accordance with Fick's law of diffusion, assuming the structure of the crypt as a tube, we hypothesized that as the depth of the crypt increases, the diffusion path length increases, and as a consequence, the resistance to the diffusion of gas increases as well. Hence, at a constant cross-sectional area, leaves with a deeper crypt should have lower water loss. Despite assumptions about the function of stomatal crypts, there are surprisingly few published studies on the physiological effects of crypts. This study evaluated the micromorphology of stomatal modifications in a range of *Banksia* species as well as the impact of stomatal crypts on leaf gas exchange. We hypothesized that as crypt depth increased, transpiration and photosynthesis would 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. Leaf cross-sections and micrographs of over 110 species of the Proteaceae family were examined. Fourteen species of Banksia, as well as *Dryandra praemorsa*, were selected for this study. 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. The current study demonstrated that crypts occurring in the epidermis of the *Banksia* species examined at different depths and widths did not impact on gas diffusion through stomata. Also, the present results showed that deeper stomatal crypts did not have a significant impact on transpiration compared with leaves that had shallower crypts. The positive relationship between leaf thickness and depth of crypts and the negative relationship between leaf thickness and stomatal density in *Banksia* species found in this study might suggest that stomatal crypts possibly act as a means of overcoming mesophyll resistance to CO_2 diffusion. Further studies are required to investigate this possibility.

Keywords: *Banksia*, Stomatal crypts, Gas-exchange

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 the carbon dioxide gained passing through them (Casson and Hetherington, 2010; 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 above a threshold 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, 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). Generally, cuticular evaporation accounts for 5 to 10% of the total leaf transpiration, depending on the magnitude of the leaf to air vapor 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). However, features such as a thick cuticle, a waxy layer and

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numerous trichomes also occur in wet environments (Brewer *et al.*, 1991; Brewer and Smith, 1997; Neinhuis and Barthlott, 1997). Thus, there is uncertainty 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 the stomata (Lee and Gates, 1964; Brodribb and Hill, 1997; Hill, 1998a; Hill, 1998b; Roberts, 2000). However, these features are also likely to affect the diffusion of CO_2 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; Naz *et al.*, 2010). 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 to the diffusion of gas should increase as well. Consequently, at a 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, transpiration and assimilation rates. Vesala (1998) stated that an increase in the thickness of the boundary layer results in a decrease in water vapor loss from the stomatal pore and also a net decrease in the number of CO_2 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 few 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. We 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. 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. 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 minutes every 3 days. One desert spoonful of non-phosphorous slow release fertilizer for Proteaceae (Protea Word, Adelaide, Australia) was applied in spring and autumn.

Electron and light microscopy: Leaf surface features were analysed by scanning electron microscopy (SEM) and light microscopy. For SEM, leaves from each species were cut in ~1 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%) 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 with an accelerating voltage of 10 kV and a standard tilt of 15° (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 3×3 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 60ºC 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 mm^2 sections of prepared cuticle. Finally, the mean number of stomata per crypt was multiplied by the mean number of crypts per mm^2 to get stomatal density.

Images of the prepared cuticle were taken at 100X magnification. Images were altered using Corel photo paint, so that the crypts were black and the surrounding cuticle white. The areas of the crypts were then calculated in μm^2 using Scion Image beta version 4.0.2 (Scion Corp.).

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 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 Lendzian, 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).

Gas exchange: Transpiration, CO_2 assimilation and stomatal conductance of the 15 species were measured using a CIRAS-2 portable infrared gas analyser (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. CO_2 concentration was 350 ppm, PFD was 650 µmol quanta $m^{-2}s^{-1}$ (which had previously been shown to be saturating for all species) and leaf temperature was 25ºC. Transpiration, stomatal conductance and photosynthetic rates were measured after 20 minutes at each VPD. Photosynthetic induction was complete in all plants prior to the start of each experiment.

Data analysis: Relationships between stomatal conductance, transpiration and photosynthesis to VPD were analysed by repeated measures ANOVA using the statistical package JMPIN, Version 4.03, 2000, SAS institute. Data for other relationships were analysed 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 and discussion

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. 1a; Table 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. 1b; Table 1). All species had sparse trichomes on the adaxial surface.

Depth of crypts varied among the 15 species, ranging from 100 µm in *B. marginata* to 425µm in *B. blechnifolia* (Fig. 1c; Table 1). The width of the entrance of crypts also varied among the 15 species, ranging from 110 µm in *B. repens* to 395µm in *B. ashbyi* (Table 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. 1d; Table 1). Leaf thickness varied from 200 ± 11 µm in *B. spinulosa* to 730 ± 24 µm in *B. blechnifolia* (Table 1).

The results of the present study showed that among 110 species of Proteaceae investigated, stomatal crypts occurred only in the genus Banksia (Table 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 our 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.

There was a significant, positive relationship between the depth of crypts and leaf thickness (r^2= 0.87,

Table 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 ± s.e., n= 15, from 5 plants.

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

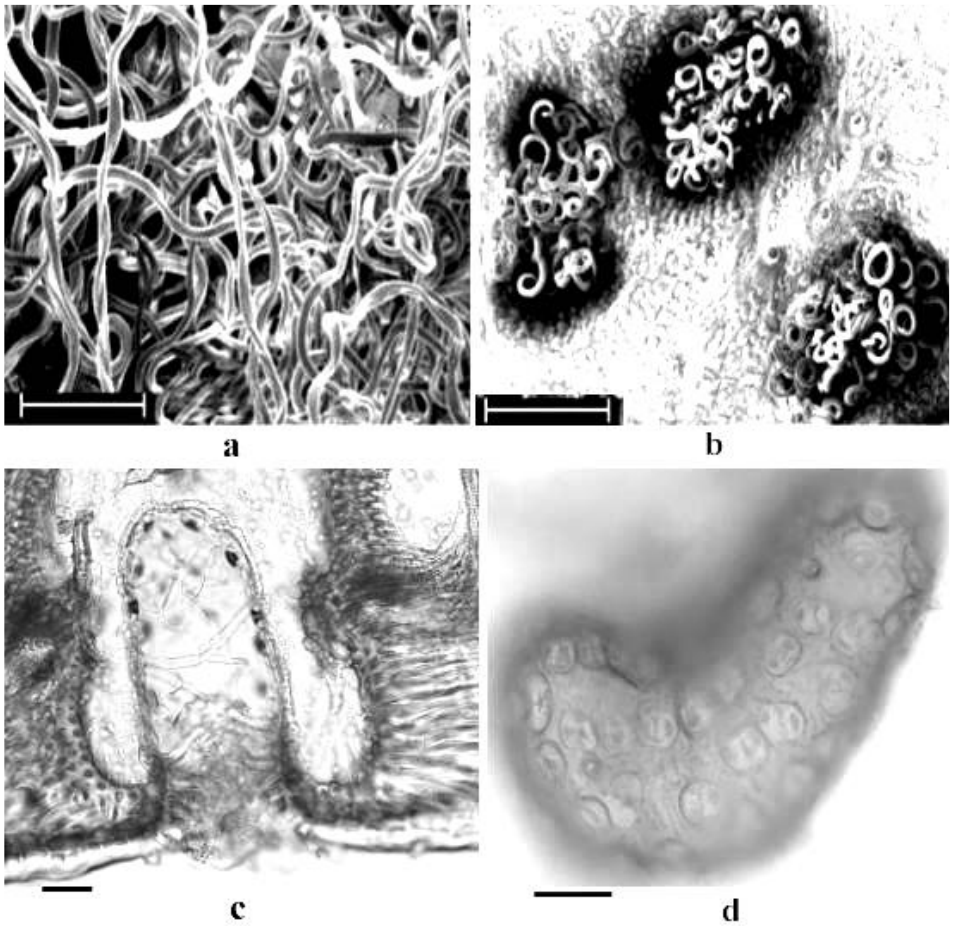

Figure 1. SEM micrographs of the abaxial (lower) leaf surface of *B. marginata* (a) and *B. baxteri* (b). The abaxial surface of *B. marginata* was covered with dense trichomes, while in *B. baxteri* trichomes occurred only inside and mostly at the entrance of the stomatal crypts. Cross-sectional view 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). The scale bar for figures (a) and (b) is 100µm and for (c) and (d) is 50µm.

P<0.0001; Fig. 2a). In contrast, there was a significant, negative relationship between stomatal density and leaf thickness (r^2= 0.44, P=0.007; Fig. 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

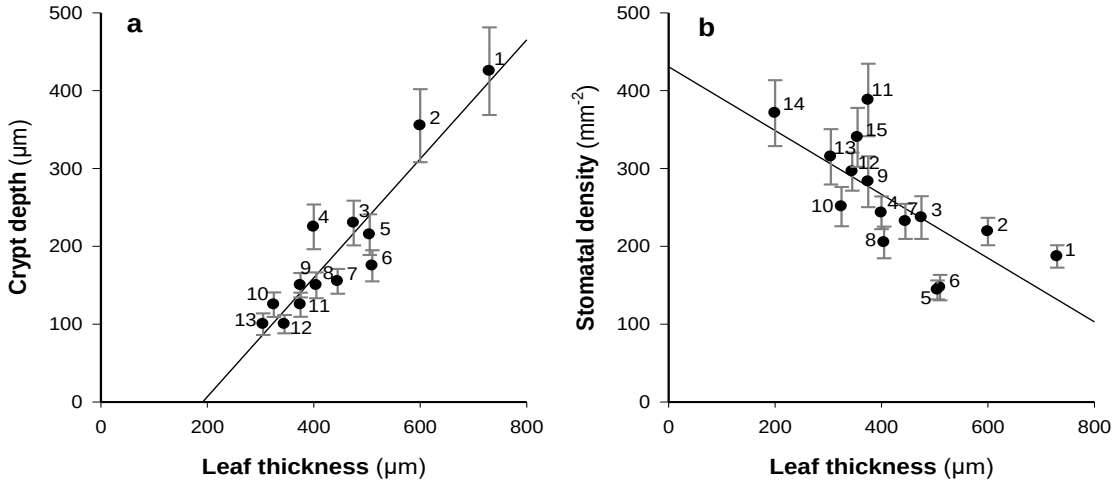

Figure 2. The relationship between leaf thickness (µm) and crypt depth (µm) in 13 *Banksia* species (a) and the relationship between leaf thickness (µm) and stomatal density (mm^{-2}) in 14 *Banksia* species and *Dryandra praemorsa* (b). Species are numbered as in table 5.1. Data points are means ± s.e.,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

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. 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. 3, slope 2). Most of this loss would have occurred across the cuticle, as the stomata would have been closed in the darkened conditions in which the experiment was conducted.

According to the literature, although cuticular transpiration accounts for 5 to 10% of total leaf transpiration (Kerstiens, 1997; Taiz and Zeiger, 2003), 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. 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. 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*.

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. 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. 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. 4c).

There was no relationship between stomatal density and either maximum transpiration rate (r^2=0.005, P= 0.80; Fig. 5a), or maximum stomatal conductance (r^2= 0.003, P= 0.85; Fig. 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. 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. 6b).

It was expected that species with shallow crypts, or lacking crypts altogether, would have higher

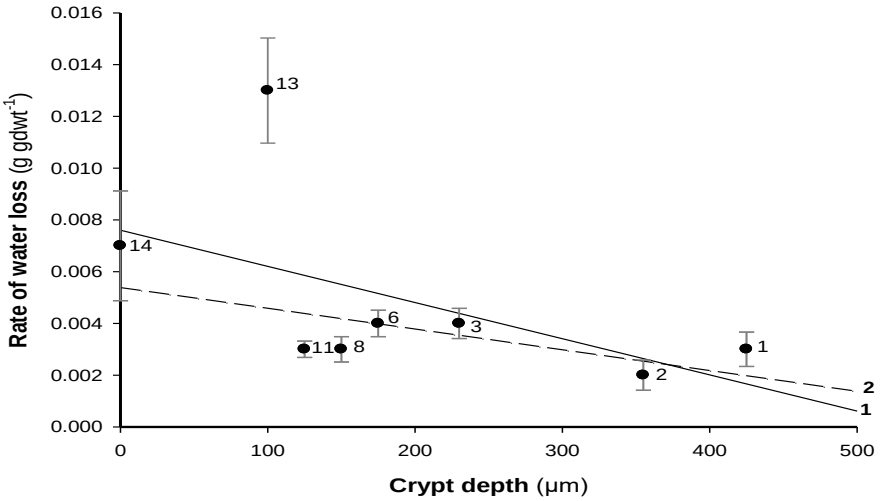

Figure 3. Cuticular water loss from detached, darkened leaves of 8 (slope 1) and 7 (slope 2) *Banksia* species with different crypt depths, including *B. blechnifolia* (1), *B. repens* (2), *B. menziesii* (3), *B. baxteri* (6), *B. praemorsa* (8), *B. speciosa* (11), *B. marginata* (13), and *B. spinulosa*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

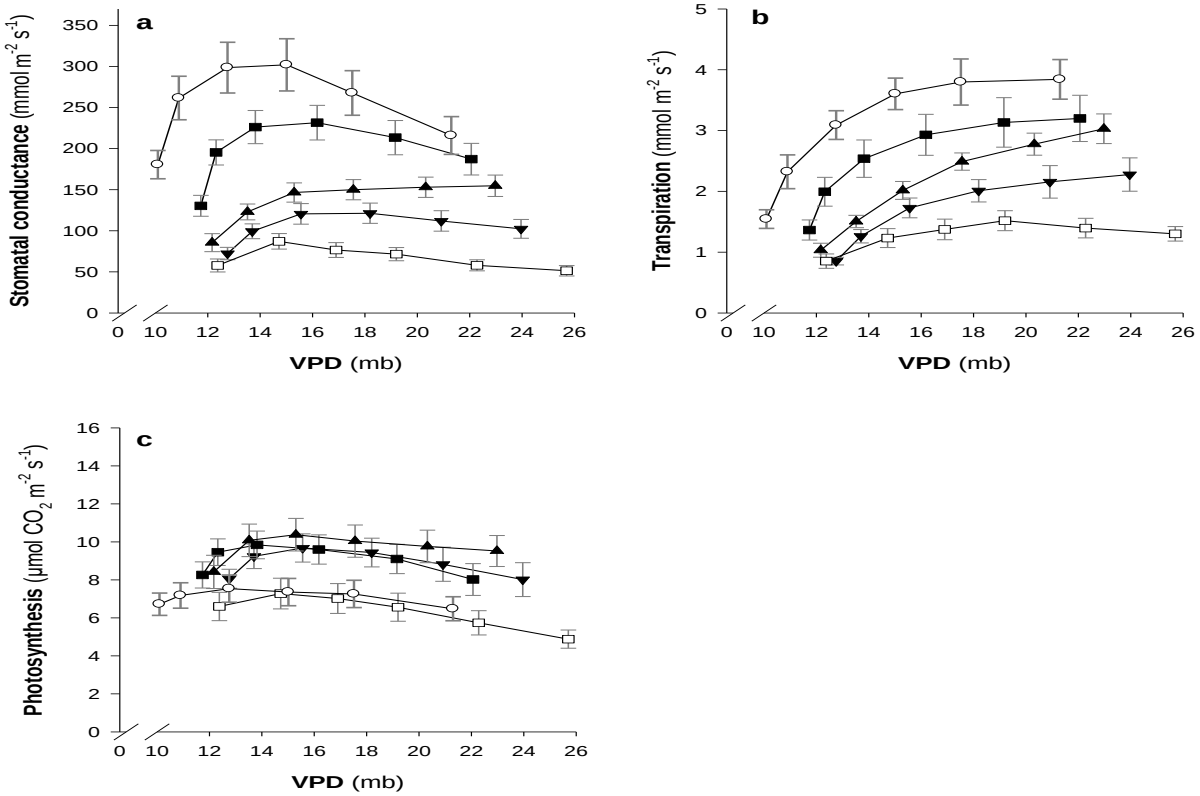

Figure 4. Representative responses of stomatal conductance (a), transpiration (b) and photosynthesis (c) to VPD (mb) in 5 of 15 study species. *Dryandra praemorsa* (○), *B. marginata* (■), *B. repens* (▲), *B. blechnifolia* (▼) and *B. spinulosa* (□). All 15 species were measured but, only 5 are shown for clarity. Data points are means ± s.e., n= 5.

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, we expected that at a given increased VPD, transpiration rates in *B. spinulosa* and *Dryandra praemorsa*, which lack crypts, should be higher than other species which possess crypts. However, the results do not support our hypothesis. As VPD increased, all species showed almost the same pattern of response to 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, *Drayandra praemorsa* did not close its stomata either, even though it lacked crypts and was expected to be more sensitive

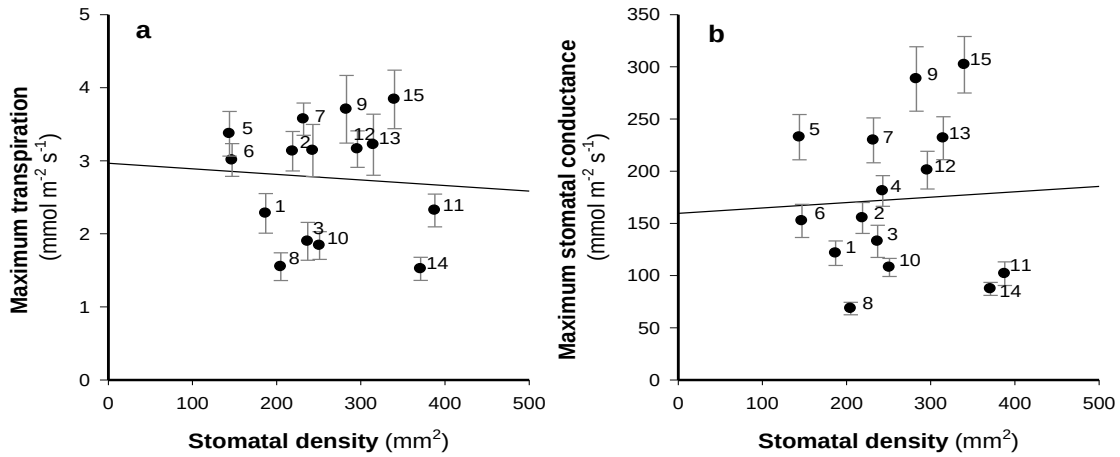

Figure 5. The relationship between stomatal density and maximum transpiration (a) and maximum stomatal conductance (b) of the 14 *Banksia* species and *Dryandra praemorsa.* Species are numbered as in table 5.1. Data points are means ± s.e., n= 5.

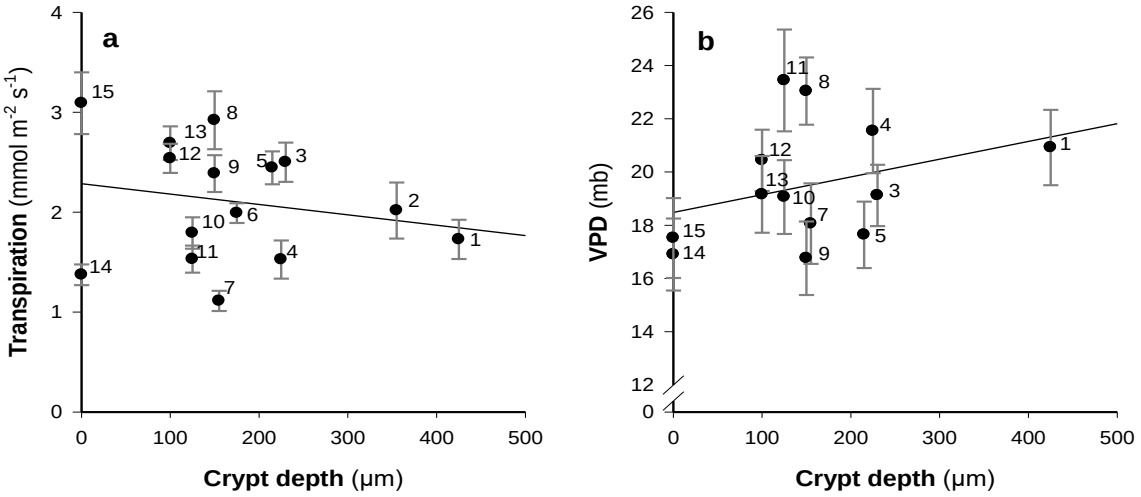

Figure 6. Relationships between crypt depth and transpiration rate at a VPD of 14 mb (a) and the VPD at which stomata began to close (b). Two species, *B. blechnifolia* and *D. praemorsa*, did not close their stomata at all in the face of increasing VPD and are not included in Fig. 5.6b. Species are numbered as in table 5.1. Data points are means ± s.e., n= 5.

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, the current 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*. Moreover, stomatal gas-exchange and secondary messenger signaling, the quota of the subcellular localization of the CO_2-binding carbonic anhydrases, the interaction with the stress hormone ABA, and the role of photosynthesis in stomatal responses to the CO_2 driver indicate new models and new open questions in CO_2 signal transduction (Engineer *et al.*, 2016).

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 among 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, 2003), the results of this study showed that there was no evidence to support this assumption in the 15 species studied. However, Meinzer

et al. (2017) reported that Species' differences in the rigor of stomatal control of plant water potential represent a chain of isohydric to anisohydric behaviours.

The current results are consistent with the findings of Matthews (2003) who modelled 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 CO_2 diffusion into leaves, probably cancelling 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. The current results are in agreement with the findings of Roth-Nebelsick *et al.* [A1](2009) who concluded that the primary function of crypts and crypt trichomes are not likely to reduce transpiration. Also, our 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 characterized by thick cuticle and epidermis and tightly packed mesophyll, all of which probably increase resistance to CO_2 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. 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 resistance to CO_2 diffusion, and as a consequence, it may increase the availability of CO_2 to photosynthetic tissues. This result supports the idea of Foteini *et al.* (2009) who proposed that crypts function to facilitate CO_2 diffusion from the abaxial surface to adaxial palisade cells in thick leaves.

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). This 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.

Conclusion

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 CO_2 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.

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