Tight control of sulfur assimilation: an adaptive mechanism for a plant from a severely phosphorus-impoverished habitat

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Summary

- *Hakea prostrata* (Proteaceae) has evolved in extremely phosphorus (P)-impoverished habitats. Unlike species that evolved in P-richer environments, it tightly controls its nitrogen (N) acquisition, matching its low protein concentration, and thus limiting its P requirement for ribosomal RNA (rRNA). Protein is a major sink for sulfur (S), but the link between low protein concentrations and S metabolism in *H. prostrata* is unknown, although this is pivotal for understanding this species’ supreme adaptation to P-impoverished soils.

- Plants were grown at different sulfate supplies for five weeks and used for nutrient and metabolite analyses.

- Total S content in *H. prostrata* was unchanged with increasing S supply, in sharp contrast with species that typically evolved in environments where P is not a major limiting nutrient. Unlike *H. prostrata*, other plants typically store excess available sulfate in vacuoles. Like other species, S-starved *H. prostrata* accumulated arginine, lysine and O-acetylserine, indicating S deficiency.

- *Hakea prostrata* tightly controls its S acquisition to match its low protein concentration and low demand for rRNA, and thus P, the largest organic P pool in leaves. We conclude that the tight control of S acquisition, like that of N, helps *H. prostrata* to survive in P-impoverished environments.

**Key words:** *Hakea prostrata*, metabolite profiling, sulfur uptake, phosphorus-use efficiency, plant nutrition, Proteaceae.
Introduction

Sulfur (S) is an essential macronutrient. It is the fourth most abundant nutrient by mass in plants after nitrogen (N), phosphorus (P) and potassium (K), and is required for plant growth and development (Nikiforova et al., 2004). Plants take up S from soil mostly as $\text{SO}_4^{2-}$ (Leustek & Saito, 1999) and transport the anion within the plant for subsequent assimilation through sulfate transporters (Rouached et al., 2009). During S assimilation, $\text{SO}_4^{2-}$ is first activated by adenylation to adenosine 5’ phosphosulfate (APS) by ATP sulfurylase (ATPS).

APS is reduced to sulfite by APS reductase (APR) and then to sulfide by sulfite reductase (Kopriva & Rennenberg, 2004). In a separate pathway, sulfite is also used in sulfolipid biosynthesis (Benning, 1998). However, sulfide is incorporated into a specific serine-derived precursor, O-acetylserine (OAS), by OAS-(thiol) lyase (OAS-TL) in the cysteine synthase complex to produce the amino acid cysteine (Wirtz et al., 2001).

O-acetylserine links S metabolism to N metabolism via serine-glycine metabolism, and to carbon metabolism through acetyl-CoA derived from pyruvate oxidation (Hell, 1997). Moreover, cysteine, being a building block for the synthesis of methionine, protein and glutathione, further interconnects S metabolism to N metabolism in plants (Hell, 1997; Nikiforova et al., 2004). Sulfur metabolism is regulated by N nutrition in plants, as N limitation reduces APR activity, resulting in decreased S assimilation (Koprivova et al., 2000). Conversely, S limitation affects N metabolism by decreasing cysteine biosynthesis, which ultimately reduces protein synthesis in the plant (Nikiforova et al., 2006).

We recently showed that N acquisition and assimilation into protein are tightly controlled in *H. prostrata* (Prodhan et al., 2016), a species that has evolved in the extremely P-impoverished habitats of south-western Australia (Lambers et al., 2011; Veneklaas et al., 2012; Lambers et al., 2015) where total P accounts for only one-twentieth (Lambers et al.,
2012b) of that in unfertilised crop or pasture soils (Hedley et al., 1982). Controlled N acquisition and thus assimilation reduces the demand for ribosomal RNA (rRNA), the largest organic P pool in leaves (Veneklaas et al., 2012). This N-acquisition strategy along with several physiological and biochemical traits underlies the remarkable adaptation of this species to a low-P environment that differs fundamentally from what is known for plants that evolved in nitrogen (N)-limited habitats (Lambers et al., 2015).

The striking physiological and biochemical adaptations in *H. prostrata* to a low-P environment include production of specialised cluster roots that mine P from inaccessible P-complexes in the root zone through carboxylate exudation (Lambers et al., 2010). In addition, this species economises the use of the P it acquires by being highly efficient and proficient in remobilising it from senescing tissues to deliver P to developing tissues (Lambers et al., 2012a). Leaf membrane lipids are also remodelled to replace P-containing phospholipids with non-P-containing lipids, which has no impact on photosynthesis (Lambers et al., 2012b). Furthermore, this species has a very low level of ribosomal RNA (rRNA) (Sulpice et al., 2014).

A low level of rRNA is associated with a low level of protein (Elser et al., 1996; Matzek & Vitousek, 2009). Therefore, the tight control of N acquisition and assimilation into protein is linked to the low level of rRNA, because most of the total N in plant goes to protein. However, this N response in *H. prostrata* likely requires modification in S metabolism, because i) there is a strong cross-talk between N and S metabolism in plants (Koprivova et al., 2000) (Nikiforova et al., 2006), and ii) the protein pool is also a sink for the majority of total S in plants (Nikiforova et al., 2005; Bimbraw, 2008; Zhang et al., 2015). However, S nutrition in *H. prostrata* has never been investigated and may reveal a link between S metabolism and the remarkable adaptation of this species to the world’s most severely P-impoverished soils. Therefore, we surmise that the tight control of N acquisition and
assimilation into protein modifies S acquisition and assimilation in *H. prostrata* to match its low protein concentration, and thus reduces its P requirement for rRNA, as an adaptation to an extremely low level of P in its P-impoverished environments.

**Materials and Methods**

**Plant growth**

Roots of soil-grown six-month-old *Hakea prostrata* R.Br. plants were washed and treated with 0.01% (v/v) sodium hypochlorite for eight min to avoid fungal contamination. The washed plants were transferred to a hydroponics tank in a temperature-controlled glasshouse at the University of Western Australia and grown during May to August 2014. The tank temperature was maintained at 18 to 20 °C. Individual plants were grown in a 5 L plastic pot containing 4 L modified Hoagland’s solution (100 µM Ca(NO$_3$)$_2$, 100 µM K$_2$SO$_4$, 2.5 µM KH$_2$PO$_4$, 27 µM MgSO$_4$, 0.1 µM MnCl$_2$, 0.1 µM ZnSO$_4$, 0.025 µM CuSO$_4$, 1.25 µM H$_3$BO$_3$, 0.0075 µM CoCl$_2$, 0.025 µM (NH$_4$)$_6$Mo$_7$O$_{24}$, 5 µM Fe-EDTA, 10 µM KCl, 33.3 µM Na$_2$O$_2$Si; pH 5.8). Before starting the treatments, plants were acclimated to the hydroponics for a six-week period with the nutrient solution changed twice a week. During the acclimation period, plants were grown on low Pi (2.5 µM) to avoid P-toxicity, because *H. prostrata* plants have evolved on the most P-impoverished soils on earth (Lambers *et al.*, 2015) and are extremely sensitive to P (Lambers *et al.*, 2013). Glasshouse temperature was maintained at 14 °C at night and 25 °C during the day on an average. The average highest light intensity was 680 µmol m$^{-2}$ s$^{-1}$ (70% transmission through the glass) between 12:00 h and 14:00 h, with sunrise at 07:15 h and sunset at 17:30 h. Minimum and maximum relative humidity inside the glasshouse were 32% during the day and 62% at night on an average.
After acclimation, $H_2PO_4^-$ and $SO_4^{2-}$ treatments were applied to the plants in a binary combination with one nutrient varying at a time. Four plants were randomly assigned to each treatment for five weeks. At the start of the treatments, a further four plants were harvested to determine the pre-treatment metabolic status of the plants. The entire nutrient solution was changed twice a week with doses of $H_2PO_4^-$ to a final concentration of 10 µM or 20 µM, and $SO_4^{2-}$ to a final concentration of 0, 77 µM or 1000 µM. Plants grown on low $SO_4^{2-}$ (77 µM) were supplied a level of $H_2PO_4^-$ (10 µM) that is standard for *H. prostrata*. The $H_2PO_4^-$ supply was increased to 20 µM for the increased $SO_4^{2-}$ supply (1000 µM) to ensure that $H_2PO_4^-$ was not limiting under the high $SO_4^{2-}$ treatment. During the treatment period, plants were visually screened for any symptoms arising from limiting or excess supply of $H_2PO_4^-$ and $SO_4^{2-}$. At the end of treatment, five to six fully-expanded mature leaves from the top of the main stem were harvested together, weighed, snap-frozen in liquid nitrogen and stored at -80 °C until later analysis. The rest of the plant was weighed immediately to determine fresh weights, oven dried at 70 °C for a week, and weighed again to determine dry weights. Concentrations of $H_2PO_4^-$, $SO_4^{2-}$, individual amino acids, total soluble protein, and chlorophyll were determined in the snap-frozen mature leaves. Total S and P concentrations were determined in oven-dried mature leaves, stems, and roots. Total S and P contents in leaves, stems and roots were calculated from the S and P concentrations and the biomass.

**Determination of nutrient elements**

Leaves, stems and roots were oven dried (70 °C, 48 h) and ground using a Teflon-coated stainless steel ball mill. An aliquot of 0.2 g ground material was acid-digested using concentrated $HNO_3:HClO_4$ (3:1) and subjected to radially configured inductively coupled plasma optical-emission spectrometry (ICP-OES) (Simultaneous Varian Vista Pro, Varian Australia Pty Ltd, Mulgrave, Victoria, Australia) equipped with an auto-sampler (A.I.
Scientific AIM-3600) and a charge-coupled device (CCD) detection system (ICP-OES; ChemCentre, Perth, WA, Australia). Concentrations of S and P were determined in leaves, stems and roots, while those of Boron (B), Molybdenum (Mo), Calcium (Ca), Potassium (K), Magnesium (Mg), Sodium (Na), Copper (Cu), Iron (Fe), Manganese (Mn) and Zinc (Zn) were determined only for leaves.

**Determination of total soluble protein**

Plant materials were ground cryogenically (Cryorobot, Labman, North Yorkshire, UK). An aliquot of 20 mg ground powder was extracted by ethanol for three times for 30 min each at 120 °C. The first and second extractions were with 250 µL and 150 µL, respectively, of 80% (v/v) ethanol buffered with 10 mM 2-(N-morpholino) ethanesulfonic acid, pH 5.9. The final extraction was carried out with 250 µL of 50% (v/v) ethanol buffered as mentioned above. The extraction was resolved by centrifugation for 10 minutes at 5200 x g after each of the above extractions. The supernatant was removed and the pellet was resuspended in 400 µL of 0.1 M NaOH by a benchtop thermal mixer at 120 °C for 30 minutes. After cooling the solution to 25 °C, the suspension was clarified by centrifugation at 10,620 x g for 10 min. Finally, total soluble protein concentration was spectrophotometrically determined in the supernatant (Bradford, 1976).

**Determination of leaf pigments**

Two ml pre-cooled (-20°C) methanol was added to an aliquot of 25 mg cryogenically ground leaf tissue. The mixture was homogenised and incubated in the dark at 4 °C. After 30 min of incubation, the mixture was centrifuged at 21,000 x g for 5 min and the supernatant was used to measure the absorbance at 470, 653 and 666 nm. The specific absorption coefficient in methanol was used to calculate chlorophyll a and b and total carotenoid concentrations in leaves (Lichtenthaler & Wellburn, 1983; Warren, 2008).
**Determination of amino acids and inorganic ions**

An aliquot of 50 mg ground leaf tissue was extracted with 360 µL pre-cooled (-20°C) extraction mixture (300 µL methanol, 30 µL 2 mg mL⁻¹ nonadecanoic acid methylester in CHCl₃, 30 µL 0.2 mg mL⁻¹¹³C₆-sorbitol in methanol) in a benchtop vortex with 550 rpm shaking at 70 °C for 15 min. The cap of the Eppendorf tubes was quickly opened and closed every minute to release the build-up pressure in the tubes. The solution was allowed to cool to 25 °C, mixed with 200 µL CHCl₃ and incubated at 37 °C with 600 rpm shaking for five min. After incubation, 400 µL of ultraliquid chromatography/mass spectrometry (ULC/MS) grade water was added to the solution and vortexed vigorously followed by centrifugation at 25 °C for 5 min at 21,000 x g. The supernatant was aliquoted in 100 µL and dried by rotary evaporation under vacuum.

For determination of individual amino acids, a vacuum-dried aliquot was dissolved in 45 µL 5 mM sodium phosphate buffer (pH 6.2) followed by vigorous mixing. Solutions were clarified by centrifugation at 21,000 x g for 15 min at 4 °C. An aliquot of 30 µL from the supernatant was used for HPLC analysis (Dionex UltiMate 3000 series HPLC equipped with a Hyperclone ODS C18 column, Thermo Scientific). Individual amino acids were determined by fluorescence detection and pre-column on-line derivatisation with O-phthalaldehyde (Lindroth & Mopper, 1979; Kim et al., 1997).

To determine ions, another vacuum-dried aliquot was dissolved in 550 µL ULC/MS grade water. H₂PO₄⁻ and SO₄²⁻ were separated and quantified by ion chromatography (Dionex ICS-3000, Dionex, Idstein, Germany). The anions were eluted with a KOH gradient up to 55 mM (KOH gradient: 0 min, 6 mM; 10 min, 45 mM; 12 min, 55 mM; 17 min, 6 mM). Data were collected and processed by chromeleon v6.8 software (Dionex). A standard curve for each ion was used to determine the corresponding ion concentration in the samples.
Determination of thiols and S-adenosyl methionine

An aliquot of 50 mg ground leaf tissue was extracted with 250 µL of 0.1 M HCl and 5.625 mg of polyvinylpolypyrrolidone (PVPP) washed with 0.1 M HCl by homogenising the sample mix in Retsch ball mill for one minute followed by centrifugation at 21,000 x g for 10 min at 4 °C. Another set of samples was spiked by adding 50 µM cysteine, 50 µM glutathione (GSH) and 50 µM S-adenosyl methionine (SAM) in 250 µL of 0.1 M HCl with the PVPP during sample extraction. Thiols were measured by a combination of monobromobimane fluorescent labelling and HPLC (Anderson, 1985; Fahey & Newton, 1987). A mixture of 20 µL of extract and 40 µL of 25 µM N-acetyl-cysteine as the internal standard was reacted with 10 µL of 8.5 mM N-ethylmorpholine and 3 µL of 30 mM tris (2-carboxyethyl) phosphine for 20 min at 37°C. After this reduction step, the reaction mix was reacted with 3 µL of 30 mM monobromobimane in acetonitrile for 20 min at 37°C in the dark. The labelling reaction was terminated by the addition of 10 µL of acetic acid and the resulting solution was subjected to HPLC analysis (Dionex UltiMate 3000 series HPLC, Thermo Scientific) equipped with a C18 column (KNAUER, Berlin, Germany). Thiols were eluted with an increasing methanol gradient comprising buffer A (0.25% acetic acid, pH 4.0) and buffer B (100% methanol) as described in Hubberten et al. (2012).

SAM was measured by HPLC with a UV detector (Dionex UltiMate 3000 series HPLC, Thermo Scientific) equipped with a Hyperclone ODS C18 column (Phenomenex). SAM was eluted with an increasing methanol gradient comprising buffer A (50 mM sodium dihydrogen phosphate and 8 mM octanesulfonic acid) and buffer B (100% methanol) as described in Zuchi et al. (2015). SAM was detected by UV absorption at 254 nm.

Data were corrected for the recovery of spikes added to plant samples at the start of extraction.
Statistics

Statistical analyses were carried out in R (R Development Core Team, 2013). Normality and equal variance of the data were tested by Shapiro-Wilk Normality test and Levene test, respectively. Log_{10} transformation of data was performed when the original data failed the assumptions of normality and equal variance tests. One-way ANOVA and Tukey’s honestly significant difference (HSD) were used to assess significant differences between the means and mean separation at p < 0.05, respectively.

Results

To determine the metabolic changes in *H. prostrata* in response to sulfur availability, we challenged established, nine-month old *H. prostrata* plants with S starvation, and a low and high supply of S. At the start of the experiment, we harvested four plants, labelled as ‘Pre-treatment’, to document the metabolic status of the plants before their exposure to the treatments. At harvest, these ‘pre-treatment’ plants were five weeks younger than the ones exposed to the treatments and had been grown on the low level of phosphorus (P) (2.5 µM) present during the hydroponics acclimation period. During the treatment period, the P level was increased to 10 µM P for the low-S (77 µM) treated plants. The P supply was two-fold higher (20 µM) for the high-S (1000 µM) treated plants to ensure that P was not limiting under the high-S treatment. We analysed both the ‘pre-treatment’ and ‘post-treatment’ plants and compared the metabolic status resulting from exposure to different S supplies.
**Hakea prostrata** tightly controls S accumulation in its organs at a set-point, despite a wide variation in sulfate supply

Remarkably, after five weeks of treatment, plants grown with 1000 µM sulfate had the same S content in their leaves, roots and stems as plants supplied with only 77 µM sulfate (Fig. 1a-c). There was an excess supply of sulfate to both sets of plants, as the total S content of the plants accounted for only 1 to 10% of the cumulative sulfate supplied over the treatment period.

Plants starved of sulfate had the same total S content in leaves, stems and roots as “pre-treatment” plants harvested five weeks earlier at the start of the treatment period (Fig. 1a-c). Thus, there was no net reallocation of S between organs or a net loss of S from the plant during the starvation treatment. By contrast, the S content in leaves, stems and roots of plants supplied with 77 µM sulfate was about twice that in the starved plants, indicating that growth within the five-week treatment period created a demand for S and that the lack of S acquisition was not simply due to the plants containing an excess of S at the start of the treatment. The S concentration was greater in leaves and stems of the pre-treatment plants compared with that of the treated plants (Fig. S1a,b), while the treated plants had 2.4- to 3.6-fold greater biomass due to five weeks more growth (Fig. 5a-c). However, like the S content, the S concentration was similar in the low-S and high-S treated plants (Fig. S1a-c).

In contrast to the situation with S, the organ concentration of total P responded strongly to only a two-fold difference in phosphate supply, as expected (Shane *et al.*, 2003) (Fig. S1e). Leaves and stems of plants supplied with 20 µM phosphate during the treatment period tended to have a two-fold greater P concentration (Fig. S1e) than those of plants supplied with 10 µM phosphate. Conversely, variation in sulfate supply had no effect on total P concentration in mature leaves, stems or roots of *H. prostrata* (Fig. S1d-f).
Sulfur metabolite concentrations do not respond to variation in sulfate supply

Regardless of sulfate starvation or supply, *H. prostrata* leaves had the same concentration of total soluble protein (TSP) (Fig. 2a), S-containing amino acids cysteine and methionine (Fig. 2c,d), S-adenosyl methionine (SAM, Fig. 2e), which is a major downstream intermediate whose production consumes 80% of total methionine compared with 20% consumed in protein synthesis (Giovanelli *et al.*, 1985), and glutathione (Fig. 2f). In addition, the concentration of these metabolites was similar to that in leaves of the pre-treatment plants (Fig. 2a,c-f). Among the non-S-containing amino acids, the concentrations of the amide amino acids asparagine and glutamine, and aspartate, the precursor of asparagine, were remarkably lower in the treated plants after the five-week treatment period compared with plants harvested before the start of the treatments (Fig. S2l-n). This reduction was similar among all three sulfate treatments, and thus independent of S supply (Fig. 2b). Being major constituents of the free amino acid pool in plants, the reduction in the concentration of these amino acids also led to an overall lower concentration of total free amino acids (TAA) in leaves of the treated plants compared with the pre-treatment plants (Fig. 2b). The leaf concentrations of the other amino acids that were profiled were about the same in leaves of plants, regardless of sulfate supply (Fig. S2a-k,o).

*Hakea prostrata* showed a sulfate-starvation response when sulfate supply was withheld

Sulfate starvation reduced the sulfate concentration in leaves compared with that in plants grown with sulfate (Fig. 3a). Interestingly, the sulfate-treated plants maintained a fairly constant leaf sulfate concentration of 20 nmol mg\(^{-1}\) FW, despite having a 13-fold range of sulfate availability in the growth medium (Fig. 3a). Furthermore, the leaf sulfate
concentration in the treated plants was similar to that in the pre-treatment plants, despite an extended growing period of five-weeks with high sulfate supply (Fig. 3a). In sharp contrast, the leaf phosphate concentration increased when the phosphate supply was increased by only two-fold (Fig. 3b).

As a typical response to sulfate-starvation, sulfate-starved *H. prostrata* accumulated arginine (Fig. 3c) and lysine (Fig. 3d) in its leaves compared with plants provided with sulfate. Leaf concentrations of these amino acids in low-S and high-S treatments were similar to those in the pre-treatment plants (Fig. 3c,d). Plants starved of sulfate also tended to have a two-fold greater leaf concentration of O-acetylserine (OAS), the non-sulfurylated precursor for cysteine biosynthesis and a marker of sulfate-starvation, than leaves of plants provided with sulfate (Fig. S3). On the other hand, plants grown on a low or high sulfate supply had a similar leaf concentration of OAS to the pre-treatment plants (Fig. S3).

Chlorophyll a and b concentrations in leaves decreased significantly under sulfate starvation in comparison with plants grown with a standard sulfate supply (Fig. 4a,b). However, increasing the sulfate supply beyond this standard level did not increase the concentration of these leaf pigments (Fig. 4a,b). The leaf concentration of total carotenoids was not responsive to sulfate supply (Fig. 4c).

*Hakea prostrata* biomass was non-responsive to short-term sulfate starvation or higher supply.

Whole-plant dry biomass increased in the treated plants by two- to three-fold compared with the pre-treatment plants during the five weeks of growth throughout the treatment period (Fig. 5a). Both shoot and root biomass increased during the treatment period (Fig. 5b,c). When the sulfate supply was withdrawn from the medium, *H. prostrata* was still able to put on as much biomass at the whole plant, shoot or root levels as when sulfate was supplied
On the other hand, *H. prostrata* had similar growth of the shoot and root at both 77 µM and 1000 µM sulfate in the growth medium (Fig. 5b,c).

Leaf molybdenum content was low when the sulfate supply was high in the medium

We surmise that the mechanism that allows *H. prostrata* to maintain a remarkably constant level of S in its organs over a wide range of sulfate supplies is to control sulfate acquisition through down-regulation of sulfate transporters in the roots (Hawkesford, 2007). To test this hypothesis, we used molybdenum as a proxy for sulfate transporter capacity, because sulfate transporters also transport molybdenum (Fitzpatrick *et al.*, 2008; Bittner, 2014). We determined molybdenum content and concentration in the leaves of *Hakea prostrata* across the sulfate treatments. Interestingly, plants grown with sulfate had only half of the molybdenum content in their leaves as plants grown without any sulfate (Fig. 6a). Furthermore, the molybdenum content was similar at both 77 µM and 1000 µM sulfate supplies, which was similar to that in the pre-treatment plants supplied with 77 µM sulfate (Fig. 6a). Not surprisingly, the lower content of molybdenum in the leaves of the sulfate-fed plants was associated with a lower concentration of molybdenum in the leaves of these plants relative to that in the sulfate-starved plants (Fig. 6b). This pattern of response of molybdenum was unique in comparison to any of the other nutrients that we profiled in the leaves of the same plants (Fig. S4a-p). Leaf content or concentration of calcium (Ca), potassium (K), magnesium (Mg), sodium (Na), copper (Cu), iron (Fe), manganese (Mn) or zinc (Zn) was non-responsive to sulfate supply during the treatment period (Fig. S4a-p). The Ca, Mg, Na, Cu, Mn and Zn content of the treated plants was also similar to that in the pre-treatment plants (Fig. S4b,f,h,j,n,p). As a result, treated plants had lower concentrations of these nutrients compared to the pre-treatment plants (Fig. S4a,e,g,i,m,o), simply because of the
dilution of these nutrients by growth (Fig. 5b). The treated plants did, however, accumulate K and Cu during growth in the treatment period, and this accumulation was independent of sulfate supply (Fig. S4c,d,k,l).

**Discussion**

Our results reveal that *H. prostrata* is exceptional in how it responds to sulfate availability compared with most other plants that have been studied. In a hydroponic culture, a 13-fold range in sulfate supply during an experimental period of five weeks resulted in no difference in S content in leaves, stems or roots of *H. prostrata*. Nearly all of the sulfate that was supplied in excess in the growth media was simply not taken up by the roots. Like S content, the average S concentration in leaves, stems or roots of *H. prostrata* was also firmly maintained, at a set-point of 1 to 3 mg g\(^{-1}\) DW, depending on the tissue, despite the large variation in sulfate supply.

The non-responsiveness of *H. prostrata* to the 13-fold range of sulfate supplies is not typical for plants. Rather, plants tend to increase internal S concentration with increasing supply of sulfate. For instance, the S concentration in leaves of sugar beet (*Beta vulgaris* cv Druid) increased 41% (1.9 to 2.6 mg g\(^{-1}\) DW) when the sulfate supply was increased only five-fold (150 to 750 µM). Leaf S concentration increased a further 66% (2.6 to 4.4 mg g\(^{-1}\) DW) when the sulfate supply was increased a further two-fold (Thomas *et al.*, 2000). This species also showed an increase in root S concentration with increasing S supply, although it was less dramatic than the increase in leaves (Thomas *et al.*, 2000). Likewise, the S concentration in leaves and roots of hydroponically-grown onion (*Allium cepa*) increased 300% (2.9 to 12.3 mg g\(^{-1}\) DW) and 600% (1.8 to 12.6 mg g\(^{-1}\) DW), respectively, when the sulfate supply was increased 40-fold (100 to 4000 µM) (McCallum & Pither-Joyce, 2002). Mungbean
(Phaseolus aureus) increased its leaf S concentration by 26% (1.5 to 1.9 mg g$^{-1}$ DW) due to increasing the S supply by four-fold (936 to 3742 µM) (Arora & Luthra, 1971). The model plant, Arabidopsis thaliana, grown in Petri dishes for 13 days with 100 or 915 µM sulfate had a whole-plant S concentration that was 72% greater at the higher supply (Nikiforova et al., 2003). Conversely, the remarkably constant total S concentration in leaves, stems and roots of H. prostrata over a 13-fold range of sulfate supply indicates that H. prostrata exhibits a tight control on its S acquisition which is fundamentally different from that found in other investigated species.

Maintaining a low S concentration in its organs is typical of the extremely P-use efficient H. prostrata. While H. prostrata plants grown here in hydroponics at the higher S supply had about 3 mg S g$^{-1}$ leaf DW, plants in their natural habitat typically have a slightly lower concentration (about 1.6 mg S g$^{-1}$ leaf DW$^*$; P.E. Hayes et al., unpublished). Although the S concentration in wild plants is above the critical limit (0.96 mg g$^{-1}$ leaf DW) required for plant growth and development (Epstein & Bloom, 2005), it is 15 to 20% lower than the median S concentration in leaves of 58 species of trees, shrubs and herbs sampled in their natural habitats (Fig. S5; Table S1).

Similar to leaf S concentration, H. prostrata also held its leaf protein concentration at a set-point. The protein concentration was non-responsive to the supply of sulfate over the range from complete starvation to an excess supply over a period of five weeks. This constant protein concentration was not unexpected, because protein is a major pool for plant S (Nikiforova et al., 2005; Bimbraw, 2008; Zhang et al., 2015). This result is consistent with a previous study (Prodhan et al., 2016) in which the protein level in leaves of H. prostrata was static and independent of N supply. In contrast, hydroponically-grown spinach (Spinacea oleracea) shows a 40% greater protein concentration in leaves at 1500 µM sulfate compared with that under S starvation (Warrilow & Hawkesford, 1998). In Brassica napus, the protein
concentration dropped by 20% in plants starved for sulfate for six days compared with that in plants that received 1500 µM sulfate in hydroponic culture (Zhang et al., 2015). Furthermore, when these plants were re-supplied with 1500 µM sulfate after S starvation for three days, the protein level increased sharply by 45%. Petri dish-grown Arabidopsis thaliana plants had a 2.5- to 3.5-fold higher protein concentration when the sulfate supply was 10-fold higher (134 to 1218 µM) (Nikiforova et al., 2005).

The distinctly different response to sulfate supply in *H. prostrata* than in other plants extends to the level of S-linked metabolites. The concentration of the first organic metabolite of S assimilation, cysteine, was independent of sulfate supply during the experimental period (Fig. 2c). In contrast, sulfate starvation results in a lower concentration of cysteine in other plants (Lencioni et al., 1997; Nikiforova et al., 2003; Krueger et al., 2010). Also in sharp contrast to other plants, the leaf methionine concentration in *H. prostrata* showed the same response as that of cysteine (Fig. 2d). In sugar beet, there was a 50% greater concentration of methionine in leaves when the sulfate supply was doubled (Thomas et al., 2000). Furthermore, the *de facto* end-product of methionine biosynthesis, S-adenosyl methionine (SAM), which consumes 80% of the methionine produced by a plant while only 20% is used in protein synthesis (Giovanelli et al., 1985; Hesse et al., 2004), did not change with increasing sulfate supply, but was rather independent of the sulfate supply (Fig. 2e). Furthermore, S-starved plants generally store N by accumulating the amides glutamine and asparagine in leaves (Migge et al., 2000). This, again, is in sharp contrast with *H. prostrata*, where the concentrations of these N-storing amino acids dropped dramatically during the five weeks of treatment regardless of the level of S or P supply. These responses, again, suggest that *H. prostrata* functions at a low level of internal N, as found by Prodhan et al. (2016). Likewise, the concentration of glutathione, a redox metabolite that contains cysteine, was unaffected by sulfate supplies of 77 µM and 1000 µM (Fig. 2f). In contrast to a response typical of other
plants (Nikiforova et al., 2005), the non-responsiveness of this S-containing metabolite to a 13-fold range in sulfate supply strongly suggests that S acquisition is tightly controlled in *H. prostrata*.

As intended, the pre-treatment growth did not satiate the sulfur requirement of the plants for the experimental period. Rather, pre-treated *H. prostrata* continued to take S from the nutrient solution over the five-week experimental period. Withdrawal of sulfate from the medium led to i) reduced sulfate concentration in leaves compared with that when sulfate was supplied (Fig. 3a), ii) accumulation of arginine and lysine (Fig. 3c,d), a sulfate-starvation response (Dijkshoorn & van Wijk, 1967), iii) a reduction in leaf photosynthetic pigment concentrations compared with plants under the standard sulfate supply (Fig. 4a,b), which has also been reported as a sulfate-starvation response in other plants (Nikiforova et al., 2005), and iv) a trend of accumulating OAS (Fig. S3), the precursor for cysteine biosynthesis and an indicator for S starvation (Watanabe et al., 2010). On the other hand, a greater supply of sulfate neither increased the abundance of S-containing metabolites in leaves nor sulfate storage in the organs, as the total S content in leaves, stems and roots was completely non-responsive to sulfate supply (Fig. 1a-c). The excess sulfate just remained as a surplus in the nutrient solution. Accordingly, the biomass at the whole plant, shoot or root level remained similar between the low-S and high-S supplies (Fig. 5a-c). Interestingly, the biomass production was also not responsive to sulfate withdrawal for the five-week treatment period (Fig. 5a-c). These results agree with a previous study that plant biomass production in *H. prostrata* can withstand a short-term perturbation in external nutrient supplies (Kuppusamy et al., 2014).

An S supply of 1000 µM led to a reduction in molybdenum content in leaves of *H. prostrata* compared with that when sulfate was withheld entirely (Fig. 6a). Likewise, the molybdenum concentration in the leaves of the sulfate-treated plants was less than that in plants grown...
without sulfate (Fig. 6b). Transport of molybdenum in the presence of sulfate has been studied in other species, where the presence of external sulfate resulted in reduced acquisition of molybdenum into roots of rice (Kannan & Ramani, 1978) and reduced translocation of sulfate from root to shoot in tomato (Stout et al., 1951). It has been reported that molybdenum can be transported by a sulfate transporter (Fitzpatrick et al., 2008; Bittner, 2014). In this study, we considered together the constant level of organ S in *H. prostrata* when provided with a high sulfate supply versus the low content of molybdenum in the leaves of the high-sulfate treated plants compared with sulfate-starved plants. However, one might envisage that the low response of *H. prostrata* to the greater availability of S was due to an excessive molybdenum uptake by the plants that led to down-regulation of the sulfate transporters. However, this is not supported by the present results. The molybdenum concentration in mature leaves of Proteaceae in their natural habitat is typically 0.01 to 0.14 µg g⁻¹ DW (Denton et al., 2007). While the present *H. prostrata* plants grown in hydroponics with a high S supply contained about 3 µg g⁻¹ leaf DW (Fig. 6), this is well below the critical concentration of 10-50 µg g⁻¹ DW molybdenum above which molybdenum toxicity likely occurs in plant tissues (Alloway, 1995). Taken together, this indicates that *H. prostrata* plants had lower leaf molybdenum concentrations than that at which toxicity occurs.

Proteaceae in south-western Australia, including *H. prostrata*, have evolved in a highly-weathered climatically-buffered infertile landscape with a Mediterranean climate (Hopper, 2009). The soils are characterised by very low levels of plant-available macro- and micronutrients including molybdenum (Lambers, 2014). Hence, molybdenum toxicity is not something this species would ever encounter in its natural habitat. Therefore, the low response of *H. prostrata* to S under the high S supply was not an adaptation to avoid molybdenum toxicity. On the other hand, the literature is clear about molybdenum being transported by sulfate transporters (Stout et al., 1951; Kannan & Ramani, 1978; Fitzpatrick et al., 2008; Bittner, 2014).
al., 2008; Bittner, 2014). In accordance, we surmise that *H. prostrata* was able to maintain a set-point of organ S concentration despite the high sulfate supply by down-regulating its sulfate transporters, which then also caused a decrease in molybdenum acquisition, as evidenced by the lower molybdenum content of leaves. In stark contrast, organ P concentration increased with increasing P supply in the growing media, reflecting the very low capacity of *H. prostrata* to down-regulate its P acquisition (Shane et al., 2004). However, even with a higher internal P status, *H. prostrata* did not translate this P response to increased need for S, as evidenced by a constant level of S in its leaves, stems and roots over a wide range of sulfate supply, and the constant concentration of total protein. These contrasting S and P responses of *H. prostrata* suggest that this species has evolved to maintain a low level of internal S, as it maintains a low level of internal N (Prodhan et al., 2016), as a result of low rRNA levels, and thus low P concentrations. This is in line with the very low level of available P in the severely P-impoverished environment inhabited of this species.

Finally, the entirety of our results as summarised in Fig. 7 indicates that *H. prostrata* tightly controls its sulfate acquisition, even when sulfate is supplied far in excess, and thus maintains a constant organ S concentration. By maintaining a set-point for total S uptake and assimilation that is independent of S supply, the plant limits production of S-containing organic metabolites, particularly the proteogenic amino acids cysteine and methionine, and total protein, which is a key pool of organic-S in the plant (Nikiforova et al., 2005; Bimbraw, 2008; Zhang et al., 2015). This strategy not only limits S use, and the energy needed for its assimilation, but, more importantly, also the requirement for P for rRNA by maintaining a constant low concentration of protein (Raven, 2013). This previously undocumented mechanism of *H. prostrata* to maintain S acquisition at a constant level is a fundamentally different strategy compared with that in other typically studied species, which have evolved in an environment where P is generally not the key limiting nutrient (Lambers et al., 2010).
Conversely, this mechanism contributes to the survival of *H. prostrata* in its severely P-impoverished environment as it enables this species to function at low levels of leaf P, including low levels of rRNA. We envisage that this novel strategy warrants further examination in the context of developing P-use-efficient crop varieties that will be required to address the challenges of diminishing non-renewable P resources (Gilbert, 2009).

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**Author contributions**

M.A.P., R.J., H.L. and P.M.F. designed research; M.A.P. performed research; M.W. and R.H. contributed expertise and analytical tools; M.A.P., R.J., M.W., H.L. and P.M.F. analysed data; M.A.P wrote the manuscript; and H.L., R.J., M.W., R.H. and P.M.F. provided editorial comments and suggestions to finalise the manuscript.
References


**Supporting items**

**Fig. S1** Total sulfur (S) and phosphorus (P) concentration in leaves, stems and roots of *Hakea prostrata* plants grown at different supplies of sulfate and phosphate.

**Fig. S2** Concentrations of the non-sulfur-containing amino acids in mature leaves of *Hakea prostrata*.

**Fig. S3** Concentrations of O-acetylserine (OAS) in mature leaves of *Hakea prostrata*.

**Fig. S4** Concentration (conc.) and content of a range of nutrients in mature leaves of *Hakea prostrata*.

**Fig. S5** Leaf sulfur concentration for flowering plants of different growth forms.
Table S1  Sulfur concentration ([S]) (mg g\(^{-1}\) dry weight) in leaves of 58 plant species grouped by their growth type (tree, shrub or herb) grown in their natural habitat.
Fig. 1 Total sulfur (S) and phosphorus (P) content in leaves, stems and roots of *Hakea prostrata*. Total amount of S in (a) leaves, (b) stems, (c) roots, Total amount of P in (d) leaves, (e) stems and (f) roots. Data are the mean ± SE of four independent replicates. The blue bars show total S and P content for plants grown at different supplies of sulfate and phosphate in hydroponics for five weeks after a six-week acclimation period. The red bar shows the corresponding value in plants harvested immediately before starting the experiment (labelled as ‘pre-treatment’) which were therefore five weeks younger than the treated plants. Significant differences among treatments were determined by ANOVA, separated by Tukey’s HSD test ($P < 0.05$), and indicated by different letters. Dry-to-fresh
weight ratio (mean ± SE) for ‘pre-treatment’, ‘limiting’, ‘standard’ and ‘high’ treatments were ‘0.23 ± 0.02’, ‘0.26 ± 0.02’, ‘0.29 ± 0.01’ and ‘0.31 ± 0.07’ for shoot; and ‘0.14 ± 0.02’, ‘0.17 ± 0.01’, ‘0.20 ± 0.01’ and ‘0.27 ± 0.02’ for root, respectively.
Fig. 2 Concentrations of central sulfur metabolites in mature leaves of *Hakea prostrata*. (a) total soluble protein (TSP), (b) total amino acids, (c) cysteine, (d) methionine, (e) S-adenosyl methionine (SAM), and (f) total glutathione. Data are the mean ± SE of four independent replicates. The blue bars show concentrations for plants grown at different supplies of sulfate and phosphate in hydroponics for five weeks after a six-week acclimation period. The red bar shows the corresponding value in plants harvested immediately before starting the experiment (labelled as ‘pre-treatment’) which were therefore five weeks younger than the treated plants. Significant differences among treatments were determined by ANOVA, separated by Tukey’s HSD test (*P* < 0.05), and indicated by different letters. Shoot dry-to-
fresh weight ratio (mean ± SE) for ‘pre-treatment’, ‘limiting’, ‘standard’ and ‘high’ treatments were ‘0.23 ± 0.02’, ‘0.26 ± 0.02’, ‘0.29 ± 0.01’ and ‘0.31 ± 0.07’, respectively.

FW, Fresh Weight.
Fig. 3 Concentration (a) sulfate, (b) phosphate, (c) arginine, and (d) lysine in mature leaves of *Hakea prostrata*. Data are the mean ± SE of four independent replicates. The blue bars show concentrations for the plants grown at the indicated supplies of sulfate and phosphate in hydroponics for five weeks after a six-week acclimation period. The red bar shows the corresponding value in plants harvested immediately before starting the experiment (labelled as ‘pre-treatment’) which were therefore five weeks younger than the treated plants. Significant differences among treatments were determined by ANOVA, separated by Tukey’s HSD test (*P* < 0.05), and indicated by different letters. Shoot dry-to-fresh weight ratio (mean ± SE) for ‘pre-treatment’, ‘limiting’, ‘standard’ and ‘high’ treatments were ‘0.23 ± 0.02’, ‘0.26 ± 0.02’, ‘0.29 ± 0.01’ and ‘0.31 ± 0.07’, respectively. FW, Fresh Weight.
**Fig. 4** Concentration of leaf photosynthetic pigments in *Hakea prostrata*. (a) Chlorophyll a, (b) Chlorophyll b, and (c) total carotenoids. Data are the mean ± SE of four independent replicates. The blue bars show concentrations for the plants grown at the indicated supplies of sulfate and phosphate in hydroponics for five weeks after a six-week acclimation period. The red bar shows the corresponding value in plants harvested immediately before starting the experiment (labelled as ‘pre-treatment’) which were therefore five weeks younger than the treated plants. Significant differences among treatments were determined by ANOVA, separated by Tukey’s HSD test ($P < 0.05$), and indicated by different letters. Shoot dry-to-fresh weight ratio (mean ± SE) for ‘pre-treatment’, ‘limiting’, ‘standard’ and ‘high’ treatments were ‘0.23 ± 0.02’, ‘0.26 ± 0.02’, ‘0.29 ± 0.01’ and ‘0.31 ± 0.07’, respectively. FW, Fresh Weight.
**Fig. 5** Biomass production of *Hakea prostrata*. (a) Plant biomass, (b) Shoot biomass and (c) Root biomass. Data are the mean ± SE of four independent replicates. The blue bars show concentrations for the plants grown at the indicated supplies of sulfate and phosphate in hydroponics for five weeks after a six-week acclimation period. The red bar shows the corresponding value in plants harvested immediately before starting the experiment (labelled as ‘pre-treatment’) which were therefore five weeks younger than the treated plants. Significant differences among treatments were determined by ANOVA, separated by Tukey’s HSD test ($P < 0.05$), and indicated by different letters. Dry-to-fresh weight ratio (mean ± SE) for ‘pre-treatment’, ‘limiting’, ‘standard’ and ‘high’ treatments were ‘$0.23 ± 0.02$’, ‘$0.26 ± 0.02$’, ‘$0.29 ± 0.01$’ and ‘$0.31 ± 0.07$’ for shoot; and ‘$0.14 ± 0.02$’, ‘$0.17 ± 0.01$’, ‘$0.20 ± 0.01$’ and ‘$0.27 ± 0.02$’ for root, respectively. DW, Dry Weight.
**Fig. 6** Leaf (a) content and (b) concentration (conc.) of molybdenum (Mo) in mature leaves in *Hakea prostrata*. Data are the mean ± SE of four independent replicates. The red bars show concentrations for plants grown at different supplies of sulfate and phosphate in hydroponics for five weeks after a six-week acclimation period. The blue bar shows the corresponding value in plants harvested immediately before starting the experiment (labelled as ‘pre-treatment’) which were therefore five weeks younger than the treated plants. Significant differences among treatments were determined by ANOVA, separated by Tukey’s HSD test ($P < 0.05$), and indicated by different letters. Shoot dry-to-fresh weight ratio (mean ± SE) for ‘pre-treatment’, ‘limiting’, ‘standard’ and ‘high’ treatments were ‘0.23 ± 0.02’, ‘0.26 ± 0.02’, ‘0.29 ± 0.01’ and ‘0.31 ± 0.07’, respectively.
Fig. 7 Diagram depicting the non-responsiveness of *Hakea prostrata* to a 13-fold variation in sulfate supply coded by colours. S, sulfur; \( \text{SO}_4^{2-} \), sulfate; 60S & 40S, ribosome subunits; rRNA, ribosomal RNA; P, phosphorus; SAM, S-adenosyl methionine.

- The majority of total S in the plant is used for protein synthesis, which requires ribosomes.
- Ribosomes comprise 65% rRNA & 35% protein; rRNA accounts for 40% of the total organic P in leaf cells.
- *Hakea prostrata* has a much lower (20%) level of rRNA in leaf cells than *Arabidopsis*.

- Despite a 13-fold difference in S supply, there was no response in:
  - sulfate concentration in leaves.
  - S-containing proteogenic amino acids, cysteine and methionine, concentration in leaves.
  - protein concentration in leaves.
  - concentration of S-containing metabolites, glutathione and SAM, in leaves.
  - total S content in leaves, stems and roots.
- Total S content in the whole plant accounted for only 1 to 10% of the cumulative supply of sulfate.
- These results suggest that *H. prostrata* tightly controlled sulfate acquisition.
- We surmise that controlled sulfate acquisition limits S assimilation and thus reduces the P requirement for rRNA for protein synthesis in *H. prostrata* as an adaptation to its severely P-impoverished habitats.