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ABSTRACT

Replacement of phospholipids with phosphorus (P)-free lipids in cellular membranes has been identified as a mechanism facilitating fast rates of photosynthesis when phosphorus availability is limited. We measured photosynthetic rates, leaf and soil P fractions, and foliar membrane lipid compositions for five species (*Geranium antrorsum, Ranunculus graniticola, Poa costiniana, Poa hiemata*, and *Veronica derwentiana*) common to two Australian subalpine ecosystems of contrasting parent material to characterize the extent to which they have adapted to long-term P availability. Our results indicate limited tolerance to reduced P, albeit adaptation strategies differ among species. Under reduced P conditions, phospholipids were replaced in foliage by galactolipids and sulfolipids, but photosynthesis was still impaired owing to reduced stomatal conductance. Accumulation of antioxidants, including carotenoids and alpha-tocopherol, in leaves with limited P supply suggests oxidative stress. Our field study shows that while subalpine Australian plants of a variety of life forms adapt to P availability by replacing phospholipids with P-free lipids in foliar membranes, this adaptation is insufficient to fully mitigate the effects of reduced P on photosynthesis.

Introduction

Nutrient deficiencies in soils are among the main factors that limit global productivity, with nitrogen (N) and phosphorus (P) being especially critical (Fisher, Badgley, and Blyth 2012). Northern hemisphere soils are typically deficient in nitrogen (Vitousek and Howarth 1991), while many southern hemisphere and tropical soils have poor availability of phosphorus because of prolonged weathering, long-term tectonic stability, and lack of glaciation events (McKenzie et al. 2004). Nutrient availability in subalpine ecosystems is also limited by characteristically and strongly acidic soils (Dakora and Phillips 2002), while adverse environmental conditions, including drought, light stress, and extremes of temperature, reinforce limitations to growth (e.g., Wahren, Williams, and Papst 1999).

Nutrient availability is a key evolutionary driver of plant anatomy and the establishment of associations between plants and other organisms. Among the earliest adaptations to enhance nutrient acquisition was the formation of symbiotic relationships between plants and fungi (Brundrett 2002). Mycorrhizal associations, present in approximately 80 percent of land plants, are estimated to provide up to 80 percent of plant P and N (van der Heijden et al. 2015).

Assessing the impacts of nutrient availability on plant physiology requires knowledge of plant requirements and capacity for within-plant allocation of nutrients (e.g., to metabolism or to storage). Nucleic acids may account for 40–60 percent of organic P in some plant tissues, of which approximately 85 percent can be RNA (Veneklaas et al. 2012). RNA content can, in turn, vary with P supply (Reef et al. 2010). Other P-containing metabolites also play major roles in metabolism. For example, availability of free orthophosphate (P_i) within the cytosol (Raven 2013) is critical to photosynthesis, while ATP is essential to cellular energy transfer, both of which are reduced when there is limited P available (Plaxton and Tran 2011).

Table 1. Climate data for the Snowy Plains (SP) and Dargo High Plains (DHP) study sites. Data are means ±SE for 2007–2012. Temperature and rainfall data were collected from eddy covariance flux towers installed at each site. Radiation data were collected from nearby bureau of meteorology stations (SP = Cabramurra; DHP = Mount Hotham airport; www.bom.gov.au).

	Site	
Climate Variable	Snowy Plains	Dargo High Plains
Min. temp. (°C)	−3.2 ± 0.4	−3.6 ± 0.2
Max. temp. (°C)	21.4 ± 0.7	22.4 ± 0.6
Total annual rainfall (mm)	552 ± 31	651 ± 62
Global solar exposure (MJ m^{-2} day^{-1})	16.9 ± 0.3	16.3 ± 0.3
Altitude (m a.s.l.)	1,514	1,540

Conversely, P stored in vacuoles is rapidly remobilized under conditions of P limitation to buffer fluctuations in cytosol P_i (Veneklaas et al. 2012).

Phospholipids are key parts of membranes and are among the most significant uses of P in plants, accounting for approximately 20 percent of foliar organic P (Veneklaas et al. 2012). Under P limitation, phospholipids can be replaced by P-free lipids (Essigmann et al. 1998), again releasing P for use in the cytosol (Tjellström et al. 2008). Replacement of phospholipids with glycolipids under limiting P supply was first reported in prokaryotes (Minnikin, Abdolrah, and Baddiley 1974), but is now a well-documented process in many higher plant species (*Arabidopsis*: Härtel, Dörmann, and Benning 2000; oat: Andersson et al. 2003; soy bean: Gaude et al. 2004; *Proteaceae*: Lambers et al. 2012). All plasma membranes, mitochondrial envelopes, and tonoplast and chloroplast membranes (Andersson et al. 2005; Härtel, Dörmann, and Benning 2001; Jouhet et al. 2004) show this plasticity, suggesting a general strategy that assists plant growth on soils of widely varying P availabilities. While galactolipids are commonly involved in the replacement of phospholipids, they are not the only possible substitute. Phospholipids can also be replaced by sulfolipids (Benning 1998; Lambers et al. 2012). Although many Australian soils are also characterized as having poor availability of sulfur (Williams and Steinbergs 1959), the "sulfur problem" is arguably not as acute as that of P.

The prevalence of old soils in Australia and many other regions suggests that many native plants are effectively oligotrophs. Morcuende et al. (2007), for example, noted that P depletion causes repression of genes involved in the light reactions of photosynthesis, chlorophyll synthesis, the Calvin cycle, and photorespiration. Overall, the responses reflected programmed down-regulation of metabolism under P stress. Morcuende et al. (2007) also observed up-regulation of genes involved in the synthesis of secondary metabolites, including the anthocyanins and phenylpropanoids characteristic of plant stress responses to high light. Some native Australian plants seemingly down-regulate photosynthetic capacity under P stress (Turnbull, Warren, and Adams 2007), with a side benefit of committing resources to photoprotective measures (for a review, see Hernández and Munné-Bosch 2015).

Soil type, and related parent material, have long been implicated in the distribution of native grasses and forbs in areas such as the Monaro and other high tablelands in Australia (Garden et al. 2001). Phosphorus status ranks highly among the properties that delineate soil types, and there are many clear examples of species preferences for either lower or higher P status. In this study we aimed to characterize the extent to which five plant species, from two subalpine Australian ecosystems of contrasting parent material, adapt to long-term P availability by modifying lipid biosynthesis—specifically replacing phospholipids with nonphospholipids—and regulating photosynthetic properties. Measurements of photosynthetic rate *in situ* were combined with laboratory analysis of soil properties, leaf P content, and leaf membrane lipids for five proximate native species: the herbs *Geranium antrorsum* and *Ranunculus graniticola*, the grasses *Poa costiniana* and *Poa hiemata*, and the sub-shrub *Veronica derwentiana*. We sought also to determine if functional types differed in effects of long-term P limitation, and thus in potential suitability to habitats of differences in P availability.

Materials and methods

Site descriptions

Our study sites were located within the Snowy Mountains in New South Wales (Snowy Plains, SP; 36° 05′36″ S, 148°31′42″ E, ~200 ha) and the High Country in Victoria (Dargo High Plains, DHP; 36°07′53″ S, 147° 10′07″ E, ~150 ha), two subalpine regions of matching climate (Table 1) and vegetation (subalpine eucalypt grassy woodland). We studied five species common to both sites: two forbs *G. antrorsum* (Carolin) and *R. graniticola* (Melville), two grass species *P. costiniana* (Vickery) and *P. hiemata* (Vickery), and one sub-shrub *V. derwentiana* (Andrews). All five species can form endomychorrizal relationships (Berch, Gamiet, and Deom 1988; Javaid 2008), although the extent of mychorrization was not characterized in this study. The two sites differed in soil P availability (Table 2) because of sedimentary parent material at SP and basaltic parent material at the DHP. Photosynthetic measurements were

Table 2. Soil properties of the Snowy Plains (SP) and Dargo High Plains (DHP) sites. Values are means ±SE (n = 5). Asterisks indicate significantly different means between sites from an unpaired t test (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$).

Soil Property	Snowy Plains	Dargo High Plains	P
Water soluble P (mg P kg^{-1} DW)	6.32 ± 0.51	9.21 ± 1.13	*
Organic P (mg P kg^{-1} DW)	3.44 ± 1.32	0.27 ± 0.08	*
Total soil P (mg P kg^{-1} DW)	9.76 ± 1.54	9.48 ± 1.19	
Carbon content (% DW)	9.6 ± 0.9	16. ± 0.4	***
Nitrogen content (% DW)	0.68 ± 0.06	1.23 ± 0.04	***
C:N ratio	14.2 ± 0.3	13.3 ± 0.2	
Sulfur content (% DW)	0.08 ± 0.004	0.12 ± 0.007	**
Dry bulk density (g cm^{-3})	0.70 ± 0.02	0.47 ± 0.02	***
Moisture-holding capacity (% water DW^{-1})	77.5 ± 3.5	108.7 ± 5.6	**
pH	3.95 ± 0.11	3.76 ± 0.02	

taken during March 2013, plant samples for membrane lipid analyses were taken in March 2014, and soil samples were collected in April 2014.

Soil properties and plant P

The top 20 cm of soil (the organic layer and underlying mineral soil) was sampled at five randomly selected points at each site, using a soil core sampler (#403.27; AMS Inc., American Falls, ID, USA). Water-soluble inorganic phosphate was measured photometrically per Netzer et al. (2016): soil was oven dried at 60°C then sieved to 4 mm, and visible roots were removed. Four grams of sieved soil were added to 30 ml of water in a tube and shaken overnight at 4°C. The tubes were centrifuged and the supernatant was removed. Then, 150 µl supernatant was added to each well of a ninety-six-well plate, together with 17 µl staining reagent. The staining reagent was a mixture of 5 mM ammonium heptamolybdate, 0.2 mM potassium antimony (III) oxide tartrate hemihydrate, and 30 mM ascorbic acid in 1.25 M sulfuric acid. The plate was incubated at room temperature for 30 min, then absorbance at 700 nm was measured (TriStar2 LB 942; Berthold Technologies, Bad Wildbad, Germany) and the concentration of phosphate (mg P kg^{-1} dry soil) in samples was calculated from potassium phosphate standard curves (Murphy and Riley 1962).

Organic P was analyzed using a method of Dijkstra et al. (2012) modified from Saunders and Williams (1955). One gram of soil was ashed in a muffle furnace at 550°C for 2 h. Then, 50 ml of 0.5 M H_2SO_4 was added to the ashed soil in a tube; 1 g of unashed milled and oven-dried (60°C) soil was added to a separate tube, and 50 ml 0.5 M H_2SO_4 was added. Tubes were shaken for 16 h then centrifuged for 10 min at 5,000 g. The P content of each tube was determined colorimetrically, using the ammonium paramolybdate/stannous chloride reagent at 690 nm (UVmini-1240; Shimadzu Scientific Instruments, Sydney, NSW, Australia), in relation to KH_2PO_4 standards. Organic P was calculated as the difference in P content between ashed and nonashed samples.

Soil pH was analysed using a 1:5 soil-to-solution ratio in both 10 mM $CaCl_2$ and water using a glass electrode (SevenMulti; Mettler-Toledo Ltd., Port Melbourne, Vic, Australia). Soil carbon and nitrogen concentrations were measured on 100 mg of dry soil, using a CHN analyser (Truspec CHN; Leco Australia, Castle Hill, NSW, Australia). Soil moisture-holding capacity was calculated by drying soil samples for 24 h at 60°C (Cassel and Nielsen 1986). A subsample of dried soil was weighed and saturated with water. This wet soil was then weighed and the percent of water per unit dry weight (DW) of soil was calculated. Soil dry-bulk density was calculated by drying soil cores of known volume for 24 h at 60°C and then weighing them (Blake and Hartge 1986).

Plant phosphorus was analyzed using a modified method of Lambers et al. (2012), and 10 mg of dry powdered leaf material was digested in 2 ml 3:1 HNO_3:$HClO_4$ for ten days. A 100 µl aliquot of the acid digest was diluted to 1 ml with MQ water. Then, 500 µl was neutralized with 560 µl 1M KOH and centrifuged at 15,000 rpm for 10 min. Analysis of phosphorus content was then the same as that of soil using the photometric method of Murphy and Riley (1962).

Photosynthesis

Photosynthetic properties were measured *in situ* under fully saturating natural insolation (>2,000 µmol photons m^{-2} s^{-1}) using a recently calibrated infrared gas analyser (IRGA; LI-6400, Li-Cor Inc., Lincoln, NE, USA) fitted with the 2 × 3 cm clear leaf chamber at a CO_2 concentration of 400 ppm and a flow rate of 350 µmol s^{-1}. Because of the nature of gas-exchange measurements (using sunlight as a light source) it was not feasible to perform A/C_i or A/Q curves. A photograph was taken of each leaf within the IRGA chamber, using a digital camera (DSC-HX9V; Sony Corp., New York, NY, USA), for the calculation of leaf area.

Membrane lipids

Crude lipids were extracted and analyzed from powdered freeze-dried plant sample using liquid chromatography time-of-flight mass spectrometry as reported by Okazaki et al. (2015).

For statistical analyses, lipids were grouped as follows: phospholipids (fourteen phosphatidylcholine [PC], two lysophosphatidylcholine [lyso-PC], nine phosphatidylethanolamine [PE], four phosphatidylglycerol [PG], and two phosphatidylinositol [PI] species); galactolipids (nine monogalactosyl diacylglycerol [MGDG] and eleven digalactosyl diacylglycerol [DGDG] species); sulfolipids (seven sulfoquinovosyl diacylglycerol [SQDG] species); glucosylceramides (six glucosylceramide [GlcCer] species); and glucuronosyldiacylglycerols (five glucuronosyldiacylglycerol [GlcADG] species). We also quantified eight diacylglycerol (DAG) and forty-two triacylglycerol (TAG) species, six chlorophylls, and two carotenoids.

Tocopherol

Alpha-tocopherol was analyzed from leaves as described by Kreuzwieser et al. (2009). Briefly, 50 mg of homogenized frozen leaf powder was added to 500 µl ice-cold 85 percent (v/v) methanol containing 10 µl 100 mg µl^{-1} ribitol as an internal standard. Samples were continuously shaken and heated to 65°C and centrifuged thereafter; 50 µl of the supernatant was dried under vacuum. Dried extracts were derivatized with 20 µl 20 mg ml^{-1} methoxyamine hydrochloride in anhydrous pyridine; incubation occurred at 30°C for 90 min and shaken at 1,400 rpm. Thereafter, 50 µl N-methyl-N-(trimethylsilyl) trifluoroacetamide (MSTFA) was added, and samples were incubated at 37°C for 30 min. Samples were centrifuged and, after transfer into GC-MS vials, analyzed on an Agilent GC/EIMS system (GC 7890A coupled to a 5975C MSD, Agilent Technologies, Palo Alto, CA, USA). Injection of 1 µl aliquots into the system occurred with an autosampler (Gerstel MPS2-XL, Gerstel, Mülheim, Germany). Analytes were separated on a capillary column (HP-5MS 5%, Agilent Technologies, Palo Alto, CA, USA) with He as a carrier gas (flow: 1 ml min^{-1}). The GC-MS run conditions described by Erxleben et al. (2012) were applied. Raw data were processed with freely available AMDIS (automated mass spectral deconvolution and identification system, version 2.69; http://www.amdis. net/). For compound identification, we used the Golm Metabolome Database (Kopka et al. 2005). Relative quantification of tocopherol contents was obtained by normalizing peak areas for sample weight and internal ribitol standard and correction for blank values.

Statistical analyses

Photosynthetic results were analyzed statistically using a two-way analysis of variance (ANOVA) with post hoc Fisher's least significant difference (LSD) test (Prism 6; Graphpad Software, La Jolla, CA, USA). Membrane lipid data were normalized by sum and scaled based on the mean and standard deviation, then the composition was analyzed using a partial least squares discriminant analysis (PLS-DA) with subsequent t tests to identify individual compounds of interest (Metaboanalyst 3.0; Xia et al. 2015). Total lipid concentration and specific compound groups were analyzed using a two-way ANOVA (Prism 6).

Results

Soil properties and plant phosphorus

As expected, soil phosphorus fractions varied between sites (Table 2). Water-soluble inorganic phosphate (P_i) was 50 percent greater at the DHP site ($p < 0.05$) while organic P was tenfold greater ($p < 0.05$) at SP. Total soil P did not differ between the two sites ($p > 0.05$). Carbon, nitrogen, and sulfur concentrations followed P_i, and were more than 50 percent greater at the DHP site (C, $p < 0.001$; N, $p < 0.001$; S, $p < 0.01$). Both C:N ratios and soil acidity were similar at both sites ($p > 0.05$). Dry bulk density was greater at SP ($p < 0.001$), while soil moisture holding capacity was greater at DHP ($P < 0.01$).

Leaf total phosphorus content was linked closely to soil P_i for herbaceous and grass species (G. antrorsum, $p < 0.01$; R. graniticola, $p < 0.01$; P. costiniana, $p < 0.01$; P. hiemata, $p < 0.01$), while the sub-shrub V. derwentiana showed little difference between sites ($p > 0.05$; Figure 1).

Photosynthesis

Light-saturated rates of photosynthesis (A_{sat}: $p < 0.001$, $F = 56.96$, Figure 2A) and stomatal conductance (g_s: $p < 0.001$, $F = 98.26$, Figure 2B) clearly followed soil P_i, and were greatest at DHP. Plant life form was important in determining responses in A_{sat} to long-term P availability, with grasses less able to use accumulated phosphorus to increase A_{sat} than forbs or shrubs. The A_{sat} of forbs and shrubs was considerably greater at the DHP than at SP (G. antrorsum, SP = 6.6 ± 1.0 µmol m^{-2} s^{-1}, DHP = 21.3 ± 2.8 µmol m^{-2} s^{-1}; R. graniticola, SP = 11.1 ± 2.5 µmol m^{-2} s^{-1}, DHP = 19.7 ± 1.4 µmol m^{-2} s^{-1}; V. derwentiana, SP = 4.1 ± 1.3 µmol m^{-2} s^{-1}, DHP = 12.1 ± 0.4 µmol m^{-2} s^{-1}), while in grasses rates
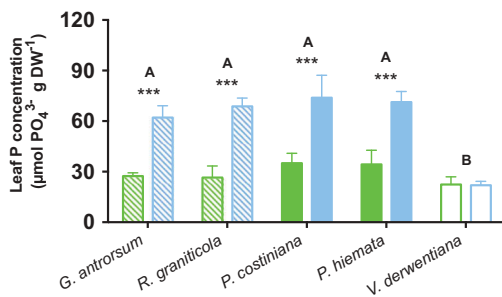
Figure 1. Leaf phosphorus concentrations for *G. antrorsum*, *R. graniticola*, *P. costiniana*, *P. hiemata*, and *V. derwentiana*. Green = SP (low P) site; blue = DHP (high P) site. Dashed bars are forbs, filled bars are grasses, and empty bars are sub-shrub species. Values are means ±SE (n = 3). Asterisks indicate significantly different means (*p < 0.05, **p < 0.01, ***p < 0.001) between sites within species; uppercase letters denote significantly different means among species identified by Fisher's LSD test (p < 0.05).
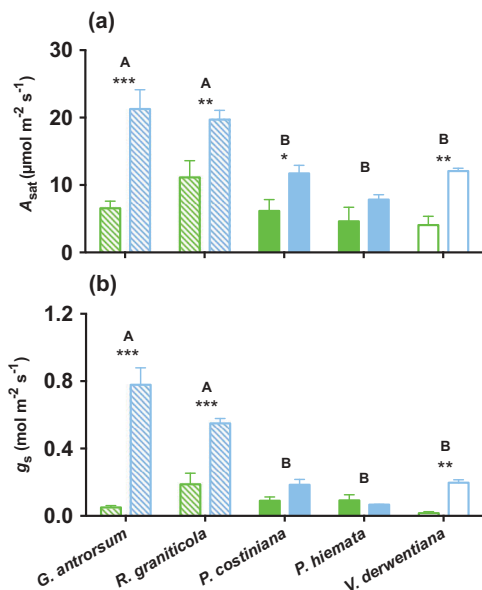
Figure 2. (A) Light-saturated photosynthetic (A_{sat}) and (B) stomatal conductance (g_s) rates of *G. antrorsum*, *R. graniticola*, *P. costiniana*, *P. hiemata*, and *V. derwentiana*. Green = SP (low P) site; blue = DHP (high P) site. Dashed bars are forbs, filled bars are grasses, and empty bars are sub-shrub species. Values are means ±SE (n = 3). Asterisks indicate significantly different means (*p < 0.05, **p < 0.01, ***p < 0.001) between sites within species; uppercase letters denote significantly different means among species identified by Fisher's LSD test (p < 0.05).

were more similar between sites (*P. costiniana*, SP = 6.2 ± 1.6 µmol m^{-2} s^{-1}, DHP = 11.7 ± 1.2 µmol m^{-2} s^{-1}; *P. hiemata*, SP = 4.6 ± 2.1 µmol m^{-2} s^{-1}, DHP = 7.8 ± 0.7 µmol m^{-2} s^{-1}).

Membrane lipids

Membrane lipid composition varied strongly with site. The PLS-DA on membrane lipid composition clearly segregated SP and DHP for all species studied (p < 0.05; Figure 3). Subsequent t tests highlighted a number of individual lipids that varied strongly with site (Tables S1–S5). Lipids in leaves on the SP generally comprised 33.3 percent galactolipids (sum of nine MGDG and eleven DGDG species), 64.9 percent phospholipids (sum of fourteen PC, two lyso-PC, nine PE, four PG, and two PI species), 1.2 percent sulfolipids (sum of seven SQDG species), and 0.6 percent GlcCers and GlcADGs (sum of six GlcCer and five GlcADG species), while those on the DHP typically comprised 30 percent galactolipids, 68.6 percent phospholipids, 0.9 percent sulfolipids, and 0.5 percent GlcCers and GlcADGs (Figure 4). Between-site differences in proportional concentrations of phospholipids were greatest in *R. graniticola* (7.4% less on the SP), *P. hiemata* (4.1% less on the SP), and *V. derwentiana* (4.2% less on the SP; Figure 4).

Foliar lipid concentrations also differed between sites, although patterns were highly variable among species. Reduced signal intensities (a proxy for lipid concentration) of the two most abundant phospholipid fractions were clear in the foliage of *R. graniticola* (−25% PC, p < 0.01; −36% PE, p < 0.001) and *P. costiniana* (−32% PC, p < 0.05; −42% PE, p < 0.05) at SP (Table 3), while there were no differences in the foliage of *G. antrorsum* and *P. hiemata* (p > 0.05). Signal intensities of almost all major membrane lipid fractions were greater in foliage of *V. derwentiana* at SP (65% PC, p < 0.01; 76% PE, p < 0.05; 162% PG, p < 0.01; 64% PI, p < 0.01; 104% MGDG, p < 0.001; 94% DGDG, p < 0.001; 88% SQDG, p < 0.001; Table 3). Signal intensities of GlcADGs were constitutively larger in foliage at SP regardless of plant life form; on average, 52 percent greater than in foliage at DHP (p < 0.05; Table 3).

Storage lipids (diacylglycerols [DAGs] and triacylglycerols [TAGs]) were reduced in concentration at SP (DAGs, p < 0.05, F = 7.39; TAGs, p < 0.001, F = 29.99; Figures 5A and 5B). Foliar concentrations of DAGs were significantly (30%) less in *P. hiemata* (p < 0.05) at SP, while the concentration of TAGs was approximately half the concentration of DHP at SP for *G. antrorsum* (p < 0.001) and *P. costiniana* (p < 0.05) and a quarter the concentration for *V. derwentiana* (p < 0.05).
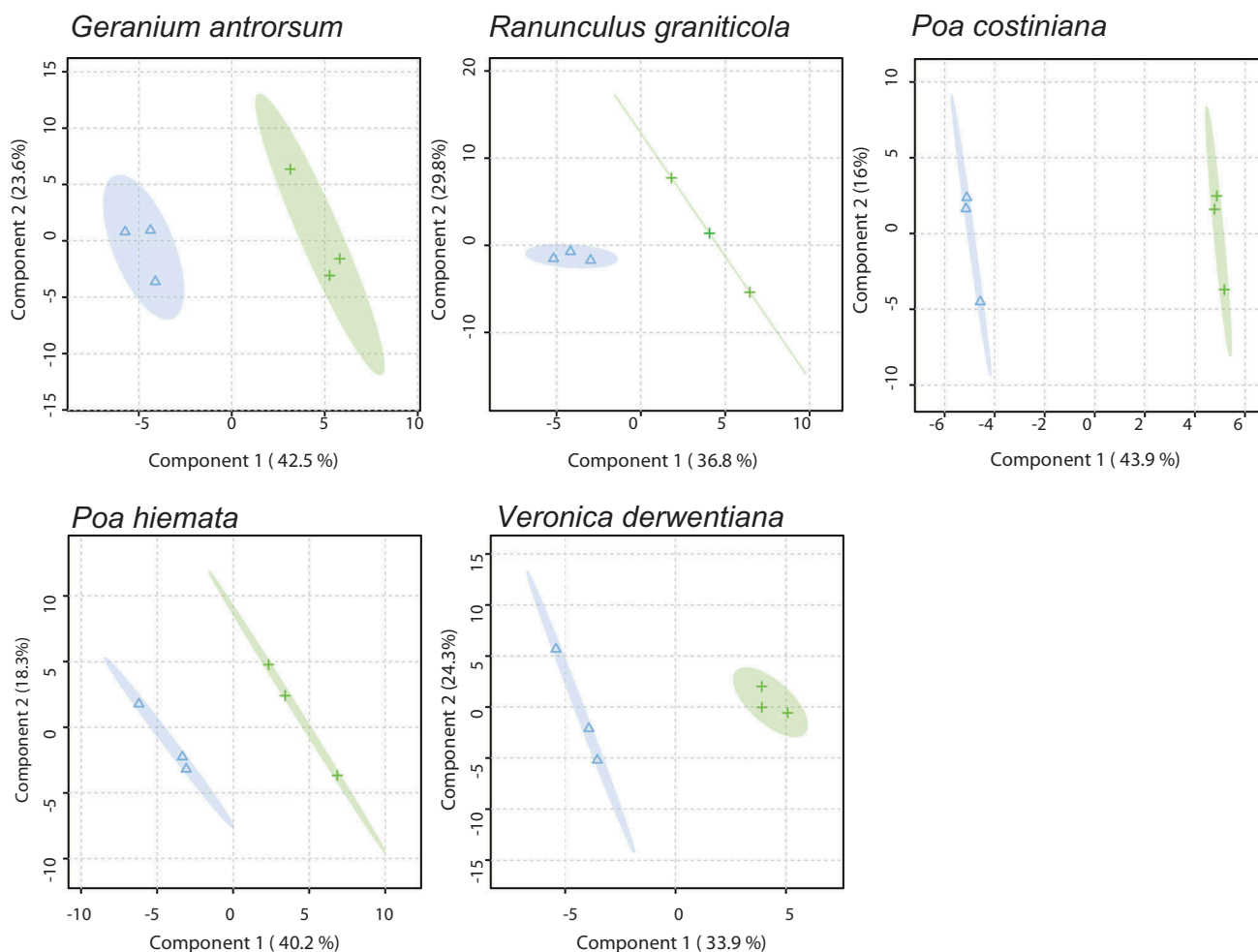
Geranium antrorsum

Ranunculus graniticola

Poa costiniana

Poa hiemata

Veronica derwentiana

Figure 3. PLS-DA score plots of the top two membrane lipid components for *G. antrorsum, R. graniticola, P. costiniana, P. hiemata*, and *V. derwentiana*. Closed green crosses = SP (low P) site; blue triangles = DHP (high P) site. Shaded areas represent 95 percent confidence intervals.

Antioxidants and pigments

G. antrorsum, R graniticola, and *V. derwentiana* were able to alter their foliar chemistry in response to long-term P availability, while this response was less clear in the grass species (Figure 5). Leaf chlorophyll content was greater at SP ($p < 0.05$, $F = 7.01$), with significantly greater concentrations found in the foliage of *G. antrorsum* (20% greater; $p < 0.05$) and *V. derwentiana* (81% greater; $p < 0.001$; Figure 5C). Foliar carotenoid concentrations were also increased in *V. derwentiana* at SP (126%, $p < 0.001$; Figure 5D). Foliar concentrations of alpha-tocopherol were greater at SP than at DHP ($p < 0.001$, $F = 42.89$; Figure 6), with significantly greater alpha-tocopherol content found in the leaves of *G.antrorsum* (91%, $p < 0.001$), *R. graniticola* (443%, $p < 0.001$), and *P. hiemata* (141%, $p < 0.05$).

Discussion

Numerous studies suggest that native Australian plants with specialized root systems can tolerate reduced soil phosphorus availability (Lambers et al. 2012; Sulpice et al. 2014), and that plants exhibit strong short-term responses to P stress (Muneer and Jeong 2015; Zhang et al. 2014). Our field-based study is unique in considering the chemical and physiological adaptations of generalist plants (i.e., *without* specialized root structures) of a variety of life forms to long-term limitation of P supply. Our results indicate a limited tolerance to shortages of P—one that is met by adaptation strategies that differ among plant life forms.

Phosphorus acquisition from the soil

Although the total P content of soils was similar at our two study sites (SP = 9.8 ± 1.5 mg P kg^{-1} DW,
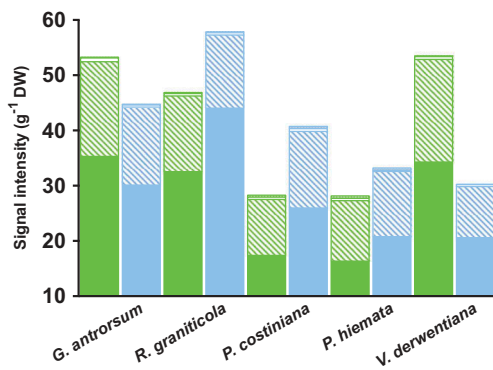
Figure 4. Total signal intensity, normalized for dry weight, for groups of lipids in the leaves of *G. antrorsum*, *R. graniticola*, *P. costiniana*, *P. hiemata*, and *V. derwentiana*. Green = SP (low P) site; blue = DHP (high P) site. The signal intensities of the different components of each group of lipids (phospholipids, filled bars; galactolipids, dashed bars; sulfolipids, empty bars; GlcCers and GlcADGs, checked bars) were summed. Values are mean signal intensities ($n = 3$).

DHP = 9.4 ± 1.2 mg P kg^{-1} DW), the sites differed in distribution among P fractions. The greater concentrations of organic P in soils at SP are likely less significant to plant nutrition because of the generally cool climates that restrict mineralization processes and the turnover of organic P, in turn reducing plant P availability (Vance, Uhde-Stone, and Allan 2003). Alternatively, water-soluble inorganic phosphate (P_i) is readily taken up by the roots (Vance, Uhde-Stone, and Allan 2003), and was considerably more abundant at DHP. Foliar P concentrations of the studied forb and grass species

were associated with P_i in the soil solution, suggesting minimal contributions of soil organic P to plant nutrition, consistent with current literature (Chiou and Lin 2011). The one exception was *V. derwentiana*, which showed similar foliar concentrations of P at both SP and DHP.

Photosynthesis

Net CO_2 assimilation rates at SP were considerably slower than those at DHP. Between-site differences were larger in the forb and shrub species than in the grass species, suggesting that photosynthesis in these species may be biochemically or diffusionally limited by P availability, while grasses may have intrinsically lower photosynthetic capacity to reduce reliance on external P. Most studies report reduced photosynthetic capacity under P stress (Jacob and Lawlor 1991; Turnbull, Warren, and Adams 2007; Warren 2011) because of both diffusional and biochemical limitations. Reduced rates of stomatal and mesophyll conductance are well documented (Singh et al. 2013; Zhang et al. 2014); however, impaired ribulose-1,5-biphosphate (RuBP) regeneration rate and reduced ribulose-1,5-biphosphate carboxylase/oxygenase (RuBisCO) carboxylation efficiency are considered to be the primary factors limiting photosynthesis (Hernández and Munné-Bosch 2015; Jacob and Lawlor 1992).

Stomatal conductance correlated closely with photosynthetic rate in all studied species. In general terms, if conductance was maintained then rates of

Table 3. Signal intensities, normalized for dry weight, for major membrane lipid fractions in the leaves of *G. antrorsum*, *R. graniticola*, *P. costiniana*, *P. hiemata*, and *V. derwentiana* at the SP and DHP sites. Values are means ±SE ($n = 3$). Lowercase letters denote significantly different means among species identified by Fisher's LSD test ($p < 0.05$); asterisks indicate significantly different means (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$) between sites within species.

|		Signal Intensity (g^{-1} DW)									
		PC		PE		PG		PI		Lyso PC	
G. antrorsum	SP	30.8[ac]	(2.0)	3.8[a]	(0.02)	0.6[a]	(0.05)	0.10[a]	(0.005)	0.02[a]	(0.002)
	DHP	26.4[ac]	(2.8)	3.1[a]	(0.38)	0.5[a]	(0.07)	0.10[a]	(0.009)	0.02[a]	(0.001)
R. graniticola	SP	28.1[a]	(4.6)**	3.4[b]	(0.71)***	0.9[b]	(0.18)	0.08[a]	(0.004)*	0.02[a]	(0.001)
	DHP	37.6[a]	(2.7)	5.3[b]	(0.55)	1.0[b]	(0.13)	0.10[a]	(0.011)	0.02[a]	(0.004)
P. costiniana	SP	15.6[b]	(0.9)*	1.4[c]	(0.18)*	0.3[ac]	(0.03)	0.02[b]	(0.003)***	0.03[a]	(0.005)***
	DHP	23.0[b]	(0.5)	2.4[c]	(0.09)	0.5[ac]	(0.06)	0.06[b]	(0.007)	0.01[a]	(0.002)
P. hiemata	SP	14.7[b]	(0.5)	1.3[c]	(0.13)	0.3[c]	(0.04)	0.02[c]	(0.005)	0.03[a]	(0.004)
	DHP	18.5[b]	(0.6)	1.8[c]	(0.06)	0.4[c]	(0.05)	0.03[c]	(0.002)	0.02[a]	(0.002)
V. derwentiana	SP	31.2[c]	(2.9)**	2.4[c]	(0.18)*	0.6[ac]	(0.06)**	0.06[b]	(0.004)**	0.01[b]	(0.003)
	DHP	18.9[c]	(1.0)	1.4[c]	(0.09)	0.2[ac]	(0.02)	0.04[b]	(0.006)	0.01[b]	(0.002)
		MGDG		**DGDG**		**SQDG**		**GlcCer**		**GlcADG**	
G. antrorsum	SP	11.8[a]	(0.9)	5.3[ac]	(0.3)	0.5[a]	(0.11)	0.12[a]	(0.027)	0.016[a]	(0.004) *
	DHP	9.7[a]	(0.9)	4.4[ac]	(0.3)	0.4[a]	(0.03)	0.13[a]	(0.015)	0.002[a]	(0.001)
R. graniticola	SP	9.1[b]	(1.0)	4.6[abc]	(0.4)	0.4[b]	(0.01)*	0.17[b]	(0.033)	0.003[a]	(0.001)
	DHP	8.7[b]	(0.5)	4.5[abc]	(0.4)	0.2[b]	(0.03)	0.14[b]	(0.013)	0.001[a]	(0.001)
P. costiniana	SP	6.3[b]	(0.3)*	3.8[ab]	(0.1)*	0.4[a]	(0.03)	0.33[b]	(0.023)	0.028[b]	(0.003)
	DHP	8.8[b]	(0.7)	5.0[ab]	(0.4)	0.5[a]	(0.05)	0.37[b]	(0.050)	0.016[b]	(0.003)
P. hiemata	SP	7.0[b]	(0.3)	3.9[b]	(0.2)	0.4[a]	(0.03)	0.39[c]	(0.043)	0.051[c]	(0.003)
	DHP	7.7[b]	(0.6)	4.1[b]	(0.3)	0.4[a]	(0.04)	0.36[c]	(0.031)	0.049[c]	(0.011)
V. derwentiana	SP	11.7[b]	(1.1)***	6.9[c]	(0.6)***	0.7[a]	(0.08)***	0.11[ac]	(0.025)	0.007[a]	(0.002)
	DHP	5.8[b]	(0.5)	3.5[c]	(0.3)	0.4[a]	(0.04)	0.04[ac]	(0.013)	0.001[a]	(0.001)
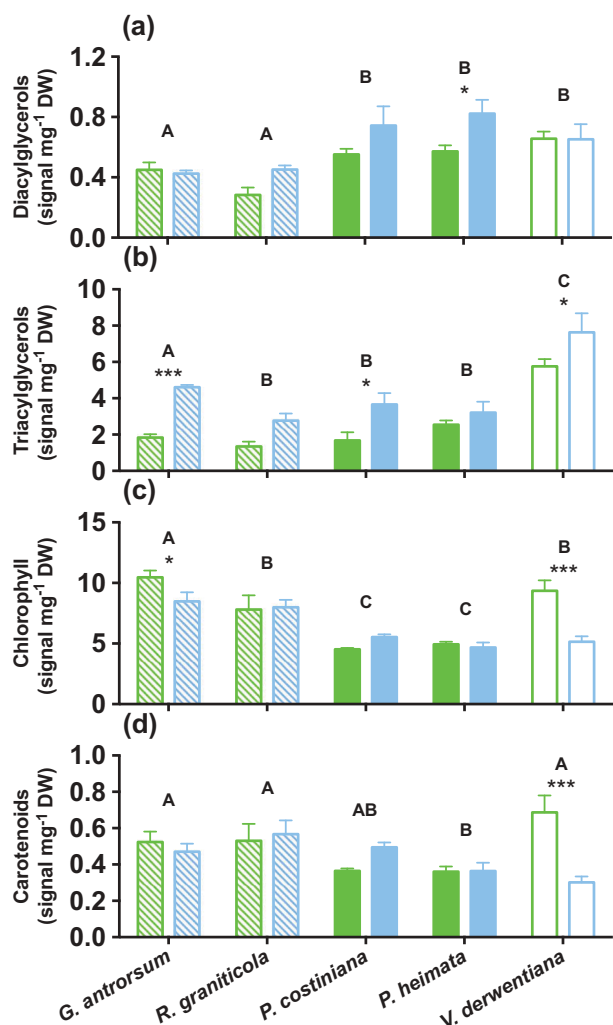
Figure 5. Leaf concentrations of (A) DAGs, (B) TAGs, (C) chlorophylls, and (D) carotenoids in *G. antrorsum*, *R. graniticola*, *P. costiniana*, *P. hiemata*, and *V. derwentiana*. Green = SP (low P) site; blue = DHP (high P) site. Dashed bars are herbaceous species, filled bars are grass species, empty bars are sub-shrub species. Values are means ±SE (*n* = 3). Asterisks indicate significantly different means between sites within each species (*P < 0.05, **P < 0.01, ***P < 0.001); uppercase letters denote significantly different means among species identified by Fisher's LSD test (P < 0.05).
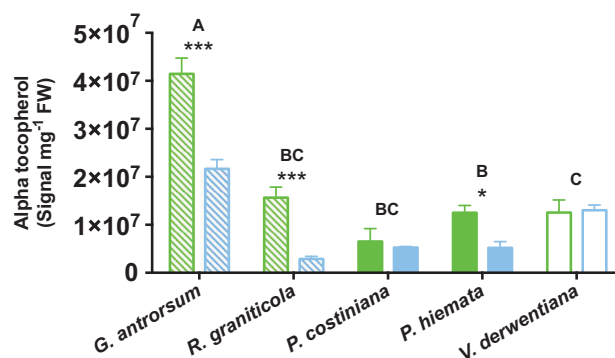

Figure 6. Concentrations of alpha-tocopherol in foliage of *G. antrorsum*, *R. graniticola*, *P. costiniana*, *P. hiemata*, and *V. derwentiana*. Green = SP (low P) site; blue = DHP (high P) site. Dashed bars are herbaceous species, filled bars are grass species, empty bars are sub-shrub species. Values are means ±SE (*n* = 3). Asterisks indicate significantly different means between sites within each species (*P < 0.05, **P < 0.01, ***P < 0.001); uppercase letters denote significantly different means among species identified by Fisher's LSD test (P < 0.05).

signaling mechanisms may be involved in photosynthetic down-regulation, glycolipid biosynthesis, phospholipid degradation, and stomatal closure.

Reduced photosynthetic capacity seems to inexorably result in increased susceptibility to photoinhibition and photooxidative stress (for review, see Hernández and Munné-Bosch 2015), particularly in alpine ecosystems that are subject to consistently strong radiation, including ultraviolet (Körner 1999). Recent genomic and proteomic studies with the model plant *Arabidopsis thaliana* argue that there is programmed down-regulation of gene expression for photosynthesis under P starvation, with simultaneous up-regulation of genes encoding photoprotective measures and systems-scavenging reactive oxygen species (Misson et al. 2005; Morcuende et al. 2007; Wu et al. 2003). Similar results have been found in other herbaceous species, including maize (Zhang et al. 2014), tomato (Muneer and Jeong 2015), and rice (Park et al. 2012).

Lipids

The replacement of membrane phospholipids with P-free lipids is a well-documented strategy of plants for allocation of internal P (Andersson et al. 2003; Li, Welti, and Wang 2006). This strategy seemed unrelated to plant life form but did vary between individual species. *R. graniticola* and *P. hiemata* substituted phospholipids with both galactolipids and sulfolipids in foliage at SP. These species also showed a lessened impact of reduced P_i availability on photosynthetic

photosynthesis varied only marginally. Diffusional limitations seem likely to be responsible for reduced rates of photosynthesis at SP. Reduced stomatal conductance, via smaller and fewer stomata, is a putative adaptation to long-term P stress (Sun et al. 2014), and could prevent direct biochemical inhibition of photosynthesis. Further work is needed to confirm this hypothesis. Chiou and Lin (2011) suggested that phosphorus starvation response (PSR) is controlled by the supply of P_i to the roots, not just systemically via internal P concentrations, implying long-distance

rate. *G. antrorsum* and *P. costiniana* had similar membrane lipid compositions between sites and greater reductions in photosynthesis. Replacement with sulfolipids is particularly important, because within plant cells this lipid group is limited to the chloroplast membrane (Benning 1998), so small increases in overall leaf sulfolipid concentration can equate to large increases in their concentration in the chloroplast membrane.

At our SP study site, all species accumulated glucuronosyldiacylglycerols (GlcADGs) in their leaves, albeit at lesser concentrations than phospholipids, galactolipids, and sulfolipids. GlcADGs have only recently been identified in plants, but are already described as critical to relieving the stress caused by P limitation (Okazaki and Saito 2014). GlcADGs accumulate under P stress in wild-type *Arabidopsis thaliana*, while GlcADG-deficient mutants showed significant growth defects under the same treatment conditions (Okazaki et al. 2013), with similar responses seen in tomato and soybean (Okazaki et al. 2015). The exact functional mechanism of these molecules remains unclear.

Lipid peroxidation is a risk under P deficiency because of greater photooxidative stress in mesophyll cells (Hernández and Munné-Bosch 2015); however, we found no evidence for this process. Instead, increased foliar concentrations of alpha-tocopherol, an important liposoluble antioxidant (Mallet et al. 1994) could prevent lipid peroxidation in forbs. Increased abundance of carotenoids, particularly in the sub-shrub *V. derwentiana*, help protect the photosynthetic apparatus from excess light and can alleviate oxidative stress via the xanthophyll cycle (Latowski, Kuczyska, and Strzalka 2011). Up-regulation of glutathione and ascorbate synthesis will also alleviate oxidative stress (Foyer and Noctor 2011).

Concentrations of DAGs and TAGs were considerably greater in the leaves of all species at DHP, and can act as a sink for excess photosynthate (Lin and Oliver 2008). Preferential resource allocation to membranes and photoprotective measures helps explain reduced concentrations in foliage at SP (Winichayakul et al. 2013).

Conclusions

Plants in subalpine Australian ecosystems seemingly optimize P use, albeit not sufficiently to negate potential reductions in photosynthesis that are the result of long-term reduced P availability. Nonetheless, P use and acquisition strategies varied among the plant species studied in response to site P availability. The two forb species altered foliar chemistry by promoting antioxidant biosynthesis and inhibiting accumulation of storage lipids. The woody

shrub species maintained consistent leaf P concentration regardless of the availability of P_i. The studied grasses reduced their reliance on exogenous P by maintaining an inherently reduced photosynthetic capacity. Reduced rates of photosynthesis at SP were arguably because of direct diffusional and biochemical limitations. Replacement of phospholipids with galactolipids and sulfolipids in foliage at SP was clear in most species, with more extensive replacement alleviating decreases in photosynthetic rate and stomatal conductance. Glucuronosyldiacylglyerol concentrations were increased in all species at SP, characteristic of P stress, while increased carotenoid and tocopherol concentrations in foliage at SP concomitantly prevent photooxidative damage that accompanies reduced P conditions. Further work is needed to assess the P acquisition strategies used by plants in these ecosystems, and the interaction of long-term reduced P availability with other environmental factors, particularly oxidative stress.

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