Nitrate-uptake restraint in Banksia spp. (Proteaceae) and Melaleuca spp. (Myrtaceae) from a severely phosphorus-impoverished environment

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Abstract

Aims South-western Australia has some of the most phosphorus (P)-impoverished soils in the world. Hakea prostrata (Proteaceae) has nitrate (NO$_3^-$)-uptake restraint, associated with its high P-use efficiency. This research explores how common this trait is in other Proteaceae and in co-occurring plant species in south-western Australia. Methods Banksia attenuata (Proteaceae), B. telmatiaea (Proteaceae), Melaleuca seriata (Myrtaceae) and M. rhaphiophylla (Myrtaceae) were grown with no NO$_3^-$, Medium (500 µM) NO$_3^-$ or High (2500 µM) NO$_3^-$ treatments with no other source of nitrogen (N). Plants were harvested after treatments. Leaf nitrate and phosphate concentrations were determined, as well as biomass, total N and total P concentrations of leaves, stems and roots separately. Results Banksia attenuata, B. telmatiaea and M. seriata had similar total N content when supplied with High NO$_3^-$ as they did when supplied with Medium NO$_3^-$.

Conclusions All four species had the same low leaf NO$_3^-$-N concentration in High and Medium NO$_3^-$ treatments, no more than 6% of leaf total N. All species had similar leaf NO$_3^-$ concentrations as those of plants growing in their natural habitat where the available soil nitrate concentration was much lower. These results are in sharp contrast with plants that are typically studied, for example Arabidopsis thaliana.

Keywords Myrtaceae · Nitrate-uptake restraint · Nitrogen · Phosphorus · Plant nutrition · Proteaceae

Introduction

South-western Australia has some of the oldest and most phosphorus (P)-impoverished soils in the world (Hopper 2009; Lambers 2014) and is a global biodiversity hot-spot (Myers et al. 2000). Phosphorus plays a pivotal role in plant productivity in south-western Australia because of low-P parent material and prolonged soil weathering (Hopper and Gioia 2004; Laliberté et al. 2012; Lambers et al. 2010). Compared with extremely low soil P-availability, the nutrient-poor soils in south-western Australia have relatively abundant available nitrogen (N) (Laliberté et al. 2012; Turner and Laliberté 2015). Soil N and...
P availability both decline during pedogenesis, but the soil N: P ratio steadily increases during long-term soil development (Turner and Laliberté 2015) which makes P the major nutrient limiting plant productivity in south-western Australia (Hopper and Gioia 2004; Laliberté et al. 2012). Native plants that evolved in this P-limiting environment have specialised P-acquisition and P-utilisation strategies, which make them highly P efficient (Hayes et al. 2021; Lambers et al. 2022).

Proteaceae is one of the dominant plant families in south-western Australia (Pate et al. 2001). Many critical traits have evolved in the Proteaceae that allow them to survive in severely P-impoverished landscapes (Lambers et al. 2015). For instance, Proteaceae acquire P from soil efficiently through their specialised cluster roots, which are composed of clusters of lateral rootlets that exude carboxylates into soil and mobilise P from poorly soluble P complexes (Shane and Lambers 2005). Proteaceae in this region also have specialised strategies of P utilisation, such as efficient remobilisation of P from senescing organs to growing organs (Lambers et al. 2012), delayed greening of young leaves (Kuppusamy et al. 2014; Lambers et al. 2012), and preferential allocation of P to photosynthetically-active mesophyll cells, rather than to epidermal cells (Hayes et al. 2018). Proteaceae leaves also have low protein and N concentrations (Hayes et al. 2014; Sulpice et al. 2014), despite the relatively high N availability compared with that of P in south-western Australia (Turner and Laliberté 2015). This results in a low demand for P in ribosomal RNA (rRNA) (Lambers et al. 2015), which is reflected by low levels of rRNA (Sulpice et al. 2014). All these traits allow Proteaceae to thrive in south-western Australia, especially on the poorest soils (Lambers et al. 2022).

Nitrate (NO$_3^-$) uptake restraint has been reported for *Hakea prostrata* (Proteaceae) (Prodhan et al. 2016), which has evolved in P-limiting environments of south-western Australia (Lambers et al. 2011, 2015). *Hakea prostrata* has all the strategies associated with efficient acquisition and utilisation of P presented above including low rRNA and protein concentrations, which reduce its demand of other nutrients, like N, sulfur (S), and micronutrients like zinc (Zn) and copper (Cu) (Prodhan et al. 2019). Nitrate restraint in *H. prostrata* was discovered in a hydroponics experiment where plant total N content and leaf NO$_3^-$ concentration were the same in plants grown with a NO$_3^-$ supply of either 5000 µM or 200 µM, both of which were in great excess to demand (Prodhan et al. 2016). This tight control of NO$_3^-$ uptake and assimilation was interpreted as an adaptive strategy to P limitation (Prodhan et al. 2019). However, the prevalence of this tight control of NO$_3^-$ uptake among other Proteaceae and in co-occurring plant families, such as the Myrtaceae, in south-western Australia is unknown.

Both soil ammonium (NH$_4^+$) and soil NO$_3^-$ availability decline during pedogenesis in south-western Australia (Turner and Laliberté 2015). Despite the slightly higher availability of NH$_4^+$ than that of NO$_3^-$ (Turner and Laliberté 2015), non-mycorrhizal species in a native *Banksia* woodland in Western Australia have been shown to exclusively take up nitrate as their N source (Pate et al. 1993). In this study, we selected two Proteaceae (*Banksia telmatiacea* and *B. attenuata*) and two Myrtaceae (*Melaleuca rhaphiophylla* and *M. seriata*) that occur naturally on P-impoverished soils in south-western Australia to determine whether they have NO$_3^-$-uptake restraint. The plants of all targeted species were grown in aerated hydroponics where NO$_3^-$ was the only source of N. Since Proteaceae have many adaptations to low-P soils, while Myrtaceae are not known for such traits, we hypothesised that the two Proteaceae would exhibit NO$_3^-$-uptake restraint, while the two Myrtaceae would not.

### Materials and methods

#### Study area and species selection

Field samples were collected in Alison Baird Reserve (32°1’19”S 115°58’52”E), a species-rich flora reserve southeast of Perth that has extremely impoverished soil (Leopold and Zhong 2019; Tauss et al. 2019). The reserve has a Mediterranean climate, with dry summers and wet winters (Gentilli 1972). It is located on the eastern edge of the Swan Coastal Plain, overlain by coastal dunes that are geomorphic residual outliers of Bassendean dune landforms (Tauss et al. 2019). Detailed information on coastal sand dune chronosequence systems in south-western Australia were provided in (Turner et al. 2018). The underlying sediments originated from the Darling Scarp towards the east and were deposited in the reserve over millions of years (Lane and Evans 2019). On top of a tall Bassendean dune, the deep sandy soils are moist in winter and dry in summer,
while at the west of the reserve there is a flat area with lower elevation where the soils are inundated in winter and dry in summer (Tauss et al. 2019).

We selected two Proteaceae (*Banksia telmatiaea* and *B. attenuata*) and two Myrtaceae (*Melaleuca rhiphiophylla* and *M. seriata*) that occur naturally in the reserve. *Banksia telmatiaea* and *M. rhiphiophylla* both occur on the flat winter-inundated area, while *B. attenuata* occurs on the winter-moist top of the tall Bassendean dune (Figs. 1 and 2). *Melaleuca seriata* occurs almost exclusively over the entire dune, but can be found in the wet area.

Sample collection from the field

Leaf samples of all species were collected for nutrient analyses, both at the winter-spring wet period (September 2019) and at the summer-autumn dry period (early April 2019 and March 2020). Most-recently fully-expanded leaves were selected from five random plants of each species in each season. Samples were snap-frozen in liquid nitrogen at harvest and stored at -80°C to be processed later.

Plant material and growing conditions

Seeds of the two *Banksia* species were collected in the reserve in 2019. Seeds of the two *Melaleuca* species were purchased (Nindethana Seed Company, Albany, WA, Australia), after collection from natural populations in Western Australia.

Seeds of all species were germinated in Petri dishes between filter papers moistened with deionised water until the primary root and cotyledons emerged (15 °C, 12 h light, 12 h dark). Seedlings were transferred to a mixture of pasteurized sand and potting mix (1:1). After six weeks of growth, seedlings of uniform size were transferred to aerated hydroponics in cooling tanks that maintained roots at 18°C to 20°C (Shane et al. 2004) to start the Pre-treatment period.
Before transferring to hydroponics, roots of seedlings were washed, and disinfected with 1% (v/v) sodium hypochlorite for 20 s. Disinfected seedlings were transferred to 5 L plastic pots containing 4 L nutrient solution (in µM, 2.5 PO$_4^{3-}$, 500 NO$_3^-$, 200 Ca$^{2+}$, 500 K$^+$, 36 SO$_4^{2-}$, 36 Mg$^{2+}$, 10 Fe-EDTA, 0.24 Mn$^{2+}$, 0.10 Zn$^{2+}$, 0.018 Cu$^{2+}$, 2.4 H$_3$BO$_3$, 50 SiO$_3^{2-}$ and 0.3 Mo$^{4+}$. pH = 5.8) (modified from Hayes et al. (2019)) with one plant per pot. The nutrient solution was replaced twice per week. The Pre-treatment ended when the plants had produced new roots and had a healthy appearance without any sign of mineral deficiency or toxicity, or any biotic stress (four weeks for M. rhaphiophylla, five weeks for M. seriata and B. telmatiaea, seven weeks for B. attenuata).

Nutrient treatments

After the Pre-treatment, five individuals from each species were randomly selected for harvest to determine the initial nutrient status. The following treatments were then applied to five randomly assigned replicate individuals from each species. All nutrients, except N and P, were provided at established levels for plant growth as previously described (Hayes et al. 2019). Nitrate was the only source of N added. The three NO$_3^-$ treatments were No NO$_3^-$, Medium (500 µM) NO$_3^-$ or High (2500 µM) NO$_3^-$, added twice weekly when the entire nutrient solution was replaced. Plants of all species received sufficient P, as KH$_2$PO$_4$, to make sure that P was not limiting for NO$_3^-$ uptake. The optimal concentration of P was determined by measuring the P concentration remaining in the nutrient solutions at each solution change. The optimal P supply was considered the concentration that was sufficient to have a small amount remaining in the solution at the next solution change, but not so high as to promote bacterial and fungal growth. The final concentrations of PO$_4^{3-}$ in the nutrient solution in each week are shown in Table 1. All NO$_3^-$ treatments for each species received the same amount of P when the solution was changed.

Fig. 2 Sampling sites of four targeted species in Alison Baird Reserve (ABR): Banksia attenuata and Melaleuca seriata on the Bassendean dune, Banksia telmatiaea and Melaleuca rhaphiophylla at the flat winter-inundated area. Elevation data of ABR was from Smith (2019). The image has been coloured to highlight the Bassendean dune.
twice a week. Treatments were applied until N deficiency symptoms started to appear in No NO$_3^-$ treatment plants, which was after 10 weeks for the two Banksia species, nine weeks for M. seriata and five weeks for M. rhaphiophylla.

Plant harvest

Approximately 1 to 3 g fresh weight of the mature leaves were harvested, weighed, immediately frozen in liquid nitrogen and stored at -80°C to be analysed later. All snap-frozen leaves were harvested between 9 am and 12 pm on sunny days. The remaining parts of the plants were harvested into leaves, stems and roots. After measuring fresh weights, plant material was oven dried at 65°C for at least 72 h and weighed again to determine dry weights and dry-to-fresh weight ratios.

Snap-frozen mature leaves were freeze dried then ground to determine NO$_3^-$ and inorganic phosphate (Pi) concentrations. Oven dried plant materials were also ground to determine total N and P concentrations. Total N and P content in each organ was calculated from the total N and P concentrations and biomass. The total biomass of leaves was calculated from the dry weight of the oven-dried leaves and the calculated dry weight of the snap-frozen leaves, which was calculated from the dry-to-fresh weight ratio and fresh weight of snap-frozen leaves.

| Week | P supply (μM) | B.t | B.a | M.r | M.s |
|------|---------------|-----|-----|-----|-----|
| 1    | 10            | 5   | 30  | 10  |
| 2    | 10            | 5   | 40  | 10  |
| 3    | 5             | 5   | 50  | 20/30 |
| 4    | 5             | 15  | 50a | 50/100 |
| 5    | 5             | 20  | 0   | 150a |
| 6    | 5             | 20  | Harvested | 0 |
| 7    | 15            | 20  | 0   |
| 8    | 20            | 30  | 0   |
| 9    | 20            | 30  | 0   |
| 10   | 20            | 30  | Harvested |
| 11   | Harvested     | Harvested |

*Determination of total nitrogen and phosphorus*

Total N concentrations were determined by combustion using an elemental analyser (Elementar Australia Pty Ltd, Sydney, Australia). Total P concentrations were determined colorimetrically using a malachite green method (Motomizu et al. 1983) after acid digestion of samples. Aliquots of 100 mg of ground freeze-dried samples were acid digested with 3 mL of concentrated HNO$_3$ at 80°C to 90°C, followed by digestion with 0.5 mL of HClO$_4$ at 130°C to 150°C, then with 1 mL of HCl at 130°C. Deionised water was added to adjust the final volume to 10 mL.

*Determination of ions*

Inorganic phosphate was extracted from 25 mg freeze-dried leaf samples by homogenising in ice-cold 1% (v/v) acetic acid (Precellys 24 Homogeniser, Thermo Fisher Scientific, Waltham, USA) taking care to keep the solution cold (Yan et al. 2019). The homogenate was clarified twice by centrifugation at 21,000×g for 15 min at 4°C. The extracts were treated with acid-washed activated charcoal to remove color interfering substances (Dayrell et al. 2021). The concentration of Pi in the final extracts was determined colorimetrically using a malachite green-based method (Motomizu et al. 1983).

Nitrate was extracted from 100 mg freeze-dried leaf samples with water while shaking at 45°C for one hour (Cataldo et al. 1975). Interfering organic substances were removed by adding 1 mL of petroleum spirit, shaking for five minutes, separating the phases by centrifugation at 5000×g for 5 min, removing the organic phase, and filtering the aqueous phase (25 mm diameter Polyethersulfone syringe filters; 0.45 μm; Sarstedt Australia Pty Ltd, Adelaide, Australia). The concentration of NO$_3^-$ in the final extracts was determined colorimetrically using a flow injection analysis system (Lachat Instruments, Milwaukee, WI, USA).

*Statistical analyses*

Differences in biomass, N and P contents and concentrations, leaf N: P ratio, leaf NO$_3^-$ and Pi concentrations among treatments were analysed by one-way analysis of variance (ANOVA) using Fisher’s least significant difference (LSD) test at $P < 0.05$ (R studio, v. 3.6.0) (R Core Team, 2022).
Results

Plant biomass

Biomass was similar between Medium NO$_3^-$ and High NO$_3^-$ treatments for *B. attenuata*, *B. telmatiaea* and *M. rhaphiophylla* (Fig. 3). For *M. seriata*, biomass in the High NO$_3^-$ treatment was lower than in the Medium NO$_3^-$ treatment due to a loss of leaves caused by slight P toxicity early in the High NO$_3^-$ treatment. Total biomass for all species under all NO$_3^-$ treatments was significantly higher than that of the Pre-treatment, indicating that the No NO$_3^-$ plants had sufficient N from the pre-treatment to sustain additional growth even when the N supply was stopped. Biomass of *Banksia* plants increased two-fold to three-fold during the treatment period, while biomass of *Melaleuca* plants increased five-fold to six-fold during the treatment period compared with the Pre-treatment plants (Fig. 3). *Banksia attenuata* and *B. telmatiaea* in High NO$_3^-$ and Medium NO$_3^-$ treatments all left behind more than 90% of the NO$_3^-$ that was supplied in the nutrient solution. *Melaleuca seriata* and *M. rhaphiophylla* in the High NO$_3^-$ treatment left behind about 87% and 69% of the total NO$_3^-$ supplied, respectively, showing there was a great excess of NO$_3^-$ available for plants during the treatment period. The N supply in the Medium NO$_3^-$ treatment was also saturating, except for *M. raphiophylla*, which exhausted the NO$_3^-$ in the Medium treatment.

Total N content

In all species, plants from the No NO$_3^-$ treatment had the same total N content as plants from the Pre-treatment, indicating that there was no loss of N during plant growth (Fig. 4). By contrast, the total N content of *Banksia* plants under Medium NO$_3^-$ treatment was 65% to 130% greater than that of N-starved plants, while total N content of *Melaleuca* plants under Medium NO$_3^-$ treatment increased even more, to at least three-fold higher than the N-starved plants (Fig. 4).

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**Fig. 3** Total biomass per plant for *Banksia attenuata*, *B. telmatiaea*, *Melaleuca rhaphiophylla* and *M. seriata* after pre-treatment (Pre) and after further growth at different supplies of nitrate. Data are means ± SE (n = 5). Significant differences among treatments were determined by ANOVA, Fisher’s least significant difference (LSD) test (P < 0.05) and indicated by different letters.
Plants of all species except _M. rhaphiophylla_ had the same total N content when supplied with High NO$_3^-$ as they did when supplied with Medium NO$_3^-$.
These three species also had the same N content in leaves, stems and roots under High NO$_3^-$ treatment as they did under Medium NO$_3^-$ treatment (Figs. S1, S3, S5, S7). The total N content of _M. rhaphiophylla_ under High NO$_3^-$ treatment was about 33% greater than that under Medium NO$_3^-$ treatment (Fig. S4). This difference in N content between treatments for _M. rhaphiophylla_ was due to a higher leaf N content in the High NO$_3^-$ treatment than in the Medium NO$_3^-$ treatment. The N content of stems and roots was the same between Medium NO$_3^-$ and High NO$_3^-$ treatments (Fig. S7).

**Leaf nitrate concentration**

The distribution of N in different organs varied among the four species (Figs. S1, S2, S3, S4, S5, S6, S7, S8). Focusing on leaf N, we found that all four species had low leaf NO$_3^-$ concentrations (Fig. 5), despite the high NO$_3^-$ supplies. Nitrate is an energetically inexpensive osmoticum to generate turgor compared with organic ions or sugars (Andrews et al. 2005). However, the four species examined did not use nitrate as an osmoticum, even in the high-NO$_3^-$ treatment, suggesting that light energy was not a major limiting factor for growth (Andrews et al. 2005). Instead, _M. seriata_, _M. rhaphiophylla_ and _B. attenuata_ plants had the same leaf NO$_3^-$ concentration when treated with Medium NO$_3^-$ or High NO$_3^-$ (Fig. 5). Remarkably, the leaf NO$_3^-$ concentrations of all four species grown in Medium NO$_3^-$ and High NO$_3^-$ treatments were similar to that of plants growing in their natural habitats, and the NO$_3^-$ in the leaves was only a small portion (0.18% to 5.86%) of total leaf N (Fig. 5). There was no significant difference in the proportion of NO$_3^-$ in total N between Medium NO$_3^-$ and High NO$_3^-$ treatments for any of the four species.
Total P content and concentration

Within a species, all plants from all \( \text{NO}_3^- \) treatments received the same P supply throughout the experiment, although the P supply varied across the experiment (Table 1). Intriguingly, in the banksias, different N supplies impacted P uptake in \textit{B. attenuata}, but not in \textit{B. telmatiaea} (Fig. 6). The total P content in \textit{B. attenuata} plants was significantly greater when plants were supplied with \( \text{NO}_3^- \) than in \( \text{NO}_3^- \)-starved plants (Fig. 6). Leaf P content in \textit{B. attenuata} was greater under High \( \text{NO}_3^- \) than Medium \( \text{NO}_3^- \) treatment, whereas the P content of roots was the same, irrespective of \( \text{NO}_3^- \) treatment (Fig. S9). The leaf P concentration of \textit{B. attenuata} was also greater under High \( \text{NO}_3^- \) than Medium \( \text{NO}_3^- \) treatment, while stem and root P concentrations were the same in both treatments (Fig. S10). In contrast to \textit{B. attenuata}, neither P content nor P concentration was affected in \textit{B. telmatiaea} by \( \text{NO}_3^- \) treatment (Figs. S11, S12). The P concentration of \textit{B. telmatiaea} leaves never exceeded 0.7 mg P g\(^{-1}\) DW, while that in \textit{B. attenuata} leaves varied from 1.0 to 3.4 mg P g\(^{-1}\) DW.

\textit{Melaleuca rhaphiophylla} had the same total P content in Medium \( \text{NO}_3^- \) and High \( \text{NO}_3^- \) treatments (Fig. S15). However, despite the total P content of the whole plant being the same among \( \text{NO}_3^- \) treatments, the \( \text{NO}_3^- \)-starved plants had a higher stem P content as well as stem P concentration than plants supplied with \( \text{NO}_3^- \) (Fig. S15). The P concentration in each organ of \textit{M. rhaphiophylla} was also the same between Medium \( \text{NO}_3^- \) and High \( \text{NO}_3^- \) treatments (Fig. S16). The P content of \textit{M. seriata}...
plants as well as leaf P content and stem P content were greater at Medium $\text{NO}_3^-$ than at High $\text{NO}_3^-$ (Fig. S13). The P concentrations in leaves, stems and roots of $M. \text{seriata}$ were the same in both the High and Medium $\text{NO}_3^-$ treatments (Fig. S14). The difference in leaf and stem P content between these two $\text{NO}_3^-$ treatments (Fig. S13) was due to lower biomass of High $\text{NO}_3^-$ plants, which was caused by transient P toxicity.

Leaf phosphate concentration

The leaf Pi concentration of $B. \text{attenuata}$ was greater under High $\text{NO}_3^-$ treatment than Medium $\text{NO}_3^-$ treatment, consistent with the leaf total P concentration in these treatments (Fig. 7). In contrast, the other three species all had the same leaf Pi concentration in High $\text{NO}_3^-$ and Medium $\text{NO}_3^-$ treatments. In addition, the two species that occur on the Bassendean dune, $B. \text{attenuata}$ and $M. \text{seriata}$, had similar leaf Pi strategies to one another: the leaf Pi concentration in both species increased when supplied with $\text{NO}_3^-$ compared with that in $\text{NO}_3^-$-starved plants, and Pi was always a major component of the P pool, representing 72% to 97% of leaf total P. The other two species, $B. \text{telmatiae}$ and $M. \text{rhaphiophylla}$, which occur in the winter-wet flat area, also had similar Pi strategies to one another: leaf Pi was highest when $\text{NO}_3^-$ was withheld (Fig. 7), and the proportion of Pi in total P in leaves was lower when plants were supplied with $\text{NO}_3^-$. Finally, for all species, the Pi and total P concentrations in leaves of plants grown in hydroponics were greater than those of plants growing in their natural habitat.

Leaf N: P ratio

There was no significant difference in leaf N : P ratio between Medium $\text{NO}_3^-$ and High $\text{NO}_3^-$ treatments for any of the species, indicating that N: P ratio was not dependent on $\text{NO}_3^-$ supply (Fig. 8). All species, except $M. \text{rhaphiophylla}$, had a lower leaf N:
P ratio when grown in hydroponics than plants collected in their natural habitat (Fig. 8). Interestingly, NO$_3^-$-treated *B. attenuata* and *M. seriata* plants, which naturally occur on the Bassendean dune, all had a leaf N: P ratio below 7.5. However, NO$_3^-$-treated *B. telmatiaea* and *M. rhaphiophylla* plants, which naturally occur in the flat and wetter area in Alison Baird Reserve, had a leaf N: P ratio of above 13, while the leaf N: P ratio of all four species from their natural habitats were all above 20 (Fig. 8).

**Discussion**

Our results showed that all targeted species exhibited NO$_3^-$-uptake restraint by not taking up more NO$_3^-$ than was needed to sustain growth. In contrast with our hypotheses, our results showed that NO$_3^-$-uptake restraint is not a trait unique to the Proteaceae, but may be a convergent trait common to many species that evolved in severely P-impoverished soils, allowing them to cope with extremely low P availability.

Nitrate-uptake restraint was first found in *H. prostrata* (Prodhan et al. 2016). The plants simply did not take up more NO$_3^-$ than needed, which is fundamentally different from plants that are typically studied (Prodhan et al. 2016, 2019). All species targeted here, except *M. rhaphiophylla*, had the same total N content when supplied with High NO$_3^-$ as they did when supplied with Medium NO$_3^-$, while total N content of *M. rhaphiophylla* increased when provided with more NO$_3^-$, the increase was only about 1.3-fold.
When the readily available NO$_3^−$ in the growing medium was increased five-fold (Fig. 4), this appears to be a minimal NO$_3^−$ uptake compared with the level of NO$_3^−$ available. Again, it suggests a tight control of NO$_3^−$ uptake compared with that in typically studied plants, where NO$_3^−$ uptake sharply increases with an increase in NO$_3^−$ availability (Greenwood and Hunt 1986; Tschoep et al. 2009). In the case of _M. raphiophylla_, the slight increase in total N content beyond the Medium NO$_3^−$ treatment may indicate that _M. raphiophylla_ may need slightly more NO$_3^−$ than supplied in the Medium NO$_3^−$ treatment to reach its static N level in hydroponics system. While total N content was greater with higher NO$_3^−$ supply in _M. raphiophylla_, even this species did not store N as NO$_3^−$, but instead converted it into organic N.

Nitrate-restraint was associated with low organ N concentration regardless of nitrate supply. The leaf and root N concentration of the two _Banksia_ spp. remained the same (below 15 mg g$^{−1}$ DW) even when NO$_3^−$ supply was higher (Figs. S2, S4). This finding contrasted with what was found for a wide range of plant species that have been studied, including trees, herbs and grasses (see Prodhant et al. 2016 for summary). The stem N concentration in _B. attenuata_ was indistinguishable between Medium and High NO$_3^−$ treatments as well. Even though the stem N concentration in _B. telmatiaeae_ was 1.5-fold higher when the plants were provided with High NO$_3^−$ than with Medium NO$_3^−$, it was still only about 20 mg g$^{−1}$ DW. In contrast, the shoot N concentration in the model plant _A. thaliana_ can increase to 75 mg g$^{−1}$ DW (Tschoep et al. 2009), and that of other species can increase to as much as 53 mg g$^{−1}$ DW (Greenwood and Hunt 1986) when supplied with more N. _Melaleuca seriata_ also had the same leaf and stem N concentration in Medium and High NO$_3^−$ treatments (Fig. S6). While the root N concentration of _M. seriata_ was about 1.5-fold higher when supplied with High NO$_3^−$ compared to Medium NO$_3^−$, it was only about 21 mg g$^{−1}$ DW. In contrast, the root N concentration of many other species can reach about 57 mg g$^{−1}$ DW when supplied with more N (Greenwood and Hunt 1986). For _M. raphiophylla_, leaf, root and stem N concentrations were no more than 1.3-fold higher under High NO$_3^−$ treatment than those under Medium NO$_3^−$ treatment. In contrast, leaf, shoot and root N concentration increased four-fold to five-fold when N supply was increased in previously studied species (Aerts 2009; Reich and Oleksyn 2004; Taylor and May 1967).

Leaf NO$_3^−$ concentrations for all four target species were non-responsive to a five-fold difference in NO$_3^−$ supply, as was the ratio of leaf NO$_3^−$ concentration to total N concentration. This was again in sharp contrast with most plant species that have been studied, where the NO$_3^−$ concentration increased up to 118-fold and the ratio of leaf NO$_3^−$ concentration to total N concentration increased up to 58-fold when NO$_3^−$ supply increased (Cox and Reisenauer 1973; North et al. 2009; Stewart et al. 1973). Despite leaf N concentrations in plants grown in hydroponics being higher than those of plants growing in their natural habitat (Fig. 5), leaf NO$_3^−$ concentrations in hydroponics here were similar to those of these plants at the field site, where the soil N concentration was much lower than that supplied in hydroponics. However, other plants studied in their natural habitats may have up to 100-fold higher leaf NO$_3^−$ concentrations when supplied with more NO$_3^−$ than naturally available in field soils (Stewart et al. 1973). Thus, the low leaf NO$_3^−$ concentrations seen here again demonstrates

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**Fig. 8** Leaf N: P ratio of _Banksia attenuata_ (B. a), _B. telmatiaeae_ (B. t), _Melaleuca raphiophylla_ (M. r) and _M. seriata_ (M. s) plants after pre-treatment and after further growth at different supplies of nitrate, and plants from their natural habitat. For Pre-, No N, Medium and High N treatments, $n=5$, while for plants at the field site, $n=10$ (five replicates collected in summer and five replicates collected in winter). Data are means ± SE. Significant differences were determined by ANOVA, Fisher’s least significant difference (LSD) test ($P<0.05$) and indicated by different letters.
that all four targeted species restrained their $\text{NO}_3^-$ uptake. Moreover, the fact that plants supplied with High $\text{NO}_3^-$ had a higher leaf N concentration than plants in the field indicated that these species do indeed take up $\text{NO}_3^-$ and plants grown in hydroponics were not limited by their $\text{NO}_3^-$-uptake capacity.

High $\text{NO}_3^-$ treatment had a fivefold higher N: P supply ratio than the Medium $\text{NO}_3^-$ treatment, yet the plants grown in these different conditions had the same leaf N: P ratio. Moreover, the $\text{NO}_3^-$-treated plants all had a much lower leaf N: P ratio than plants grown in their natural habitat. This non-responsiveness of leaf N: P ratio to high $\text{NO}_3^-$ supplies again indicated that the four targeted species have $\text{NO}_3^-$-uptake restraint. The N: P ratio of less than 20 in hydroponics also indicated that P was not limiting $\text{NO}_3^-$ uptake.

The four investigated species are non-mycorrhizal, and non-mycorrhizal species in a native Banksia woodland in Western Australia exclusively take up nitrate as their N source (Pate et al. 1993). However, plants take up N from soluble inorganic sources in the soil solution as well as some organic N (Lambers and Oliveira 2019). Plants growing in hypoxic soil conditions such as paddy rice take up $\text{NH}_4^+$ as the main source of N, whereas plants adapted to aerobic soils where nitrification occurs tend to prefer $\text{NO}_3^-$ (Maathuis 2009; Nadelhoffer et al. 1984). In addition, nitrate is highly mobile in soil, and thus can easily be taken up by roots, while ammonia may be immobilised or nitrified by soil microorganisms (Pate et al. 1993; Shearer et al. 1974). While we do not know the source of N the targeted species prefer, the available evidence suggests that they are all likely to access nitrate in their native habitats. The plants studied here were grown in aerated hydroponics where $\text{NO}_3^-$ was the only source of N. They all showed good growth and had higher total leaf N concentrations compared with plants in their natural habitat. This confirms that the plants were capable of taking up nitrate and using it as the sole source of N. They all exhibited low leaf $\text{NO}_3^-$ concentrations, even when they had access to an excess of $\text{NO}_3^-$, indicating that they all exhibited $\text{NO}_3^-$-uptake restraint. Taken together, these results indicate that the actual form on N that these plants prefer, which is unknown, did not impact their $\text{NO}_3^-$ uptake capacity.

Nitrate-uptake restraint is likely another P-efficiency strategy for plants that evolved in severely P-impoverished environments (Prodhan et al. 2016, 2019). Walker and Syers (1976) proposed that all terrestrial ecosystems begin with a finite amount of P in parent rock, which gradually weathers, releasing P that is eventually eroded, leached or occluded. Therefore, P limitation of plant productivity increases in ecosystems as soils age (Lambers et al. 2008; Vitousek et al. 2010). South-western Australia has some of the world’s oldest soils that are derived from parent material with low P concentrations. They have also been highly weathered and leached, making them extremely P impoverished (Hopper 2009; Lambers et al. 2010). In these extremely P-impoverished landscapes, P plays a key role in limiting plant productivity (Hopper and Gioia 2004; Laliberté et al. 2012). In contrast to many N-limited ecosystems in the world, P is in the ‘driver’s seat’ and has provided the selective pressure for plant evolution in south-western Australia (Hayes et al. 2021; Prodhan et al. 2019). Nitrate-uptake restraint would not only force plants to function at low protein concentrations that would reduce the demand for P (Lambers et al. 2015), but may also save metabolic energy by not using energy to transport unneeded $\text{NO}_3^-$ across cell membranes.

Proteaceae, one of the dominant plant families in the P-impoverished ecosystems of south-western Australia, have numerous strategies to adapt to extreme P limitation. The P efficiency traits that allow Myrtaceae to be another dominant plant family in these P-limiting ecosystems are not well established. We can now add nitrate restraint as a possibly general adaptation. The highly efficient use of P decreases the requirements for N, allowing plants with $\text{NO}_3^-$ restraint to economize further.

We also found that species from contrasting habitats functioned differently with respect to $\text{NO}_3^-$ supplies. Banksia attenuata and M. seriata, which naturally occur in the severely P-impoverished Bassendean sand on the dune at our study site, took up more P when supplied with medium and high $\text{NO}_3^-$, compared to the $\text{NO}_3^-$-starved plants. Conversely, B. telmatiaea and M. raphiophylla, which occur in the winter-wet area in the reserve, where soils are somewhat richer (Gao et al. 2020; Leopold and Zhong 2019), had similar total plant P content for all three treatments. This led to relatively lower leaf N: P ratios in B attenuata and M. seriata than in B. telmatiaea and M. raphiophylla. Banksia attenuata and M. seriata grown in hydroponics also had higher leaf Pi concentrations than plants from their natural habitats, in contrast with B. telmatiaea and...
M. rhaphiophylla, which had similar leaf Pi concentrations with plants from their natural habitats. The higher Pi concentration in the two species that occur naturally on the dune of Bassendean sand suggested that they have a lower capacity to down-regulate their P-uptake. We surmise that this difference reflects the difference in P availability of their natural habitats. The plant-available P in soil on the Bassendean dune was lower than at the winter-wet areas of the reserve (Gao et al. 2020; Leopold and Zhong 2019). In a more fertile habitat, a greater capacity to down-regulate the P-uptake system would prevent P toxicity, whereas in severely P-impoverished habitats, down regulation would not be essential. Moreover, the lack of down regulation might actually be associated with a lower P-remobilisation efficiency from senescent leaves, as suggested by de Campos et al. (2013).

Based on our results, we conclude that B. attenuata, B. telmatiaea, M. rhaphiophylla and M. seriata all exhibited NO$_3^-$-uptake restraint to different degrees. None of these species stored N as NO$_3^-$, but instead converted it to organic N. However, the adaptation of these species to low P availability in their natural habitat seemed to impact on how they responded to different NO$_3^-$ supplies. Thus, NO$_3^-$-uptake restraint may be a convergent trait common to many species that evolved in severely P-impoverished soils, allowing them to cope with extremely low P availability.

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Data availability The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

Declarations

Competing interests The authors declare that they have no conflict of interest.

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