Does pubescence increase uptake of nutrient deposition on leaves?

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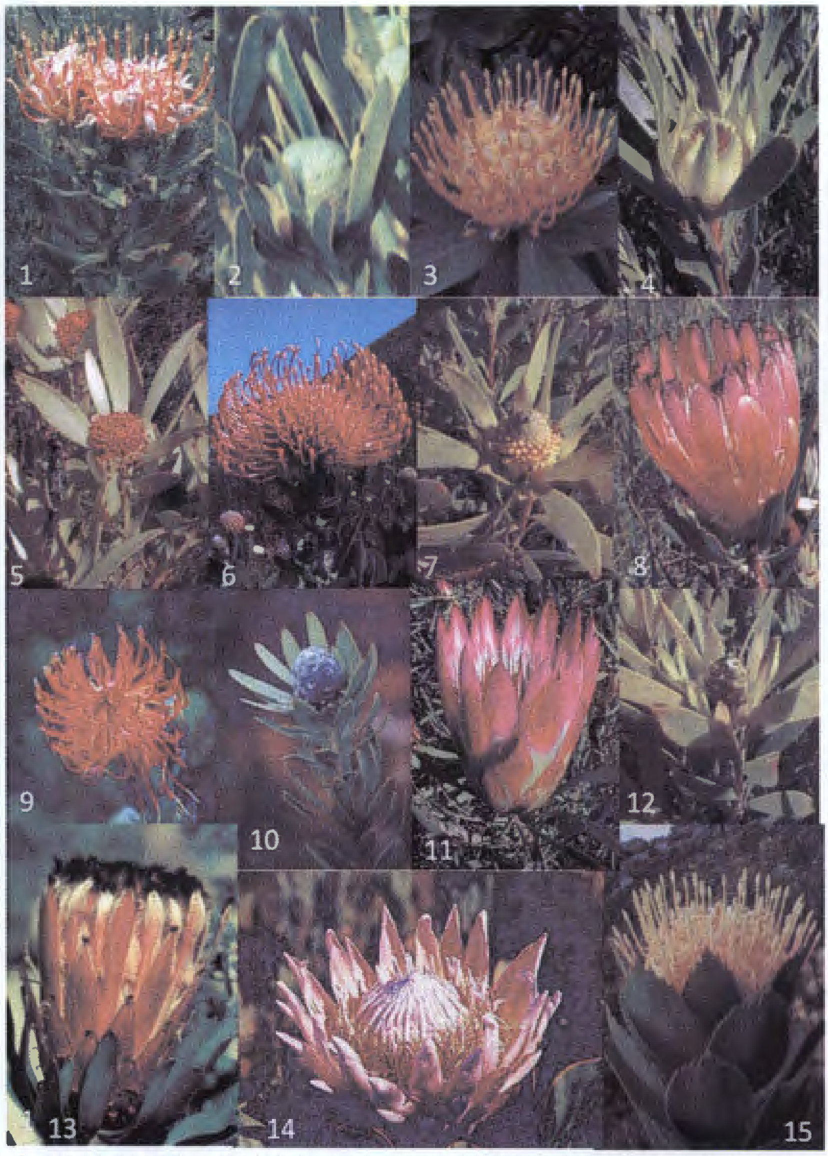

1 – Ls glabrum 2 – Ld meridianum (female) 3 – Ls conocarpodendron 4 – Ld meridianum (male) 5 – Ld discolor 6 – Ls cordifolium, 7 – Ld coniferum (male) 8 – P burchellii 9 – Ls reflexum 10 – Ld pubescens (female) 11 – P repens red 12 – Ld coniferum (female) 13 – P neriifolia, 14 – P cynaroides 15 – Ls conocarpodendron subsp viridum

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Summary

1. Leaf hairs have been associated with reducing environmental stress by increasing reflectance and the thickness of the boundary layer, however, such effects are more obvious in species with dense trichome layers. The adaptive functions for species with sparse leaf hair cover, such as in the Cape Proteaceae species of the Cape Floristic Region (CFR), remain unknown.
2. We postulated that leaf hairs of the Proteaceae in the CFR facilitate both nutrient absorption and water retention on the leaf surface. Leaf hair distribution is expected to show a pattern relative to the higher levels of atmospheric nutrients with proximity to the coast.
3. We followed a three pronged approach, this involved (i) evaluating the relationship of leaf pubescence with leaf surface water holding capacity, water uptake and nitrogen absorption from wet deposition for 12 Proteaceae species from the CFR, under controlled conditions; (ii) evaluating the relationship between leaf pubescence and plant height of 39 Proteaceae species from the CFR and (iii) comparing visual scores of pubescence with the relative proximity of the plants to the coast (oceanic index/continentality) of 205 Proteaceae species.
4. There was a positive correlation between leaf pubescence (0 to 8 $\times 10^6$ m^{-2}) and water holding capacity for the 12 experimental Proteaceae species, however, water uptake did not vary with hairiness. $^{15}NO_3^-$, $^{15}NH_4^+$ and ^{15}N-glycine solutions were each independently applied as wet deposition to the 12 experimental Proteaceae species. The uptake of glycine showed a positive correlation with hairiness overall and for *Protea*,

whereas nitrate and ammonium uptake were positively correlated only with hairiness of *Leucospermum*.

5. Average plant height was positively correlated with leaf hair density of the 39 Proteaceae species. The visual score of pubescence was significantly different for each genus and there was no correlation between leaf pubescence and distance from the coast for the 205 field Proteaceae species.
6. Leaf hairs probably play a role in reducing environmental stresses, however, it is also possible that they play a role in water retention and nutrient absorption. Hairs on leaves vary in arrangement and morphological characteristics, thus they accordingly.probably serve different functions .

Key-words: fynbos, glycine, leaf hair, Leucadendron, Leucospermum, nitrogen, nutrients, Protea, Proteaceae, trichomes.

Abbreviations: Cape Floristic Region, CFR; Leucadendron, Ld.; Leucospermum, Ls.; nitrogen, N; phylogenetically independent contrast, PIC; phosphorous, P; Protea, Pr..; Surface area, SA.

Introduction

The Cape Floristic Region (CFR) lies on the south-western tip of the Western Province, South Africa, has a Mediterranean climate and is one of the floristic hotspots of the world consisting largely of small-leaved sclerophyllous shrubs (Mucina & Rutherford 2006). Fynbos, the CFR's dominant vegetation type, grows on a mosaic of nutrient poor, particularly with regard to N and P, Table Mountain Group Sandstone-derived soils and less commonly, on limestone (Kruger et al. 1983; Mucina & Rutherford 2006). Fynbos has a unique and large diversity of plant species thriving on extremely low nutrient concentrations (Cowling et al. 1996; Cowling et al. 2008). Fynbos plants have adapted many mechanisms for dealing with these low nutrient conditions e.g. serotiny (Groom & Lamont 2009), sclerophylly (Cowling & Campbell 1983; Specht & Rundel 1990), cluster roots and mycorrhizal associations (Lambers et al. 2008). However, these alone do not suffice to explain the proliferation of species and the high nutrient content of some plants.

In mediterranean-type ecosystems, such as the CFR, vegetation structure and function is largely determined by nutrients, in particular nitrogen (N) and phosphorous (P) (Stock & Lewis 1986). Overall, not only are the CFR soils highly weathered as well as P and N depleted but there are also large losses of N and P due to fires (Stock & Lewis 1986). Fires play an important role in the CFR with a return interval of 5 to 25 years (Thuiller et al. 2004) and can affect soil-N contents directly by volatilization of organic N and the deposition of ash (Stock & Lewis 1986). A large amount of total above ground biomass is consumed by fire and N is lost to the atmosphere, possibly adding to the loss of N from the ecosystem because N flushes after a fire are temporary and rapidly disappear (Brown & Mitchell; Stock & Lewis 1986). How does fynbos continue to proliferate on the highly weathered, as well as P and N depleted, soils of the CFR? What other sources of nutrients are there? Where and how are the plants on this nutrient poor substrate getting their nutrients from?

Another possible source of nutrients is atmospheric deposition. Nutrient deposition via precipitation (Brown et al. 1984), dust (aeolian; Soderberg & Compton 2007), ash (Stock & Lewis 1986), industrial pollution (Wilson et al. 2008) and fog (Marloth 1904) are some of the various sources of atmospheric deposition that have been quantified, recorded and investigated for the CFR. This highly P depleted ancient soil of the CFR (Lambers et al. 2008) receives an estimated 0.19 kg ha^{-1} yr^{-1} P deposition (coastal, Brown et al. 1984), which is 5-10 % of extractable soil P and thus a significant atmospheric contribution to both soluble and bulk fractions of soil P (Soderberg & Compton 2007). N is the next most limiting nutrient and the overall N deposition measurements vary from 1.46 to 13.5 kg N ha^{-1}yr^{-1} (van Wyk et al. 1992; Wilson et al. 2008; Stock and Lewis 1986). In other ecosystems ammonia and nitrate accumulate on leaf surfaces from the dry deposition of both gases and particles (Davidson & Wu 1990; Bobbink et al. 1992) and a large body of evidence comparing bulk deposition to canopy throughfall has suggested an uptake of N and other reactive compounds via the leaf surface (Sparks 2009). Tree leaves have been shown to be able to assimilate nitrate and ammonium ions from simulated wet deposition (Garten et al. 1998) and therefore it is likely that plants in a nutrient limited environment could have adapted the ability to assimilate atmospheric nutrients through their leaves. Mechanisms of N uptake through the leaves are still unclear although various paths have been proposed e.g. diffusion through stomata and cuticular transport (Wittwer 1955; Peuke et al. 1998; Hannstein et al. 1999; Sparks 2009).

Not only N and P are available through the atmosphere. Significant amounts of other nutrients such as K and Ca are also deposited (Soderberg & Compton 2007). The plants accumulate large amounts of K, Ca and Mg, which can be accounted for in the dissolved fraction of wet deposition to the ecosystem, whereas Fe, Al, and Mn are increased by weathering of bedrock and deposited mineral dust (Soderberg & Compton 2007). Furthermore, washout of marine salts and mineral dust particles constitute an additional important source of nutrients in the CFR (Soderberg & Compton 2007). The fynbos ecosystem is effective at retaining these nutrients (Soderberg &

Compton 2007). It is not clear when and how wet and dry nutrient deposition is an important input to ecosystems (Paterson & Mackay 1994) and remains to be evaluated for the CFR.

There are three possible forms of nutrient deposition from the atmosphere: wet, dry and cloud deposition (Lovett 1994). While it seems reasonable to expect that plants inhabiting the coastal regions of the Cape might use fog water, few investigations have explicitly demonstrated or quantified the extent to which fog is used by different fynbos species. Marloth (1904) investigated the interception of fog by reed-like plants on Table Mountain in South Africa and estimated that there would be at least 3810 mm of water as a consequence of condensation of moisture (exclusive of rain) during the summer season alone. Such fog uptake has been investigated in a study of a mediterranean coastal Californian forest where it was shown to be at least as significant as nutrient input from rain (Azevedo & Morgan 1974). The formation of fog under warm climatic conditions increases concentrations of nutrients (Ingraham & Matthews 1990) and these condensation nuclei and dissolved gases may become available to the plants, as in the mediterranean coast of California (Azevedo & Morgan 1974). Indeed, some plant leaf shapes may have evolved to facilitate the interception and collection of fog (Went 1955) on leaf surfaces and direct dissolved nutrients to the ground for uptake (Azevedo & Morgan 1974). Although it has been suggested that plants may absorb water vapor directly from a saturated atmosphere (Breazeale et al. 1950; Azevedo & Morgan 1974) it is unlikely. More probable is the uptake of atmospheric nutrients dissolved in wetted leaf surfaces (Azevedo & Morgan 1974). The vigorous wave action of the ocean surf is a major source of condensation nuclei for fog and rain near the coast (Boyce 1952). As these aerosols are transported inland they diminish

(Azevedo & Morgan 1974), thus perhaps the adaptation of foliage to take up atmospheric nutrients diminishes with increasing distance from the coast.

Increasing food production by using fertilizer and the increase in fossil fuel combustion has led to an increased nutrient input into the biosphere each year as a result of human activities (Matson et al. 1999; Sparks 2009; Holland et al. 1999). Reactive N gases return back to the earth's surface and this atmospheric deposition of N is increasing (Sparks 2009) with an estimated doubling in total deposition of reactive N by 2050 compared with the early 1990s (Galloway et al. 2004; Phoenix et al. 2006). Such increases in N deposition are a major ecological threat as availability of nutrients is a key factor in determining plant community composition and the stability of vegetation in many natural and semi-natural ecosystems which are dependent upon low soil fertility (Bobbink et al. 1998; Peuke et al. 1998; Phoenix et al. 2006). Although it seems like an important source of N for plants, there is a need for a greater understanding of the N deposition impacts on vegetation (Phoenix et al. 2006). A significant increase in nutrients could negatively affect plant diversity (Bobbink et al. 1998). Therefore a diversity hotspot such as the CFR would stand under threat as the predicted increases in N deposition exceed 10 kg N ha^{-1} yr^{-1-} within ca. 40 % of the CFR by 2050 (Phoenix et al. 2006).

A common feature of mediterranean climates is leaf pubescence, however the adaptive value of these leaf hairs is not completely clear (Ehleringer 1984). There are various hypotheses as to the functions of hair (Ehleringer 1984). Hairs are said to increase the thickness of the boundary layer around leaves and increase reflectance of solar radiation (Ehleringer 1984). An increase in pubescence increases leaf reflectance (Gausman & Cardenas 1969; Agrawal 2006) and thus reduces the leaf temperature consequently decoupling the leaf from the atmosphere and reducing transpiration rate (Ehleringer 1984). Although leaf reflectance does increase with pubescence in many species, there are exceptions and the effects of reflectance are likely to be dependent on density and thickness of the indumentums (Ehleringer 1984) as well as the length, arrangement and posture of the hairs (Gausman & Cardenas 2009). Differences in hair characteristics and arrangement are possibly a consequence of different functions. Leaf hair may increase the thickness of the boundary layer, thereby decoupling the leaf from the atmosphere and reducing the transpiration rate (Ehleringer & Mooney 1978; Ehleringer 1984; Agrawal, 2006; Benz & Martin 2006). Thus the boundary layer plays a large role in controlling water and heat loss (Schuepp 1993). As Johnson (1975) said the "evolutionary significance of trichomes have been the subject of much speculation..." and the understanding of leaf trichome functions are still misunderstood.

Leaf hair has also been shown to be an adaptation to herbivory by serving as a physical barrier to animal feeding or by emitting toxic or repellent compounds (Ehleringer 1984; Traw & Bergelson 2003; Agrawal 2006; Hanley et al. 2007). An increase in hair density on new leaves in response to insect damage has been observed in many plant species (Traw & Bergelson 2003). Tannins, phenolics and hair have been said to form the effective defense system of Proteas against insect herbivory (Wright & Giliomee 1991), however, leaf predation is almost non-existent in the Proteaceae (http://protea.worldonline.co.za). It seems unlikely that Proteaceae pubescence plays a role in defense as there is little recorded evidence of predation on Proteaceae leaves (http://protea.worldonline.co.za). Furthermore, there should not be a priori expectation that hair has the same function for all plants and that the function is mutually exclusive.

A critical adaptation for inhabiting the epiphytic niche is undoubtedly the ability to absorb water and nutrients directly through the foliar trichomes, as in Bromeliads (Benzig & Burt 1970), where entire shoots are covered in absorptive trichomes (Benzig et al. 1985) or for example foliar trichomes of *Tillandsia* which have the primary function of liquid and nutrient absorption (Benz and Martin 2006). Some Bromeliaceae leaf surfaces are hydrophobic and possess highly irregular microrelief to reduce the adhesion and spread of water on the leaf blade so as to prevent the growth of other organisms on the leaves in the moist environment (Pierce et al. 2001). Differences in the foliar permeability of bromeliad leaves are associated with the type of trichomes and the frequency of their distribution over the leaf surface (Benzig et al. 1976). In general the trichome functions of Bromeliads are well understood (Benzig et al. 1976). However the functions of leaf hairs for many other plant families remains unclear. These hair-like appendages extending from the aerial tissue occur in a multitude of forms and most likely fulfill multiple roles in plant physiology and ecology (Wagner et al. 2004; Hanley et al. 2007).

Cape Proteaceae has an exceptionally broad range of leaf hairiness. Increased pubescence may have an effect on surface area of Proteaceae leaves for trapping nutrients from atmospheric deposition. In order to investigate the ability of leaf hairs to increase the uptake of nutrients from deposition, we experimentally applied nutrients in atmospheric concentrations by wet deposition to leaves of varying hairiness and investigate whether the hairy leaves were more effective at absorbing nutrients from wet deposition. We hypothesized that hairs were effective at increasing atmospheric nutrient deposition uptake and increasing the water holding capacity of the leaves. Therefore the hairier leaves could hold more water on their surface and absorb a larger amount of foliar applied nutrients. Nutrient assimilation consequences of variation in leaf hair density were measured across 12 species of *Protea* L. (*P.*), *Leucadendron* R. Br. (*Ld.*) and *Leucospermum* R. Br. (*Ls.*), which display a range of leaf hairiness.

Material and Methods

Plant cultivation and environmental conditions

Twelve Proteaceae species of variable hairiness were obtained from Kirstenbosh Nursery, Cape Town. Plants had been grown in a 1:1 mixture of sand and compost in 2 L plastic bags. Species were selected from each of the three genera: *Leucadendron, Leucospermum* and *Protea* with varying degrees of leaf pubescence. They were then transferred to the greenhouse at the University of Cape Town (UCT) and watered once a day. The plants were maintained in the greenhouse and were only put in the temperature-controlled growth chamber set to 25°C with humidity of *ca.* 21% and irradiance of 1 000 μmol m^{-2} s^{-1} immediately prior to the experiment. A further 25 Proteaceae species were grown in the greenhouse and used for hair counts.

Leaf trait and biomass measurements

For all 37 Proteaceae species the average height was obtained from the Rebelo (2001). Hair density was determined by coating both surfaces of the leaves with nail polish, peeling this off, and counting the hair impressions on the peels in three random fields of view at 400 X magnification on a transmission light microscope. The hair density and average plant height for the 37 Proteaceae species were correlated with each other.

Water holding capacity and leaf hair characteristics

To determine the water carrying capacity of the leaves with different hair densities, leaves from each replicate of the 12 experimental species were collected. These leaves were then weighed immediately after being removed from the plants and again after rinsing in water and draining. The leaves were then dried at 80°C for 48 hours in a forced convection oven and then weighed. The leaf hair density of the 12 Proteaceae species was then correlated with the water holding capacity of the leaves.

To document the leaf hair arrangement and characteristics images of the leaf hairs of the 12 Proteaceae species were taken using a Nikon stereoscopic zoom microscope (SMZ1500) and digital camera (Nikon DS Camera Control Unit DS-U2 & DS-5M camera head) at 112.5 X magnification.

Nitrogen absorption

The $Na^{15}NO_3$, $^{15}NH_4Cl$ and ^{15}N-Glycine solutions were applied to a leaf of each plant by dipping a filter paper in one of the three solutions, folding this around the leaf and covering it with a plastic sleeve, which was kept in place with two microscope slides and two paper clips. 100ml of 1mM $Na^{15}NO_3$, $^{15}NH_4Cl$ and ^{15}N-Glycine (98 atom% enrichment, Isotec, Miamisburg, Ohio, USA). The ^{15}N-Glycine, $Na^{15}NO_3$ and then $^{15}NH_4Cl$ were each applied (10h00) to the youngest fully expanded leaf of each replicate and collected in the same order after 4 h. One leaf of each replicate plant was collected before applying nutrients and another leaf from each plant after all treated leaves had been collected i.e. after 4- 5 hours.

The dried leaves were ground to a fine powder using a Retsch MM200 ball bearing mill (Retsch, Haan, Germany) at a frequency of 25/5. For the non-treated leaves approximately 12mg of ground leaf was weighed out and for treated leaves 7 mg was weighed out using a scale (AND GH-202, Japan). Leaf $\delta^{15}N$ was determined on an isotope ratio mass spectrometer (Finnegan Mat 252, Bremen, Germany) with a N elemental analyser (NA 1500NC, Carlo-Erba, Milan, Italy). The fraction of label ($\Delta^{15}N$) that contributed to $\delta^{15}N$ signal in leaves of each species after labeling was calculated using the following formula:

$$\Delta^{15}N = \delta^{15}N_{\text{(after)}} - \delta^{15}N_{\text{(before)}}$$

Where $\delta^{15}N_{\text{(after)}}$ is the average signal in the leaves of a species after labeling and $\delta^{15}N_{\text{(before)}}$ is the average signal in the species before labeling.

Deuterium treated leaves

30ml of heavy water were mixed using 450µl of 99 atom% D_2O (Aldrich, St Louis, MO, USA). This was applied to a leaf of each *Protea* species in the same way as the N solutions were. After four hours the leaves were harvested, weighed, put in vials sealed with Parafilm to eliminate evaporation and stored at 4°C until water extraction and analysis. The leaf weight was obtained by weighing the airtight vials before and after putting the harvested leaf in. Water was extracted from leaves by cryogenic distillation (West et al. 2006). Extracted water was reduced to H_2 gas using the closed tube zinc reduction method (Coleman et al. 1982). Isotope ratios were measured with a Delta XP mass spectrometer (Finnigan MAT GmbH, Bremen, Germany) at the University of Cape Town (UCT) and were expressed as δ^2H (in ‰) relative to the Standard Mean Ocean Water (V-SMOW) , following the equation:

$$\Delta^2H = [(R_{sample}/ R_{standard})-1] \text{ x } 100$$

where R is the ratio of $^{2}H/^{1}H$. Analytical precision on internal standards was 1.4%

The fraction of label ($\Delta^{2}H$) that contributed to $\delta^{2}H$ signal in leaves of each species after labeling was calculated using the following formula:

$$\Delta^{2}H = \delta^{2}H_{\text{(after)}} - \delta^{2}H_{\text{(before)}}$$

Where $\delta^{2}H_{\text{(after)}}$ is the signal in the leaves after labeling and $\delta^{2}H_{\text{(before)}}$ is the signal before labeling.

Field plants

For a comparison of pubescence between the three genera a visual score of hairiness from Rebelo (2001) was used. The visual score of hairiness had been assigned by rating the plants pubescence in the field with the naked eye. The four categories were delimited, as follows: hairless - 0, hairless when mature - 1, hairy – 2 and hairy or hairless - 3. Furthermore, the average oceanic index of the three genera were compared. The oceanic index (continentality) is a simplified measure of the distance from the coast of the species natural distribution: zero would be a plant that occurs at the coast, whereas four would be a plant that occurs on the inland edges of the CFR.The species' oceanic indexes were characterized by querying appropriate databases using the species' distribution data obtained from the Protea Atlas Project (Rebelo 2001). All queries were done using an ARC Geographic Information System, with 1' x 1' grid, giving, for each species, a mean, standard deviation and range.

Statistical analysis

Total average hair (x 10^{6} m^{-2}) was correlated with water holding capacity of leaf surface (g g^{-1}); $\Delta^{15}N$ of NO_3, NH_4 and glycine treated leaves, as well as average plant height using

STATISTICA ver. 8 (StatSoft Inc., Tulsa, OK, USA). All values of water holding capacity, $\Delta^{15}N$ of NO_3, NH_4 and glycine were logged and then a one-way analyses of variance (ANOVA) test was performed on these variables in relation to differences between hairiness of species, using STATISTICA ver. 8. One-way analyses of variance (ANOVA) were performed using STATISTICA ver. 8 between continentality and genus, as well as haircode and genus, followed by a post-hoc Fisher HSD test.

Phylogenetic contrast analysis

A tree of the 12 species used in the experiment was generated with the known Proteaceae phylogenies (Barker et al., 2002; Barker et al. 2004; Barraclough & Reeves, 2005). Branch lengths were assumed to be equal and assigned a value of 1.0. A phylogenetically independent contrast analysis was used to determine the significance of correlations, taking phylogenetic history into account. This allowed for the evaluation of trait relationships, in which contrast signify differences between trait values of two sister taxa (Felsenstein 1985). The PDAP:PDTREE module (Midford et al. 2005) of Mesquite (Version 2.5, Maddison & Maddison 2008) was used to conduct PIC's, which calculates independent contrast over the phylogenetic tree. Since the *Ld. pubescens* relative position within the *Leucadendron* clade is unknown, all 5 possible trees were generated over which correlations were performed. Thus for these allometric correlations, ranges of correlation coefficients and p-values were used. The relative position of two of the four *Leucospermum* species within the clade, were unknown and therefore it was kept as a polytomy. Although PIC analysis was used to test the significance of the correlations, these graphs were not presented as they misrepresent the form of the relationships. Thus PIC correlation coefficients and P-values were presented in the figures legends, although, a-historical graphs were drawn to illustrate relationships.

Results

Leaf trait and hair characteristics

The 12 Proteaceae species used for the wet deposition experiments and $\Delta^{15}N$ analyses represented a wide range of hairiness, with hair density varying between 0 and ca. 1.5 $\times 10^9$ hair m^{-2} (Table 1). Since some plant material was young (*ca.* 1 year old) some leaf pubescence did not directly correspond with those of plants in the field situation and furthermore the field plants were only scored with the naked eye (Rebelo 2001). Not only was there a wide range in hair density but also in hair posture, arrangement and length (Figs 1a and 1b). All four of the *Leucospermum* species had curly hair. *Ls. glabrum* had short curly hairs versus *Ls. conocarpodendron*, which had longer but also curly hairs. Both *Ls. cordifolium* and *Ls. reflexum* had curly hair, however *Ls. reflexum* also had sparsely distributed long straight hairs. In contrast *Leucadendron* had straight hairs, varying from very sparsely distributed, as in *Ld. discolor* and *Ld. coniferum,* through crisscrossing moderately hairy *Ld. pubescence* to the thick mat of flat straight hairs of *Ld. meridianum*. The *Protea* species had a much lower hair density overall then either of the other two generas, from no hairs in *P. cynăroides* to a moderate density of straight hairs in *P. burchellii.*

In the photographs of *Ld. coniferum, Ld. pubescence* and *P. repens-red* it appeared as if these leaves had no hairs, however, on closer inspection with the microscope, sparse hair was indeed observed (Table 1). *Ld. meridianum* was the only leaf where the hairs were so dense the cells and stomata were not visible. As average plant height increases so does the hair density (A-historical $r = 0.38$, $P < 0.05$) for all 37 Cape Proteaceae species.

Water holding capacity

Over all three genera there was a positive relationship between leaf hair density and leaf surface water holding capacity (A-historical $r = 0.65$, $P = 0.00$). For each genus (Fig. 2) clear positive relationship between hair density and water holding capacity was found, *Protea* (A- historical, $r = 0.87$, $P = 0.00$, Fig. 2 a), *Leucadendron* (A-historical, $r = 0.89$, $P = 0.00$, Fig. 2b) and *Leucospermum* (A-historical, $r = 0.88$, $P = 0.05$, Fig. 2 c).

Nitrogen absorption and water uptake

No correlative relationship over all three genera was evident between $\Delta^{15}N$ and leaf hairiness for either nitrate treated leaves (Historical $r = 0.44$ to 0.56, $P = 0.03$ to 0.08, Fig. 3) or ammonium treated leaves (Historical $r = 0.18$ to 0.4, $P = 0.11$ to 0.29, Fig. 4). The change in $\Delta^{15}N$ uptake with leaf pubescence showed the same trend for each genus with nitrate and ammonium (Figs 3 and 4) although ammonium uptake exceeded nitrate uptake ($t = -3.29$, $P = 0.00$). There was a positive correlation between *Leucospermum* and nitrate/ammonium uptake, whereas there was no correlation between ammonium or nitrate and hairiness of *Leucadendron* (A-historical, nitrate: $r = 0.1$, $P = 0.76$; ammonium: $r = -0.24$, $P = 0.46$) or *Protea* leaves (A-historical, nitrate: $r = -0.39$, $P = 0.21$; ammonium: $r = -0.17$, $P = 0.59$). $\Delta^{15}N$ is positively correlated with leaf pubescence of *Leucospermum* for nitrate treated leaves (A-historical, $r = 0.74$, $P = 0.01$) as well as ammonium treated leaves (A-historical, $r = 0.75$, $P = 0.005$). $\Delta^{15}N$ only showed a positive relationship with hair density of all species from all genera for the ^{15}N-Glycine treated leaves

(Historical $r = 0.66$ to 0.74, $P = 0.02$ to 0.03, Fig.6) and here it was only within the *Protea* genera that the positive correlation held (A-historical data $r = 0.6$, $P = 0.04$, Fig. 5 a). There was no correlative relationship between glycine assimilation and hairiness of *Leucadendron* leaves (A-historical $r = 0.54$, $P = 0.13$) or *Leucospermum* leaves (A-historical $r = 0.84$, $P = 0.07$). The lack of points in the *Leucospermum* and *Leucadendron* correlations was due to missing data (Figs 5b and 5c).

There was no relationship between hairiness and water uptake for the *Protea* (A-historical $r = 0.289$, $P > 0.05$, Fig. 7). The D_2O treatment, however, was successful and the leaves did take up a lot of the labeled water that was applied, with an average 1×10^4 fold increase in δ^2H of the leaves before treatment (Fig. 7).

Plant and environmental conditions of field plants

The visual score of hairiness differed significantly between the three Proteaceae genera (A-historical $F = 21.56$, $P = 0.00$, Fig. 6). *Leucospermum* had a significantly higher average haircode than *Leucadendron* (A-historical $F = 21.56$, $P = 0.00$) or *Protea* (A-historical $F = 21.56$, $P = 0.00$) and *Leucadendron* had a significantly higher haircode than *Protea* (A-historical $F = 21.56$, $P = 0.004$).

The *Protea* species were more inland than the *Leucospermum* (A-historical $F = 4.08$, $P = 0.002$, Fig. 9). There was no significant difference in the distribution of *Protea* and *Leucadendron* (A-historical $F = 4.08$, $P = 0.144$, Fig. 9) in relation to distance from the coast, nor was there a significant difference in the *Leucadendron* and *Leucospermum* (A-historical $F = 4.08$, $P = 0.053$,

Fig. 9) distribution in relation to distance from the coast. Furthermore, there was correlation between the visual score of hairiness and distance from the coast (A-historical $F = 2.50$, $P = 0.06$).

Discussion

Failure to detect a consistent relationship in this investigation between species' leaf hair density and the $\Delta^{15}N$ of leaves shows that leaf hair density was not consistently linked to the uptake of nutrients. However, this does not exclude the possibility that leaf hairs do play some sort of role in nutrient capture, considering that water holding capacity had a positive relationship with hairiness over all three genera. Furthermore, in evaluating the significance of leaf hair density with water holding capacity, as well as water and nutrient uptake of 12 Cape Proteaceae species, proximity to the coast was also considered. Whilst recognizing the fundamental importance of incorporating phylogenetic history in all of our analyses (cf. Felsenstein 1985), efforts to do so were limited by the paucity of suitable phylogenetic studies for our study taxa.

Water is needed for the absorption of nutrients, therefore establishing whether leaf hairs enhance the water holding capacity of leaves was investigated because this allows the dissolution of nutrients and increased trapping of aerosols. Furthermore, soluble forms of nutrients are more efficiently absorbed by the plant (Wittwer 1955), therefore nutrients not in solution of surface water are not as effectively absorbed. Apart from a few exceptions, the comparison of hairiness and water holding capacity overall three genera, *Leucadendron*, *Leucospermum* and *Protea,* revealed that hairier species are more likely to hold onto larger amounts of water (Fig. 2). Exceptions such as that of *Ld. meridianum* do not show an increase in water holding capacity although it has a very high trichome density. This can possibly be explained by the very different hair arrangement and posture of *Ld. meridianum* (Fig 1 b), which are much denser and likely to be less penetrable by water, than those of the other species. The leaf cells and stomata are not as visible through the hair as in the other leaves. The prostrate dense layer of long hairs appears to create a smoother surface. These findings are consistent with research by Savé et al. (2000), who found that leaves have higher water retention if the water of the leaf surface is segregated into patches, whereas leaves with a "lifting strategy" have dense trichomes preventing surface moisture from reaching the leaf epidermis (Brewer & Smith 1997; Savé et al. 2000), such as *Ld. meridianum*. This suggests that hairiness is of principle importance in influencing the water carrying capacity of leaves (Brewer & Smith 1997). However, over the whole spectrum of pubescence tested, the uptake of water was invariable and did not vary significantly with leaf hair density. Thus hairiness seemingly had no effect on water uptake, which confirms Brewer and Smith's (1997) findings that trichomes can reduce the amount of moisture in direct contact with epidermal tissue. Perhaps this is as a result of our methodology in trapping the water on the leaves instead of allowing water to evaporate off as physiology and morphology would allow. This would possibly result in an increased water uptake with increased pubescence. Therefore, if hair had no effect on the uptake of water, which is needed for the absorption of nutrients and in which the nutrients are dissolved, the question remains do the hairs increase the uptake of nutrients deposited in the Cape Proteaceae species?

Mineral nutrient availability and acquisition of nutrients are enhanced by water therefore although the uptake of water did not increase with pubescence (Fig. 7), perhaps the increased moisture would suffice to increase nutrient uptake in those leaves with a greater pubescence and thus also leaf water retention. Continuous water layers connect the leaf surface to the mesophyll through the stomata during wetting, allowing the diffusion of ions in addition to cuticular diffusion (Buckhardt & Eiden 1994; Peuke et al. 1998). However the relationship with hairiness and nutrient uptake was not as clear (Figs 3 – 5).

Glycine uptake improved with hairiness overall (Fig. 6), however this did not hold across all genera (Fig. 5), which was possibly due to the lack of species data because not all Δ^{15}N values were retrieved. *Protea* showed a positive relationship between glycine uptake and hairiness. No data was lacking for *Protea*, however, for *Leucadendron*, on the other hand, there were only values for three of the four species and for *Leucospermum* only two of the four species results were retrieved (Fig. 4) and these genera showed no relationship with glycine uptake. Therefore, it is not the lack of data that causes the positive correlation between hairiness and glycine uptake and one can assume this is a real relationship. There were significant increases in glycine uptake from the least hairy to the most hairy for each genus, however, in order to determine whether the pattern generally holds true for Proteaceae, further investigation is necessary. Glycine is a common form of atmospheric reduced organic nitrogen; its source is both oceanic aerosols and agricultural sources (Neff et al. 2002; Paerl et al. 2002). Since the Cape Proteaceae occur in the CFR, which is for a large part bordered by coast (Mucina & Rutherford 2006), it is possible that glycine is a common source of atmospheric organic nitrogen to these plants and thus they are well adapted to the uptake of atmospheric glycine. Furthermore, organic nitrogen forms an important input of nitrogen to ecosystems (Neff et al. 2002) therefore plant organic nitrogen transporters might be more active.

Ammonium and nitrate are both compounds assimilated by the normal plant N assimilation pathways (Sparks 2009). The significantly larger amount of ammonium assimilated is in accordance with previous research (Boyce et al. 1996), although atmospheric concentration of ammonium were much lower than nitrate concentrations (Neff et al. 2002). Recent interpretations of NO_3^- and NH_4^+ ion transport suggest cuticular diffusion as the main pathway (Peuke et al. 1998) and thus suggest that the positive charge of the plant cuticle at pH >3 favors cation e.g. NH_4^+ transport (Tyre et al. 1990).

The only positive relationship between hairiness and nitrate or ammonium uptake was found in the *Leucospermum*, which is the genera for which all leaf hairs are curly and there was a large variation in pubescence. Coiled hairs might be a morphological adaptation to leaf wetting, which enhances the effect of surface moisture as opposed to limiting it, which was the proposed function of trichomes in montane and subalpine plants of Wyoming, USA (Brewer & Smith 1997). This proposed enhanced wettability of *Leucospermum* leaves as a result of coiled trichomes, might also explain the positive relationship between hairiness and nitrate/ ammonium assimilation. The only *Protea* species (*Pr. burchellii*) with an obvious hair cover had straight hairs and with this came a significant decrease in nitrate absorption and a decrease in ammonium absorption (Figs 3a and 4a). Thus, possibly the uptake of nitrate and ammonium can be associated with coiled hair, as these are the only hairs that had a consistent significant positive effect on nitrate and ammonium assimilation. However, this could also be due to phylogenetic constraints of trichome function, as nitrate and ammonium uptake was significantly increased with some of the other species that had straight hair. For example *Ld. meridianum* and *coniferum* for nitrate uptake (Fig 3b) and *Ld. coniferum* for the ammonium uptake (Fig 4b). *Ld. meridianum* is the hairiest *Leucadendron* and thus it is consistent with the proposed hypothesis that increased pubescence leads to increased nutrient uptake. However, *Ld. coniferum,* does not have high trichome cover and it is only the second hairiest of the four species, yet it showed the highest nitrate and ammonium assimilation. Therefore, for *Leucadendron* it is unclear what caused the significant increase of nitrate uptake in the two species, perhaps it is due to cuticular differences. *Protea* showed an even more surprising result with the hairiest species (*P. burchellii*) taking up significantly less nitrate than any of the other species did (Fig. 3a), similarly it had the second lowest ammonium uptake. The results for the *Leucadendron* and *Protea* species indicate that hairiness does not increase the uptake of atmospheric nutrients and that there are other more influential adaptations to increase nutrient uptake through the leaf surface.

Deposition mostly consists of organic forms of nitrogen such as amino acids e.g. glycine and organic nitrates (Neff et al. 2002) and therefore nitrate, ammonium and glycine transporters should be expressed and active, thus allowing the uptake of these nutrients. Perhaps the effect of increased water retention of the hairier leaves only has an effect over time as those leaves holding onto more water for longer because of the greater trichome density are at an advantage. For this investigation all leaves were exposed to the wet deposition for the same amount of time, however, a future study should test the effect of better water retention of the hairier leaves on water uptake by spraying or dripping the nutrients on and harvesting leaves after water has evaporated from the leaf surface.

It was expected that those species occurring closer to the coast would have a higher hair density as an adaptation to take up nutrients brought in from the ocean in the atmosphere, however, there was no correlation with hairiness and distance from the coast. Atmospheric organic nitrogen is significantly greater close to oceanic environments, however, there are other natural sources of atmospheric nitrogen such as atmospheric dust, bacteria and organic debris (Neff et al. 2002). Perhaps in the CFR the oceanic input of nutrients is not significantly more than the terrestrial contribution of organic nitrogen to the atmosphere and thus there is no relationship between foliar nutrient uptake adaptations and distance from the coast. Perhaps because the deposition of oceanic aerosols at the coast enrich these soils (Brown et al. 1984), the plants at the coast thus rely on soil enrichment, whereas further inland the plants have to rely more on fog and atmospheric aerosol deposition. Therefore possibly hair is regained more inland or maybe only when there is regular fog.

On the other hand hairiness was positively correlated with height (Fig. 10) and previous studies point out that taller plants such as trees due to their height, have an advantage at intercepting more airborne particulate N than smaller plants and therefore benefit most from N deposition (Kellman & Carty 1986). Perhaps the hairs on the taller plants play a more important role in nutrient absorption. Hairs possibly have different functions for different plants and perhaps even different leaf hair types (e.g. curly versus straight and long versus short) have different functions. Further research should investigate this and it is important in future studies on leaf hairiness to record hair type and characteristics.

Conclusion

Hairier leaves were associated with a higher water holding capacity on the leaf surface but not water uptake It is likely that leaf hairs play a role in determining the water carrying capacity of leaves. It is possible that one of the principle functions of leaf hairs in the CFR Proteaceae family is to control water holding capacity of the leaves. There was a less consistent relationship with leaf hairiness and nitrate or ammonium uptake, however there was a correlation with glycine uptake, possibly because glycine is an abundant form of the common organic nitrogen in the CFR's atmosphere, these transporters are more active and expressed. It was concluded that the water carrying capacity of leaves was not the only function of leaf hair but that they also could play a role in the uptake of common nutrients in the atmosphere such as organic N like glycine. There was no relationship with hairiness and distance from the coast as was expected, possibly because the nitrogen input of terrestrial activities are as significant as oceanic input. However, there was a positive correlation with average plant height. Larger plants have previously been said to have an advantage at accessing atmospheric nutrients. Leaf hairs of the Proteaceae species probably have multiple functions; perhaps these functions are related to the type of hair e.g. long or short and curly or straight. Therefore further studies should lay emphasis on hair type and characteristics.

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Table 1. Leaf characters, habitat, altitudinal range and plant height of 12 *Proteaceae* species used. Data is from Rebelo (2001). Pubescence was scored with the naked eye, although many of the species had sparse hair visible under a microscope.

Species	**Leaf dimensions**		**Pubescence**		**Habitat**	**Altitude range (m)**	**Height (m)**
	average width (mm)	**average maximum length(mm)**	**haircode**	**Measured * (# m^{-2})**			
Ls glabrum	30 - 50	80 - 120	hairless	718±83.49	cool, southern slopes on peaty soils	150 - 500	2.5
Ld discolor	15 - 17 (males) 17 - 20 (females)	30 - 35 (males) 40 - 48 (females)	young leaves hairy with a tuft of hairs on tip	4±3.86	Rocky sandstone soils	450 - 500	2 (rarely 4)
Pr cynaroides	20 - 30	120 - 300	hairless	0	extremely varied	0 - 1500	0.3 - 2
Ld coniferum	7 (males) 9.5 (females)	77 (males) 83 (females)	Hairy juvenile, hairless mature	135±20.42	Windblown sands	0 - 300	4
Ls conocarpodendron	25 50	60 - 115	dense layer of short hairs, interspersed with long hairs	205±23.48	Granite and sandstone soils, dry slopes facing north and west	0 - 160	3-5
Pr repens-red	5 - 18	50 - 150	hairless	12±6.68	extremely varied	0 - 1500	4.5
Ld pubescens	3 - 7 (males) 7 - 15 (females)	16 - 28 (males) 25 - 57 (females)	hairless or with silvery hairs	424±13.92	sandstone and quartzite soils	60 - 1700	0.6 - 2.5
Ls cordifolium	20 - 45	20 - 80	Juvenile slightly hairy, hairless mature	911±103.63	Sandstone derived soils	30 - 500	1.5
Pr neriifolia	14 - 30	100 - 180	Hairy juvenile, hairless mature	19±10.21	Sandstone and occasionally granite soils	0 -1300	1.5-3
Ld meridianum	7	40	silver hairs to hairless	4724±803.49	limestone soils	0 - 200	2
Ls reflexum	5 - 13	20 - 55	dense, crispy hairs with 2 or three glandular teeth	3431±544.43	near streams or sandstone soils	1000 - 2000	4
Pr burchellii	7 - 20	70 - 170	hairless	123±32.97	varied but favoring richer soils	100 - 850	1 - 2

* Measurement from plants obtained from Kirstenbosch Nursery

Figure Legends

Figure 1a. Microscope images of hair on abaxial side of first six Proteaceae species acquired from the Kirstenbosch Nursery Cape Town. From left to right downwards: *Ls. glabrum, Ld. discolor, Pr. cynaroides, Ld. coniferum, Ls, conocarpodendron* and *Pr. repens-red* Bars 200 µm.

Figure 1b. Microscope images of hair on abaxial side of last six Proteaceae species acquired from the Kirstenbosch Nursery, Cape Town. From left to right downwards: *Ld. pubescens, Ls. cordifolium, Pr. neriifolia, Ld. meridianum, Ls. reflexum* and *Pr. burchellii.* Bars 200 µm.

Figure 2. Correlation between total (abaxial + adaxial) leaf hair density of 12 Proteaceae species compared with log of their water holding capacity, for all three genera: a) *Protea* species (P = 0.00, r = 0.87 to); b) *Leucadendron* species (P = 0.00 , r = 0.89) and c) *Leucospermum* species. (P = 0.05, r = 0.88). Symbols and bars represent means ±S.E. (n=3). The a-historical Pearson correlation coefficient (r) and letters indicating significant differences are shown on the graph.

Figure 3. The relationship of total (abaxial + adaxial) hairiness of 12 Proteaceae species with fraction of nitrate label (Δ^{15}N) taken up by treated leaves a) for *Protea* species b) *Leucadendron* species and c) *Leucospermum* species. Considered over all three genera there is no relationship between leaf hair density and Δ^{15}N of nitrate treated leaves (PIC P = 0.03 to 0.08, r = 0.044 to 0.56). Symbols and bars represent species mean ± S.E. (n=3). The a-historical Pearson correlation coefficient (r) and letters indicating significant differences are shown on the graph.

Figure 4. The variation in measures of total (abaxial + adaxial) hairiness of 12 Proteaceae species with Δ^{15}N of ammonium treated leaves a) for *Protea* species b) *Leucadendron* species and c) *Leucospermum* species. Considered over all three genera there is no relationship between leaf hair density and Δ^{15}N of ammonium treated leaves (PIC P = 0.11 to 0.29, r = 0.18 to 0.39).

Symbols and bars represent species mean ± S.E. (n=3). The a-historical Pearson correlation coefficient (*r*) and letters indicating significant differences are shown on the graph.

Figure 5. The relationship between total (abaxial + adaxial) hairiness of 12 Proteaceae species and the fraction of glycine label ($\Delta^{15}N$) taken up by treated leaves a) for *Protea* species b) *Leucadendron* species and c) *Leucospermum* species. Considered over all three genera there is no relationship between leaf hair density and $\Delta^{15}N$ of glycine treated leaves (PIC $P = 0.02$ to 0.03, $r = 0.66$ to 0.74). Symbols and bars represent species mean ± S.E. (n=3). The a-historical Pearson correlation coefficient (*r*) and letters indicating significant differences are shown on the graph.

Figure 6. Correlations between total (abaxial + adaxial) hairiness of 12 Proteaceae species and fraction of glycine label ($\Delta^{15}N$) taken up by treated leaves. Symbols represent species replicates. Linear regression lines are shown with the same color code as symbols, the dashed line is the linear regression over all three genera and the a-historical Pearson correlation coefficient (*r*) is shown on graph (PIC $P = 0.02$ to 0.03, $r = 0.66$ to 0.74).

Figure 7. The fraction of deuterated water (Δ^2H) that contributed to δ^2H signal in leaves of each species after labeling in relation to total (abaxial + adaxial) hairiness of four *Protea* species. Symbols and bars represent species mean ± S.E. (n = 3).

Figure 8. Distribution of hairiness of leaves of three Proteaceae genera of the CFR are all significantly different ($F(1,2) = 21.55$, $P = 0.00$). Bars represent 100% of Ld – *Leucadendron* (n = 89), Pr – *Protea* (n = 68), and Ls – *Leucospermum* (n = 47) respectively. Letters mark significant differences according to Fisher HSD test.

Figure 9. Distance from coast (Oceanic Index, 0 = on the coast to 4 = furthest inland) and hairiness of three genera of Proteaceae of the CFR. Symbols and bars represent mean ± S.E. for: Ld – *Leucadendron* (n = 89), Pr – *Protea* (n = 71), and Ls – *Leucospermum* (n = 47). Letters mark significant differences according to Fisher HSD test.

Figure 10. Total hair (abaxial + adaxial) correlated with the average height for 37 Proteaceae species of the CFR. Considered over all 37 species there is a positive relationship between hairiness and plant height (A-historical $r = 0.38$, $P < 0.05$).

Fig. 1 a

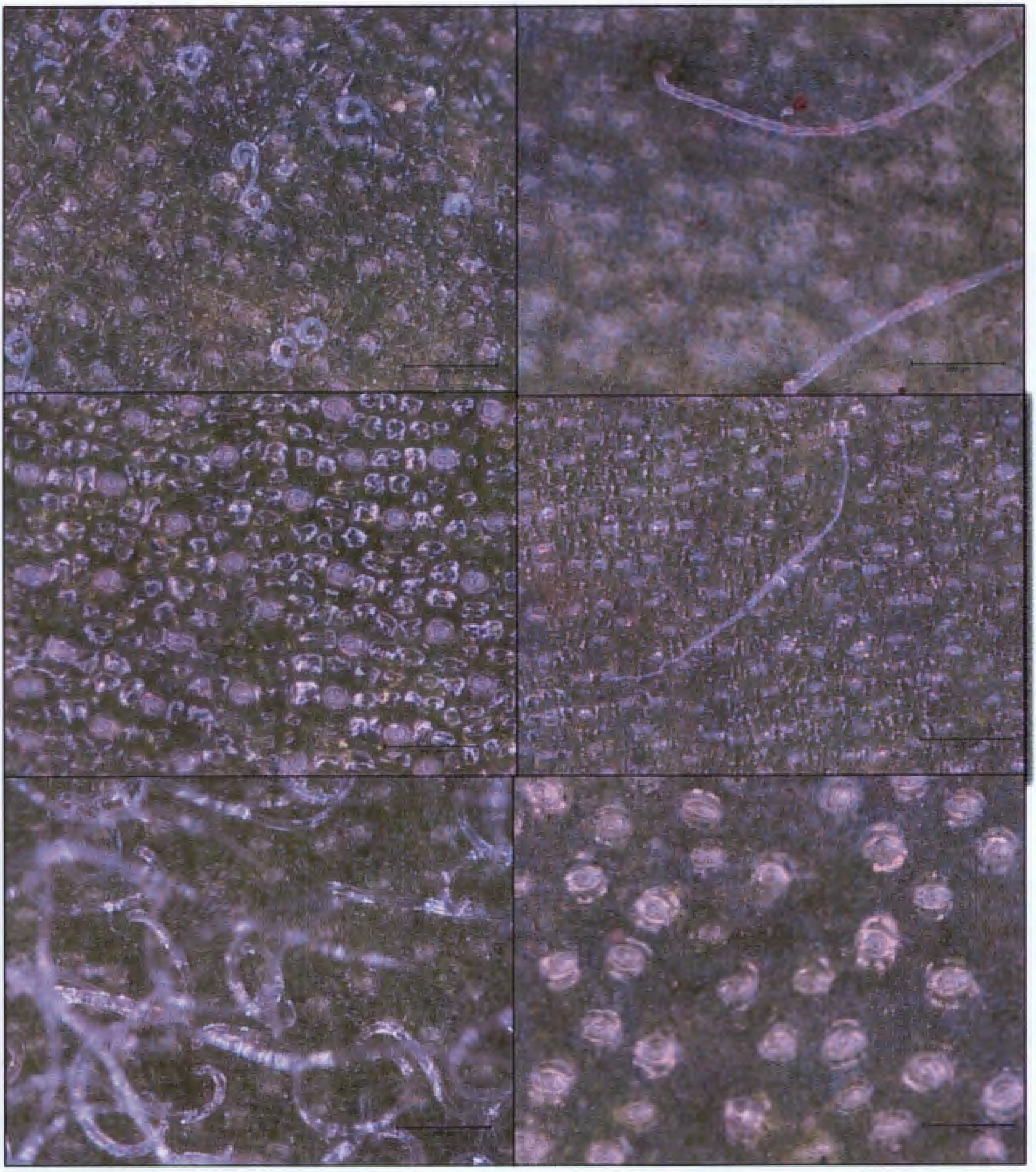


Fig. 1 b

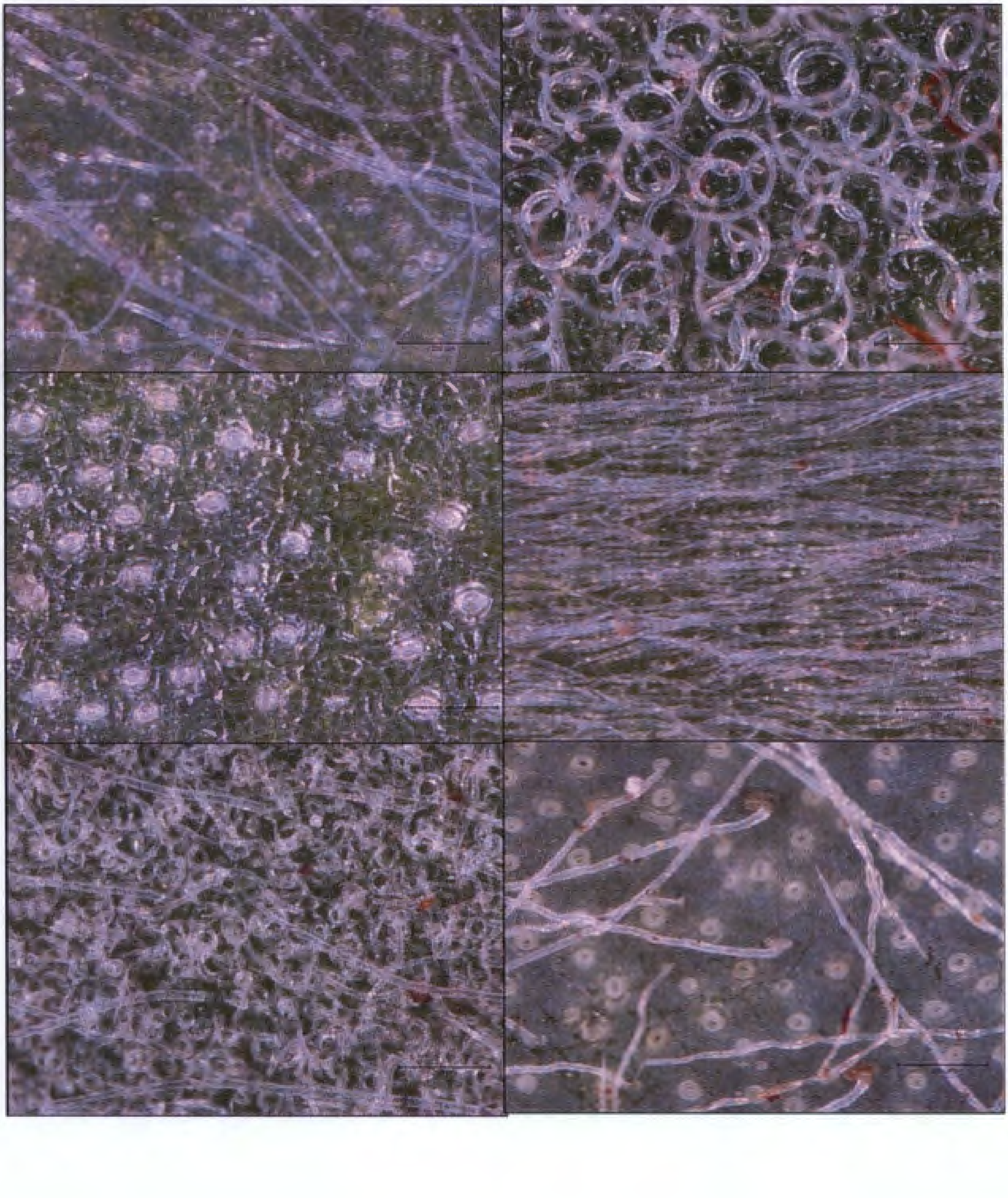


Fig. 2

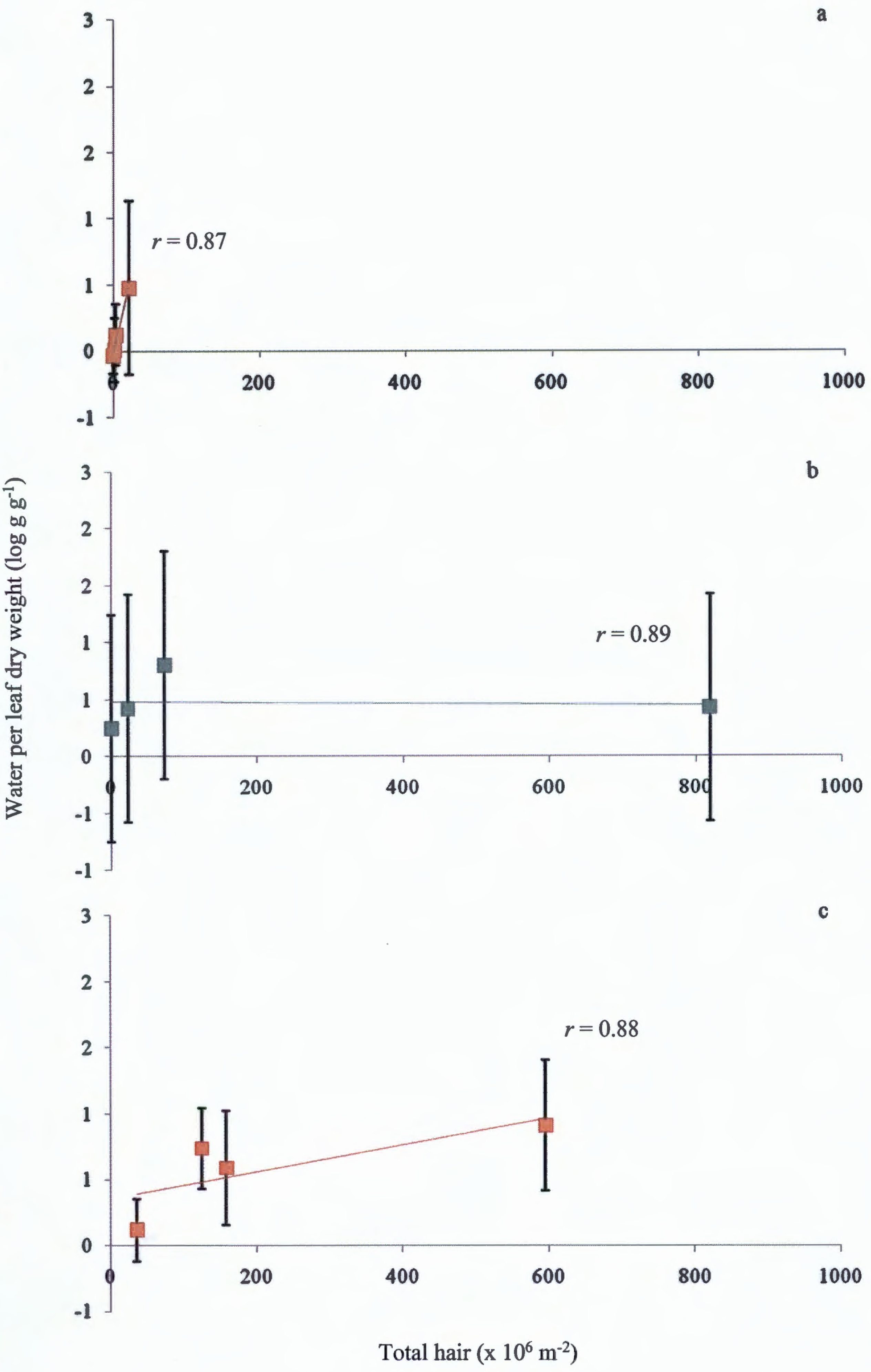

a
3
2
2
1
r = 0.87
1
0
0
200
400
600
800
1000
-1
b
3
2
2
1
r = 0.89
1
0
0
200
400
600
800
1000
-1
-1
c
3
2
2
r = 0.88
1
1
0
0
200
400
600
800
1000
-1
Water per leaf dry weight (log g g^{-1})
Total hair (x 10^6 m^{-2})

Fig. 3

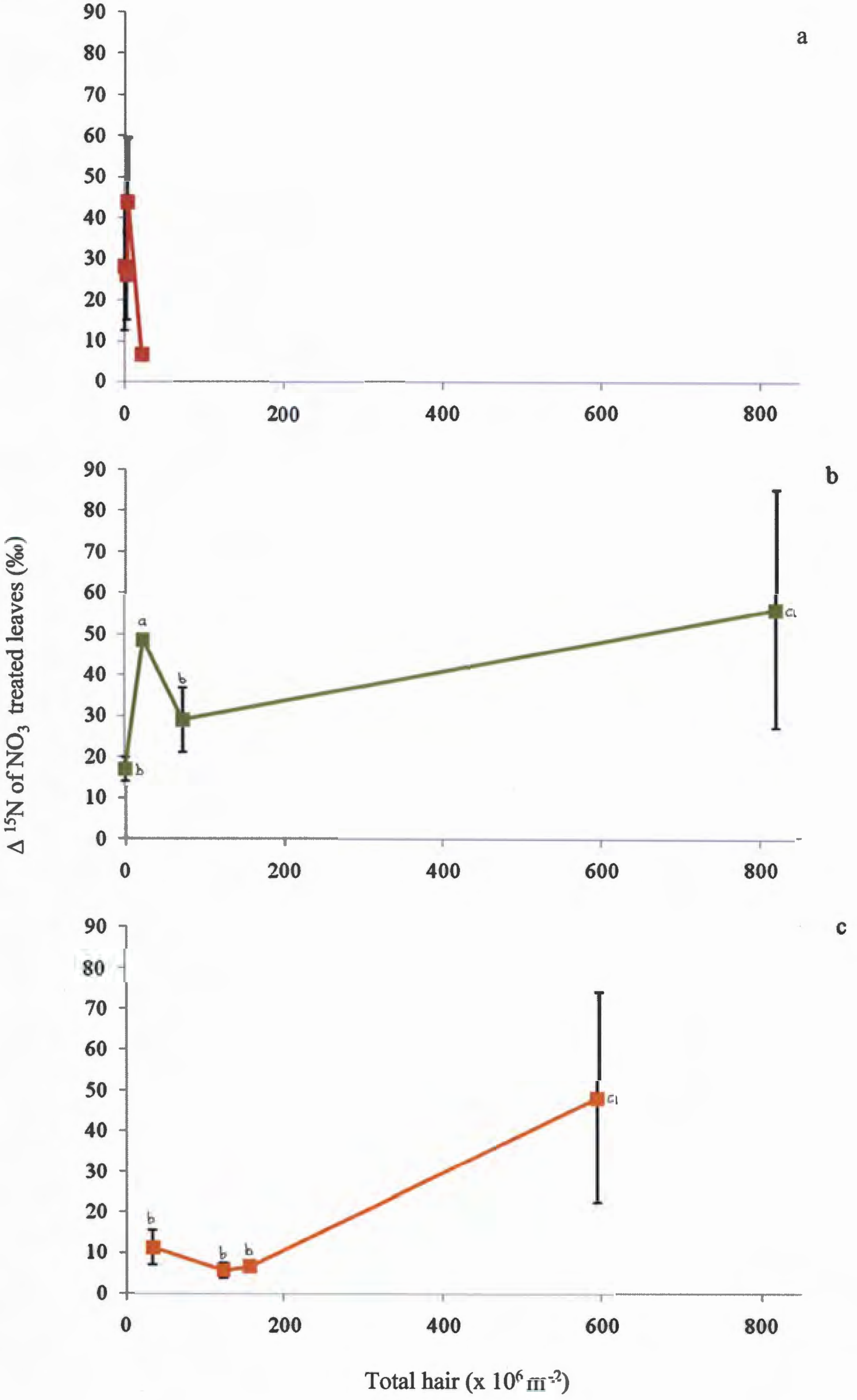

a
b
c
90
80
70
60
50
40
30
20
10
0
0
200
400
600
800
a
b
b
a
b
b
b
a
Δ ^{15}N of NO_3 treated leaves (‰)
Total hair (x 10^6 m^{-2})

Fig.4

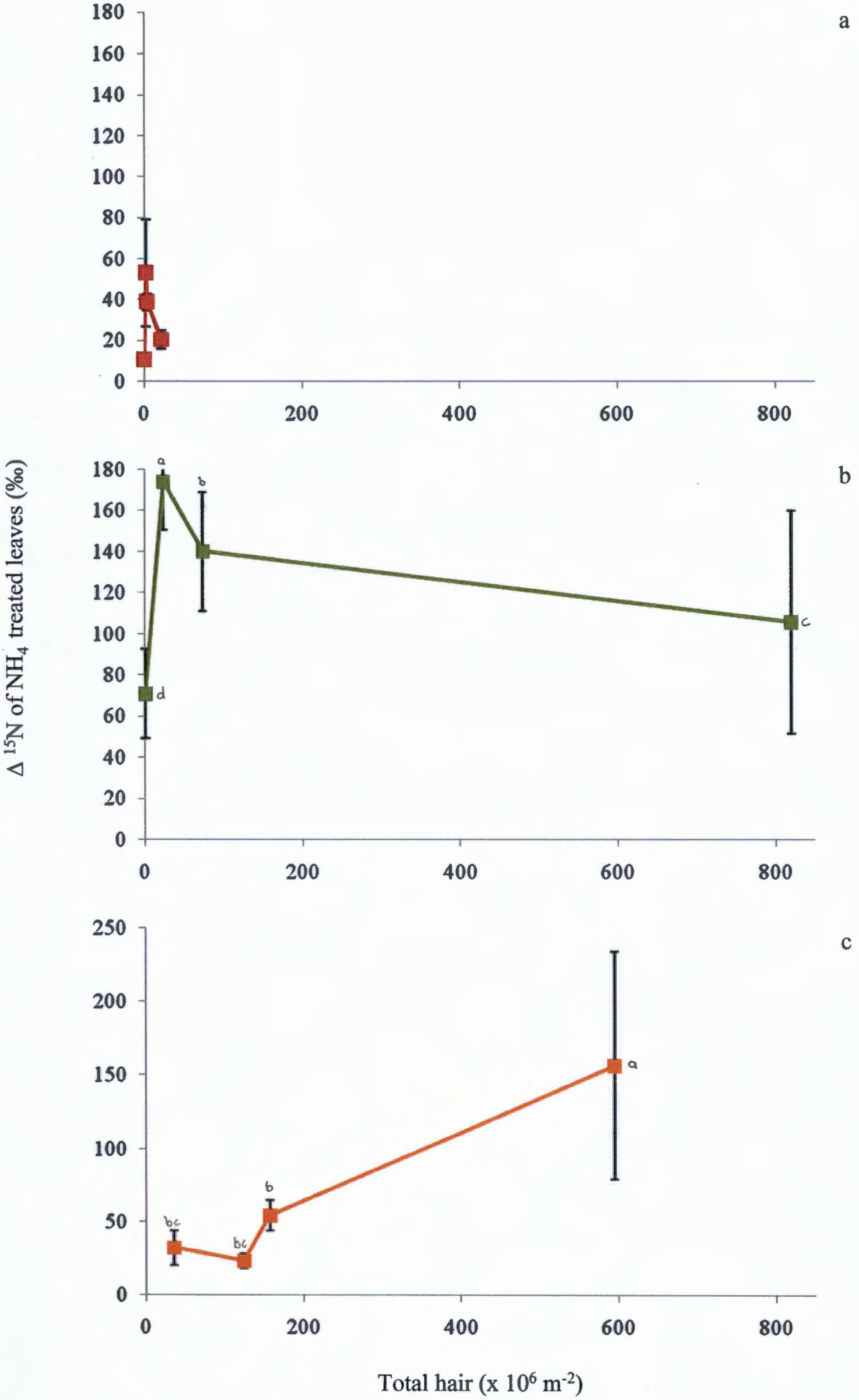

a
180
160
140
120
100
80
60
40
20
0
0
200
400
600
800
b
a
b
d
c
$\Delta\ ^{15}N$ of NH_4^+ treated leaves (‰)
c
250
200
150
100
50
0
bc
bc
b
a
Total hair ($\times 10^6\ m^{-2}$)

Fig. 5

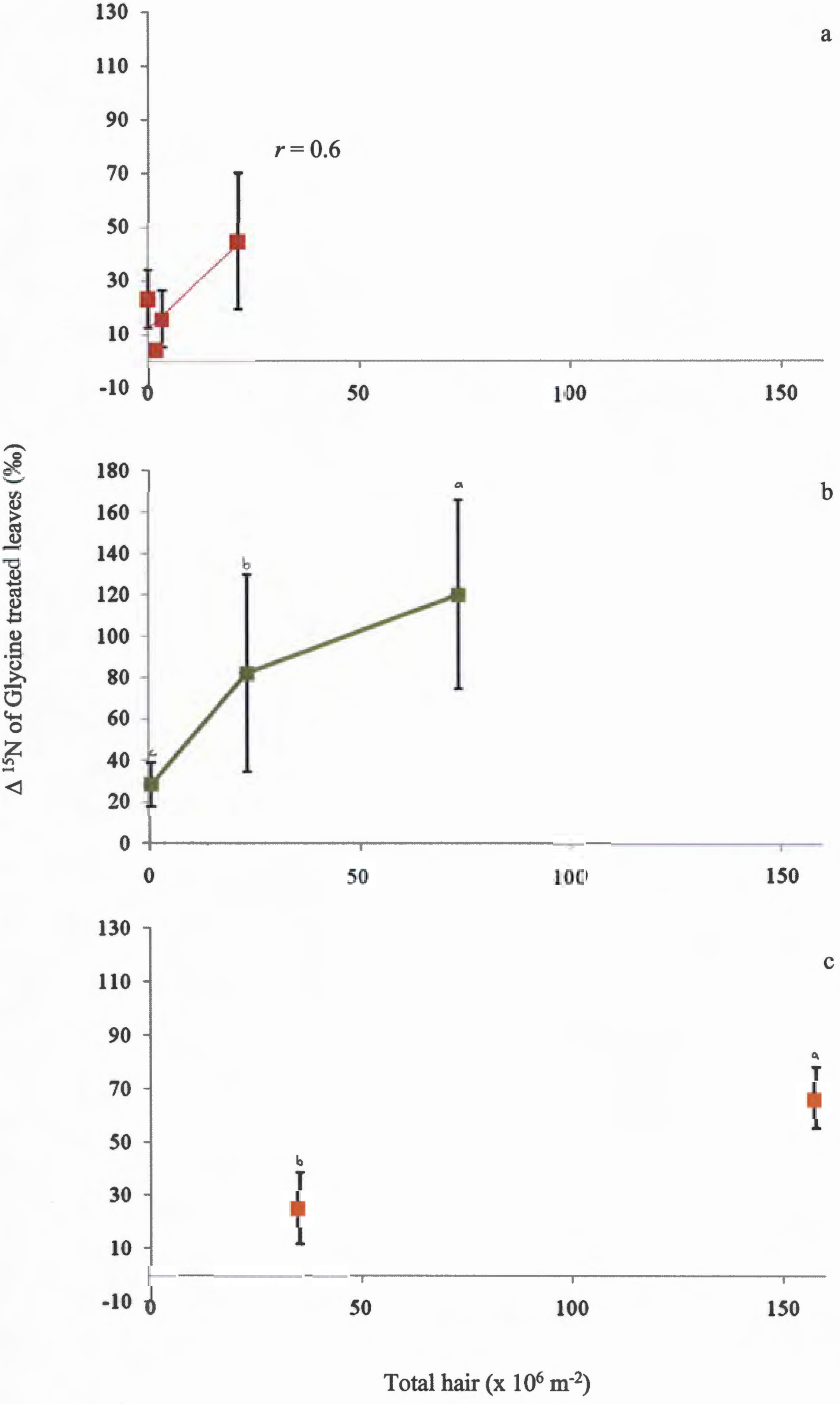

a
r = 0.6
130
110
90
70
50
30
10
-10
0
50
100
150
b
a
b
c
180
160
140
120
100
80
60
40
20
0
0
50
100
150
c
a
b
130
110
90
70
50
30
10
-10
0
50
100
150
Δ ^{15}N of Glycine treated leaves (‰)
Total hair (x 10^6 m^{-2})

Fig. 6

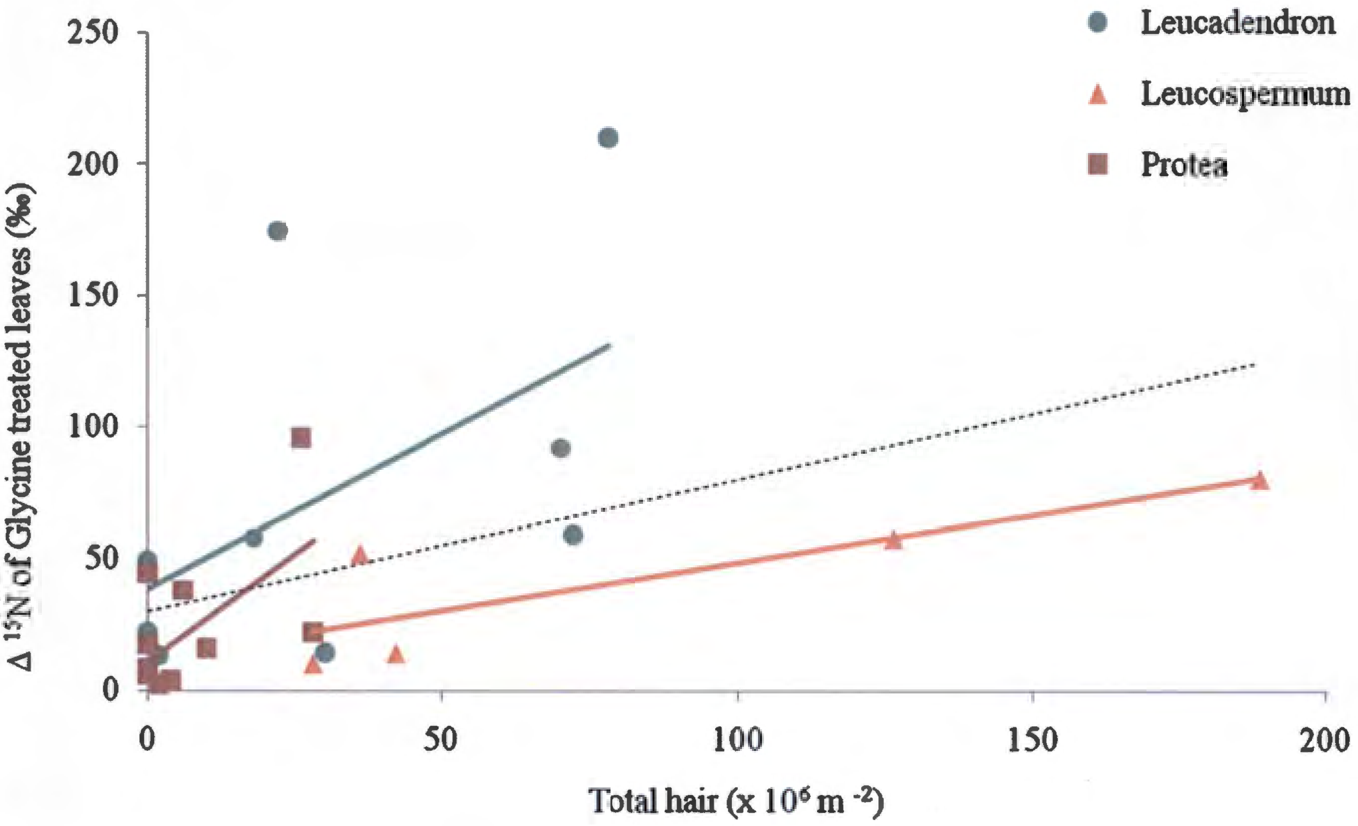
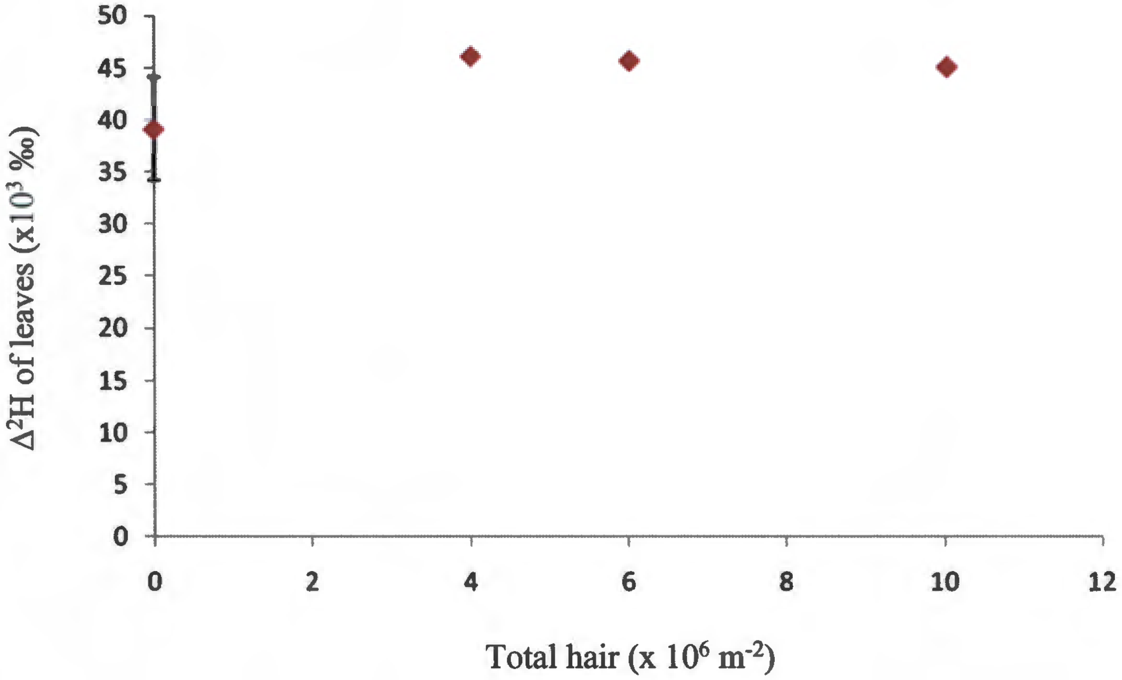

● Leucadendron
▲ Leucospermum
■ Protea
250
200
150
100
50
0
Δ ^{15}N of Glycine treated leaves (‰)
0
50
100
150
200
Total hair (x 10^6 m $^{-2}$)

Fig. 7



50
45
40
35
30
25
20
15
10
5
0
Δ^2H of leaves (x10^3 ‰)
0
2
4
6
8
10
12
Total hair (x 10^6 m^{-2})

Fig. 8

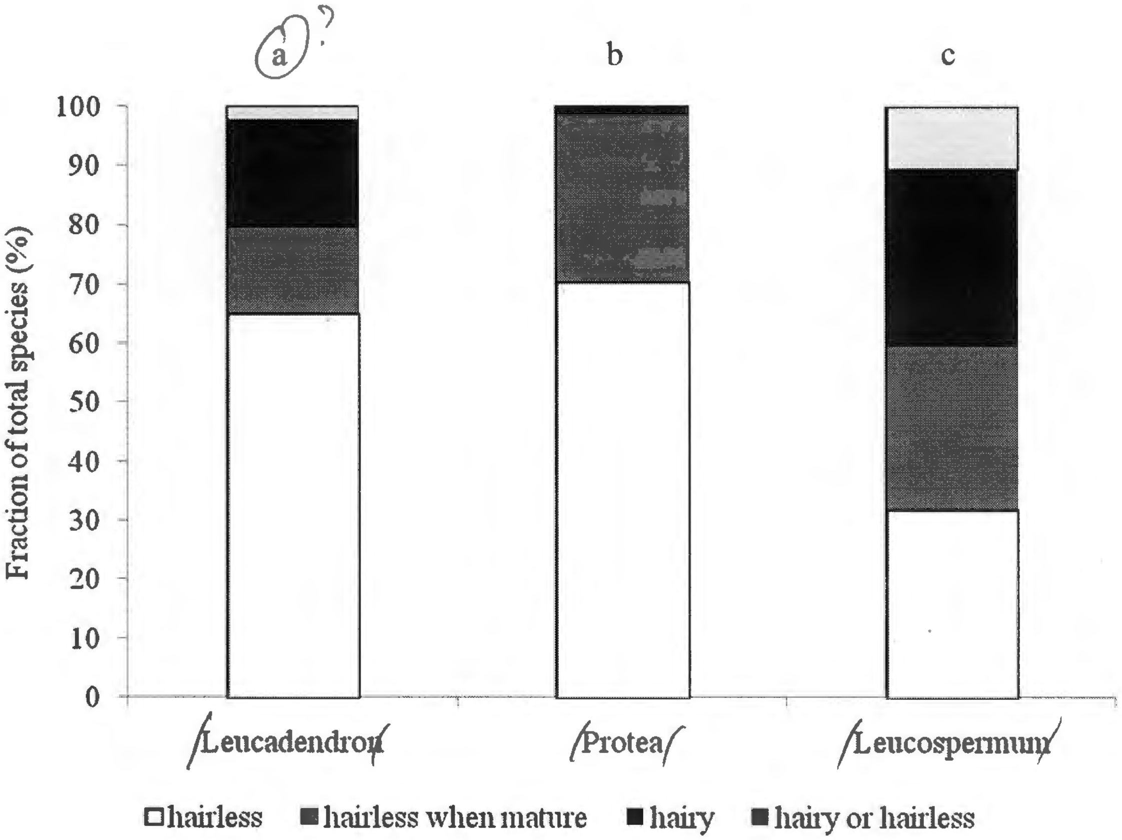

a
?
b
c
Fraction of total species (%)
100
90
80
70
60
50
40
30
20
10
0
Leucadendron
Protea
Leucospermum
□ hairless
▨ hairless when mature
■ hairy
▨ hairy or hairless

Fig. 9

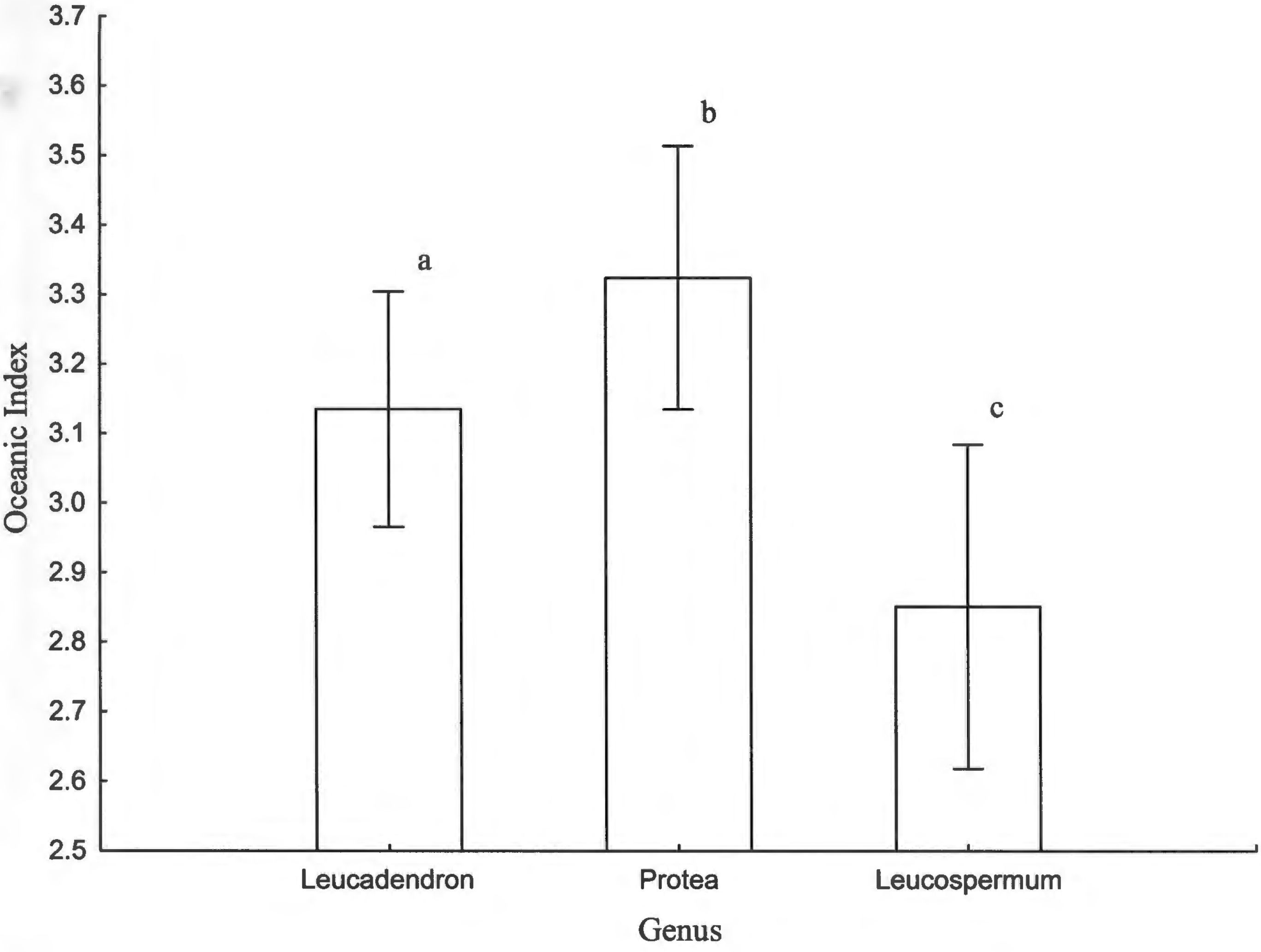
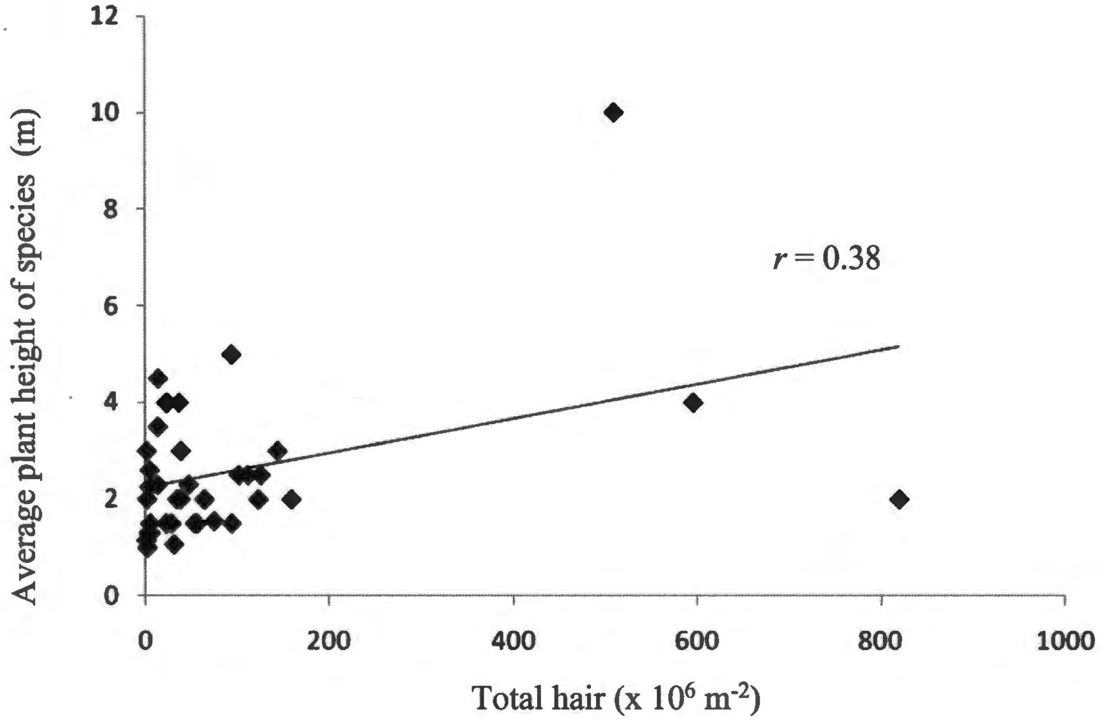

3.7
3.6
3.5
3.4
3.3
3.2
3.1
3.0
2.9
2.8
2.7
2.6
2.5
Oceanic Index
a
b
c
Leucadendron
Protea
Leucospermum
Genus

Fig. 10



12
10
8
6
4
2
0
Average plant height of species (m)
0
200
400
600
800
1000
Total hair (x 10^6 m^{-2})
$r = 0.38$