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Diagnostic tools for nutrition status in *Eucalyptus globulus*: changes in leaves, xylem and phloem sap compounds according to N-, P-, and K-withdrawal or salt application

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Abstract

Key message **The results presented here demonstrate the capacity for phloem sap as a diagnostic tool for monitoring the nutritional status of plants and highlight the processes of remobilisation and source–sink dynamics.**

Abstract It is essential to develop methods that accurately reflect plant nutritional status for growth. In a greenhouse experiment with potted eucalyptus, we studied the effects of withdrawing single macronutrients N, P, or K, or salt application on the abundance of components in phloem, xylem and leaves to identify and refine methods to rapidly and effectively assess the nutrient status of *Eucalyptus globulus* trees. Clear effects on growth (and photosynthesis) were found for "−N" treatment and NaCl application. Effects of nutrient withdrawal were detected indicating our methodologies are useful for nutrient availabilities below the threshold of growth responses. While "−P" resulted in significantly lower P in xylem sap, the corresponding effect for "−K" on K in xylem sap was not found, although Ca and Na increased. Salt application increased Na in xylem sap sixfold. In leaf material "−N" reduced N. Surprisingly, "+NaCl", "−P" and "−K" did not change the corresponding elements in leaves. Additionally, "−P"-treatment had the tendency to affect C-, N-, K- and particularly P-increment (n.s.). In phloem sap, "+NaCl" resulted in several effects, most prominently doubling Na concentration. Importantly, "−N" increased amino acids in phloem sap (n.s.). Statistically significant relationships between concentrations in leaves and phloem sap were found for δ^{13}C and amino acids. Within the results obtained from phloem sap, sugars and bivalent cations (Ca and Mg) are correlated with that obtained from the leaves as well as Na and P. The role and comparative advantages of phloem sap as a diagnostic tool for nutritional status are discussed.

Abbreviations
"−N"	N-withdrawal
"−P"	P-withdrawal
"−K"	K-withdrawal
"+NaCl"	Salt application

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Introduction

Developing efficient tools for the rapid and reliable assessment of plant nutritional status is beneficial for both monitoring plant health and to provide diagnostic information to inform management of nutrient supplements. The availability of water, light and mineral nutrients is essential for plant biomass production as CO_2 is sequestered into photoassimilates to the extent that nutrients, temperature or water availability permits (Körner 2015). Thus, understanding plant nutrient status is a vital component of managing plant nutrition.

Nutritional supplements (fertiliser) are used to correct for nutrient deficiencies, primarily consisting of N, P and

K, which affect final biomass production and yield. N, P, and K are together with S, Mg and Ca the most important nutrients for plants and play essential roles in almost all categories of plant physiology (Maathuis 2009; White and Brown 2010). N is often the most critical element, since it is taken up at highest rates and accounts up to 2–5% of plant dry matter (Marschner 1995). N is the key constituent of amino acids, proteins, nucleotides and chlorophyll (Maathuis 2009); therefore, its availability influences their synthesis. P is often a limiting nutrient for plant growth across the world, as soils are frequently deficient in soluble forms of inorganic phosphate (Pi) which can be taken up and assimilated by plants (Yang and Finnegan 2010). K is the most abundant cation in plants (Luan et al. 2009) and plays an important role in osmoregulation, cell expansion, stomatal opening and activity of enzymes (Maathuis 2009). Similarly, in a number of studies, the impacts of potassium as well as of calcium on wood formation have been shown (Fromm 2010). The manufacture and supply of a fertiliser are costly and if provided in excess, greatly influences the sustainability of many agricultural practices. This can potentially lead to the loss of arable land (Luan et al. 2009) and associated environmental pollution such as eutrophication and contamination of water resources.

Both the deficiency and excessive abundance of minerals can impede biomass accumulation. The most common excess of a mineral is that of sodium chloride (NaCl), the most common form of salinity. About 5–7% of land used for agriculture is affected by salt (Shabala and Cuin 2008; Munns and Tester 2008; White and Brown 2010). In Australia, salinization of agricultural land is an important issue (Shabala and Cuin 2008) and is a major impediment to agricultural practices, particularly in temperate regions. Whilst Na^+ and Cl^- may play a useful role in osmotic and ionic adjustments, in excess they will cause toxic effects on plants that are well characterised (Munns and Tester 2008). Salt inhibits water and nutrient uptake, referred to as "the osmotic or water-deficit effect of salinity". Additionally, Na^+ and Cl^- may enter the xylem stream and damage leaves ("the salt-specific/ion-excess effect of salinity", Munns 2005). The latter 'ion effect' may be mitigated by plants with the help of mechanisms excluding toxic ions from the xylem or recycling from leaf tissue. Reclamation of salt-affected land by woody vegetation has been proposed, including by species of Eucalyptus (Polle and Chen 2015) suggesting that improved monitoring to detect salinization of soils will be of great value.

The degree of nutrient supply affects the translocation, allocation and distribution of assimilates and nutrients between the different organs and tissues of the plant (Hermans et al. 2006; Maathuis 2009; Peuke 2010). Water, nutrients, chemical signals and metabolic products are transported via xylem and phloem leading to the suggestion

of their use in developing monitoring tools to assess plant water and nutrient status. By improving our understanding of nutrient transport systems and their coupling to environmental change, we may be able to identify methods to detect nutrient imbalances such as those already characterised for water availability (Merchant et al. 2010).

Tasmanian blue gum (*Eucalyptus globulus*) is widely used for timber and pulp production, and planted in temperate regions of the Australian, African and South American continents. For tree species, assessing nutrient status traditionally encompasses leaf-level components; however, such approaches can suffer from natural inconsistencies—due to longevity, spatial variation across the canopy, leaf age and/or tree age, and sampling bias due to canopy access. Visual analysis of tree nutrient status is a cost-effective method; however, such findings often deviate from trends demonstrated through measured foliar nutrient concentrations. Additionally, visual symptoms are limited to detection long after the onset of deficiencies or toxicities.

Eucalyptus globulus has been shown to maintain 'bleeding' after incisions into the phloem representing a potentially useful avenue to monitor water and nutritional status of the trees by analysing phloem sap composition (Pate et al. 1998). Phloem analysis is a non-destructive measure, which has the potential to reflect the current availability of nutrients to all plant components, and provide an assessment of plant nutritional status (Peuke 2010). The use of phloem information to assess the physiological status of *E. globulus* has been demonstrated in both controlled and field studies. For example, the naturally occurring carbon isotope abundance ($\delta^{13}C$) of phloem sap has been used to predict tree water status (e.g. Tausz et al. 2008; Pate and Arthur 1998; Merchant et al. 2010, 2011). In addition, a strong correlation has been found between the $\delta^{13}C$ and sugar concentration of the phloem sap (Tausz et al. 2008; Pate and Arthur 1998; Merchant et al. 2011). This allows for a simple field measure of phloem sugar concentration, using a hand-held refractometer, to assess tree water status (Tausz et al. 2008). Similarly, concentrations of sugars and amino acids in the phloem can be utilised to assess the nitrogen status of *E. globulus* plantations (Pate and Arthur 1998). It is, therefore, valuable to further our understanding of how phloem information reflects tree nutrient status to improve plantation management decisions.

In a previous study, we investigated the effects of water availability—flooding or drought—on growth and biomass production in seedlings of *E. globulus* (Merchant et al. 2010). In the present study, we investigate the effects of alteration of nutrient supply with phloem composition and the elicitation of changes in physiology under similar environmental conditions. A prominent aim of our studies was to introduce analysis of phloem sap as an indicator for plant (nutrition) status. Plants were exposed to full nutrient

solution with selected removal of individual nutrients N, P, or K (withdrawal) or the addition of 100 mM NaCl. Using this experimental framework, we aimed to identify suitable methods to assess the nutrient status of *E. globulus* trees shortly after or before the onset of growth inhibition. For this purpose, we addressed the following questions: (1) can we detect macronutrient deficiencies/toxicities in phloem exudates? (2) Are macronutrient deficiencies better reflected in phloem sap than leaf material? (3) What components of phloem sap nutrient content best reflect nutrient imbalance? And (4) can we detect compensation of nutrient withdrawal in other plant/metabolite components? Combined, these analyses may enable the detection of nutrient limitation prior to the onset of growth inhibition and provide a more convenient, cost-effective methodology to assess tree nutrient status.

Materials and methods

Plant material and cultivation

Tasmanian blue gum (*Eucalyptus globulus* LABILL.) saplings (ca. 26 cm height, 2.8 mm stem diameter) were provided by a commercial nursery. At the end of October 2006, one plant per 9-L container was potted in a mixture of 80:20 potting soil and dry river sand. On average, the substrate could store 0.95 ± 0.12 L water per pot (~ 10.5%). Plants were watered daily to the field capacity of the soil by drip irrigation and raised under greenhouse conditions in Perth, Australia (31° 57′S, 115° 52′E). Glasshouse temperature conditions were set at 28/15 °C (day/night). Illumination was subject to daily variation in conditions regularly reaching 1000 μmol m^{-2} s^{-1}. Before treatments were started, plants were supplied weekly with a water-soluble fertiliser ("Thrive", Yates Australia, DuluxGroup Pty Ltd, Padstow, NSW 2211, Australia; NPK: 27%, 5.5%, 9% plus Mn, Fe, Cu and trace elements).

After 12 weeks, nutrient withdrawal treatments commenced. Plants were on average 132 ± 14 cm high and the stem diameter was 8.81 ± 0.95 mm. The pots were rinsed with excess tap water for 3 days to remove nutrients from the substrate and fertiliser application was stopped. Afterwards, plants were supplied every Monday and Friday with 1 L of a nutrient solution (following drip irrigation) optimised for *Eucalyptus* (Macfarlane et al. 2005): $CaCl_2$ 700 μM, NaH_2PO_4 500 μM, K_2SO_4 1400 μM, $MgSO_4$ 750 μM, H_3BO_4 40 μM, $MnSO_4$ 15 μM, Fe–Na-EDTA 25 μM, $ZnSO_4$ 1 μM, $CuSO_4$ 1 μM, $CoSO_4$ 0.3 μM, Na_2MoO_4 0.15 μM, NH_4NO_3 6000 μM. Each treatment had the following specification (for entire composition of the nutrient solution see Supplemental Table 1):

- controls ($n = 18$) were supplied with full nutrient solution.
- "−N": N-withdrawal ($n = 8$) NH_4NO_3 was omitted.
- "−P": P-withdrawal ($n = 8$) NaH_2PO_4 was omitted.
- "−K": K-withdrawal ($n = 8$) K_2SO_4 was omitted.
- "+NaCl" salt treatment ($n = 8$) plus 100 mM NaCl (in the first treatment only 50 mM NaCl, but strength was supplied in the second addition to avoid osmotic stress).

Growth monitoring

Stem diameter at the root–shoot interface and the height of all plants were measured weekly to calculate increments of diameter and height [actual value minus last week value per last week value (%)] and for monitoring growth. At the beginning of the experiment, 12 plants were harvested prior to the application of treatments (pre-treatment) and the leaf, stem and root dry weights were determined for each plant. At the end of the treatment period, leaf, stem and root dry weights were determined for each plant. Average growth measurements for each plant component are expressed at the average increment in dry mass calculated as the difference between a plant organ dry weight and that of the pre-treatment harvested plants.

Harvesting of plant material

6 weeks after the commencement of treatments, gas exchange measurements (see below) were measured, followed by harvest of all plant components. First, ten fully expanded leaves were collected from the canopy with leaves taken from several heights. Leaves were immediately microwaved as per Popp et al. (1996) and then oven dried at 75 °C up to constant weight and ground with a ball mill. Samples were then kept at − 80 °C awaiting analysis. The harvested plants, 14 control plants and 7 treated plants each (extreme individuals from each treatment/population were excluded), were divided into leaves, stems, and roots, and fresh weight was determined. Every plant part was carefully washed with water or sorbitol (50 mol m^{-3}) in the case of roots to avoid leaching of solutes.

Gas exchange

Three fully expanded non-senescent well-illuminated leaves from the upper canopy were measured for maximum rates of photosynthesis (A_{max}, μmol m^{-2} s^{-1}) and stomatal conductance (mmol m^{-2} s^{-1}) five times across the course of the day and averaged for each parameter. Gas exchange was measured in situ on attached leaves between 10:00 h and 14:00 h. Measurements were made with a CIRAS-1 (PPS Systems, Amesbury, MA 01913 USA) portable infra-red gas analyser fitted with automatic broad-leaf cuvette. The

gas exchange system was automatically controlled $[CO_2]$ at 350–360 ppm and photosynthetic active radiation at 1000 ± 9 µmol m^{-2} s^{-1}. The cuvette temperature was at ambient 28 ± 2 °C.

Sampling of xylem and phloem saps

Phloem sieve tube sap was collected 1 day before plant harvest by shallow incisions into the bark of the hypocotyl at a height of approximately 3 cm above the base according to Pate et al. (1974) and Merchant et al. (2010). Phloem sap bled from incisions for a period of 4–6 h, mostly between the hours of 11 am and 2 pm. Phloem sap droplets formed on the surface of the cut were collected in a glass disposable pipette and bulked into a single microtube.

Xylem sap was obtained as root pressure exudate at the time of harvest (Jeschke and Pate 1991; Alexou and Peuke 2013). The shoot was removed at 2 cm above the soil surface and then around 1 cm of bark removed below the cut, and washed with deionised water. A silicon tube was fitted to the stump to avoid contamination with phloem sap. The initial µLs of xylem sap were removed (around 40 µL).

All saps—phloem and xylem—were transferred to 4 °C during the collection period then frozen at −80 °C.

Sample extractions

For carbohydrate and amino acid analyses, soluble compounds were extracted from approximately 40 mg of dried leaf material with methanol–chloroform–water (MCW, 12:5:3) as detailed in Merchant et al. (2006). The water fraction of the extraction solution included an internal standard of 0.1% xylitol. Extracts were stored at −80 °C. For the analysis of carbon isotope composition of the soluble extract in leaves (δ^{13}Csol), 40 mg of ground leaf material was weighed into a 2-mL microtube to which 1 mL of hot, deionised water was added and incubated for 1 h at 75 °C. Samples were centrifuged at 11,400g for 3 min and 800 µL of the supernatant transferred to a 2-mL microtube.

Analysis of phloem sap and leaf extracts for soluble carbohydrates

Carbohydrates were separated and quantified using gas chromatography according to Merchant et al. (2006). GC analysis was performed using a Shimadzu 17A series gas chromatograph using a DB1 (30 m) column. Peak integration was made using Class VP analysis software (Shimadzu Corporation Limited, Kyoto, Japan). For further details, see Merchant et al. (2010).

Carbon isotope analysis

Four tissues or plant extracts were measured for isotopic composition in this study. For further details, see Merchant et al. (2010). Isotope ratio mass spectrometry (IRMS) was used to determine the ratio of ^{13}C/^{12}C in samples. Samples were analysed on an Isochrom mass spectrometer (Micromass, Manchester, UK) coupled to a Carlo Erba elemental analyser (CE Instruments, Milan). Samples were dropped from an AS200 autosampler and combusted by Dumas combustion in a furnace kept at 1060 °C. Carbon isotope ratios are expressed in delta-notation, where δ^{13}C $= R_{sample}/R_{standard} - 1$, and R is the ratio of ^{13}C to ^{12}C in a sample and standard (VPDB), respectively. δ^{15}N values reported here were defined as δ^{15}N $(‰) = (R_{sample}/R_{standard})$ $- 1$, where R_{sample} and $R_{standard}$ (0.0036765) are ^{15}N/^{14}N ratios in sample and atmospheric N$_2$, respectively. We used IAEA-600 (caffein), IAEA-N-1 (ammonium sulphate), IAEA-NO-3 and various in-house standards as reference material.

Amino acid and inorganic ion analyses

Sample preparation for amino acid analysis followed that described for phloem sap and leaf sugars. For leaf extracts, samples for amino acid analysis were removed prior to deionising with the mixed bed resin. For amino acids in phloem, 25 µl of sap was dried under vacuum into a 2-mL glass vial (Merchant et al. 2010). Amino acid derivatives were separated by capillary gas chromatography on a 5% diphenyl–95% dimethyl polysiloxane stationary phase (30 m long × 0.25 mm ID × 0.25 µm film thickness; Rtx-5SilMS, Restek, Bellfonte, USA). The column eluent was ionised by electron impact (70 eV) and mass spectra were collected from 100 to 600 amu (GCMS-QP2010Plus, Shimadzu, Kyoto, Japan).

Major cations in leaf extracts were determined using inductively coupled plasma-optical photoemission spectroscopy (ICP-OES). For further details, see Merchant et al. (2010).

ABA analysis

Freeze-dried tissue samples were homogenised and extracted in 80% methanol. Extracts were passed through a Sep Pak C18-cartridge. Methanol was removed under reduced pressure and the aqueous residue partitioned three times against ethyl acetate at pH 3.0. The ethyl acetate of the combined organic fractions was removed under reduced pressure. The residue was suspended in TBS buffer (Tris buffered saline; 150 mol m^{-3} NaCl 1 mol m^{-3} MgCl$_2$ and 50 mol m^{-3} Tris; pH 7.8) and subjected to an immunological ABA assay (ELISA) as described earlier (Peuke et al. 1994). For phloem

and xylem saps, the Sep-Pak C18 purification step was omitted. The aqueous phase after partitioning against ethyl acetate was hydrolysed for 1 h at room temperature with 1 M NaOH. This fraction was acidified with concentrated hydrochloric acid to pH 3 and partitioned three times against ethyl acetate. The accuracy of the ELISA was verified for Ricinus (Peuke et al. 1994). Recoveries of ABA during purification procedures were checked routinely using radioactive ABA and found to be more than 95%.

Statistics

Concentrations of solutes in plant tissues as well as growth parameters were obtained from 7 (treatments) and 14 (controls) plants, cultivated in individual pots in each single treatment. Values are given as means ± SE. Statistical calculations were performed with SAS (V9.4, SAS Institute Inc., Cary, NC, USA) non-linear regression analysis with Sigma Plot (V12.5, Systat Software Inc., San Jose, CA, USA). One-way analysis of variance (ANOVA, model: "treatment") was performed using the general linear model (GLM) procedure. The adjustment of multiple comparisons according to the Dunnett test (in comparison with the control) was chosen for the p values and confidence limits for the differences of least squares of the means (LS-means). Analysis of correlation was performed using the CORR procedure of SAS (K_p: and p value for H_0: $K_p = 0$). Regression analyses (REG procedure of SAS) was used to test a linear model which included an intercept ($y = a + b \times x$). In circumstances where the slope was both significant and correlated ($r^2 > 0.5$) but the intercept was not significant (H_0: estimates = 0), a model without the intercept was calculated ($y = b \times x$) and r^2 redefined. Only significant estimates (slope as well as intercept) are presented in the figures with non-significant estimates

or models eliminated. Minimum r^2 was set at 0.5. Mean values of the different treatments or studies were used for regression analyses.

Results

Plant growth

During the experimental period, total biomass of control plants increased by more than 80% from 113 to 211 g (data not shown). Due to N-withdrawal from the nutrient solution, reductions in leaf (80% of the control) and stem increment (71%) were observed (Table 1). However in contrast, root increment increased slightly but not significantly (124%), so that the total plant increment was not affected under N-withdrawal. The shoot to root ratio was reduced to 79%, but, these observations were statistically not significant. Within the stem of eucalyptus grown without N supply, weekly height increment was stronger and significantly reduced (69%) compared to the diameter increment (89%). Withdrawal of P and K, respectively, caused no significant changes of growth increment during the experimental period. Application of salt (100 mM) reduced the growth of all plant organs with leaves (80%), stems (86%), and roots (73%, not significant) so that the total plant growth increment was only 80% of the controls. Salt application increased significantly the height to diameter ratio (114% of the control, Table 1).

Gas exchange and ABA

Stomatal conductance was not affected in treated eucalyptus compared to the control (Table 1). However, the withdrawal

Table 1 Growth and gas exchange parameters in 5-month-old Tasmanian blue gum (*Eucalyptus globulus* Labill.) saplings after 6 weeks of nutrient withdrawal ("−N", "−P", "−K") or salt application, (control: full strength nutrient solution)

Treatment	Control	"−N"		"−P"		"−K"		"+NaCl"	
	Mean ± SE	Mean ± SE	Sig	Mean ± SE	Sig	Mean ± SE	Sig	Mean ± SE	Sig
Leaf increment [g DW (6 weeks)$^{-1}$]	28.1 ± 4.1	22.3 ± 5.2	*	27.1 ± 4.2	n.s.	29.3 ± 4.1	n.s.	22.3 ± 4.5	*
Stem increment [g DW (6 weeks)$^{-1}$]	35.1 ± 4.1	25.1 ± 3.6	***	31.8 ± 3.7	n.s.	33.9 ± 3.1	n.s.	30.1 ± 4.8	*
Root increment [g DW (6 weeks)$^{-1}$]	28.5 ± 9.2	35.3 ± 11.1	n.s.	21.1 ± 3.5	n.s.	34.6 ± 6.1	n.s.	20.9 ± 8.3	n.s.
Total increment [g DW (6 weeks)$^{-1}$]	91.7 ± 15.9	82.8 ± 18.2	n.s.	79.9 ± 9.7	n.s.	97.9 ± 8.8	n.s.	73.3 ± 15.8	**
Shoot to root ratio [DW DW^{-1}]	3.23 ± 0.38	2.54 ± 0.35	n.s.	3.13 ± 0.19	n.s.	2.59 ± 0.31	n.s.	3.49 ± 0.56	n.s.
Height to diameter ratio [cm mm^{-1}]	14.8 ± 0.4	14.8 ± 0.40	n.s.	14.6 ± 0.68	n.s.	16.0 ± 0.50	n.s.	16.9 ± 0.5	*
Height increment [% week^{-1}]	13.2 ± 0.9	9.03 ± 0.99	*	10.8 ± 0.83	n.s.	13.7 ± 0.9	n.s.	13.4 ± 1.3	n.s.
Diameter increment [% week^{-1}]	0.73 ± 0.04	0.65 ± 0.06	n.s.	0.81 ± 0.03	n.s.	0.66 ± 0.07	n.s.	0.77 ± 0.06	n.s.
Stomatal conductance [mmol m^{-2} s^{-1}]	219 ± 19	153 ± 21	n.s.	244 ± 42	n.s.	194 ± 18	n.s.	153 ± 15	n.s.
Photosynthesis [μmol m^{-2} s^{-1}]	8.85 ± 0.59	5.94 ± 0.53	*	9.20 ± 0.91	n.s.	7.84 ± 0.34	n.s.	7.55 ± 0.38	n.s.

Mean values (LS-means) ± standard errors are shown, significant differences to the controls are indicated by asterisk: *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$ (n.s. not significant)

of N (73% of the control) and K (93%) or application of salt (73%) tended to decrease stomatal conductance. The photosynthetic rate in eucalyptus without N supply was reduced to 68% of the control. Stomatal conductance also showed a tendency to decrease due to K-withdrawal (90%) or application of salt (87%). Surprisingly, minus P seemed to slightly increase gas exchange. In leaves of eucalyptus supplied nutrients without N or added salt application, ABA decreased to 68% or 67% of the control, respectively (Table 3). Additionally, the other treatments tended to reduce ABA in leaves. In contrast, in phloem sap ABA increased in all treatments, but only the withdrawal of N was significant (157% of the control, Table 4).

Xylem sap composition

In xylem sap of plants under "−P" conditions, bivalent cations –Ca and Mg– increased and monovalent cations decreased (Table 2). However, only the strong decrease of P (12% of controls) was statistically significant in P-limited plants. Following withdrawal of K, cations in xylem sap increased, however, only the changes in xylem sap Mg (262%) and Na (166%) were statistically significant. Surprisingly, the element excluded from supply—K—was least and not significantly affected (159%). In salt-treated plants, all cations in xylem sap with the exception of K− were strongly increased: Ca (262%), Mg (497%) and in excess

Table 2 Solute concentrations in root pressure xylem saps of 5-month-old Tasmanian blue gum (*Eucalyptus globulus* LABILL.) saplings after 6 weeks of nutrient withdrawal ("−N", "−P", "−K") or salt application

Treatment	Control	"−N"		"−P"		"−K"		"+NaCl"	
	Mean ± SE	Mean ± SE	Sig	Mean ± SE	Sig	Mean ± SE	Sig	Mean ± SE	Sig
Xylem Ca [mg L^{-1}]	7.05 ± 2.2	n.m.	n.m.	14.7 ± 4.7	n.s.	18.4 ± 5.8	*	58.2 ± 20.9	**
Xylem K [mg L^{-1}]	80.1 ± 17.9	n.m.	n.m.	57.1 ± 9.6	n.s.	127 ± 47	n.s.	335 ± 108	n.s.
Xylem Mg [mg L^{-1}]	9.64 ± 2.99	n.m.	n.m.	11.9 ± 4.4	n.s.	16.4 ± 5.7	n.s.	47.9 ± 18.0	*
Xylem Na [mg L^{-1}]	66.7 ± 13.6	n.m.	n.m.	63.9 ± 4.6	n.s.	111 ± 19	*	410 ± 85	***
Xylem P [mg L^{-1}]	29.4 ± 6.1	n.m.	n.m.	3.58 ± 0.57	***	42.2 ± 15.0	n.s.	41.5 ± 12.7	n.s.
Xylem S [mg L^{-1}]	14.1 ± 11	n.m.	n.m.	1.39 ± 0.85	n.s.	2.57 ± 1.23	n.s.	6.46 ± 1.90	n.s.

For further details see legend Table 1

Table 3 Concentrations of chemical compounds in leaf material of 5-month-old Tasmanian blue gum (*Eucalyptus globulus* LABILL.) saplings after 6 weeks of nutrient withdrawal ("−N", "−P", "−K") or salt application

Treatment	Control	"−N"		"−P"		"−K"		"+NaCl"	
	Mean ± SE	Mean ± SE	Sig	Mean ± SE	Sig	Mean ± SE	Sig	Mean ± SE	Sig
Leaf bulk δ^{13}C [$^0/_{00}$]	−30.8 ± 0.1	−30.87 ± 0.2	n.s.	−30.6 ± 0.2	n.s.	−30.9 ± 0.2	n.s.	−30.6 ± 0.3	n.s.
Leaf soluble δ^{13}C [$^0/_{00}$]	−31.6 ± 0.1	−32.1 ± 0.2	*	−31.6 ± 0.2	n.s.	−31.7 ± 0.3	n.s.	−31.3 ± 0.4	n.s.
Leaf sugar δ^{13}C [$^0/_{00}$]	−31.2 ± 0.2	−31.8 ± 0.2	n.s.	−31.1 ± 0.2	n.s.	−31.5 ± 0.3	n.s.	−30.5 ± 0.4	n.s.
Leaf δ^{15}N [$^0/_{00}$]	4.36 ± 0.12	1.93 ± 0.31	***	3.91 ± 0.21	*	4.21 ± 0.13	n.s.	4.26 ± 0.19	n.s.
Leaf %N by weight	1.60 ± 0.08	1.04 ± 0.08	*	1.45 ± 0.07	n.s.	1.33 ± 0.06	*	1.51 ± 0.14	n.s.
Leaf amino acids [µg mL^{-1}]	43.0 ± 5.8	78.7 ± 12.9	*	28.2 ± 5.7	n.s.	35.0 ± 8.9	n.s.	40.9 ± 7.4	n.s.
Leaf ABA [nmol g^{-1} DW]	2.84 ± 0.20	1.93 ± 0.10	**	2.38 ± 0.11	n.s.	2.23 ± 0.17	n.s.	1.89 ± 0.08	**
Leaf fructose [mg g^{-1} DW]	4.55 ± 0.28	4.08 ± 0.29	n.s.	4.41 ± 0.23	n.s.	5.14 ± 0.24	n.s.	4.56 ± 0.23	n.s.
Leaf glucose [mg g^{-1} DW]	7.15 ± 0.39	5.44 ± 0.20	**	6.27 ± 0.32	n.s.	6.32 ± 0.28	n.s.	6.7 ± 0.3	n.s
Leaf myo--inositol [mg g^{-1} DW]	5.35 ± 0.29	4.01 ± 0.28	**	4.39 ± 0.37	*	4.56 ± 0.3	*	5.36 ± 0.46	n.s.
Leaf sucrose [mg g^{-1} DW]	1.91 ± 0.14	2.07 ± 0.25	n.s.	1.84 ± 0.18	n.s.	2.70 ± 0.38	*	2.84 ± 0.73	*
Leaf raffinose [mg g^{-1} DW]	2.57 ± 0.27	1.55 ± 0.51	*	2.13 ± 0.46	n.s.	2.34 ± 0.60	n.s.	2.86 ± 1.04	n.s.
Leaf Ca [mg g^{-1} DW]	4.09 ± 0.43	4.09 ± 0.19	n.s.	3.89 ± 0.49	n.s.	4.91 ± 0.51	n.s.	2.96 ± 0.23	n.s.
Leaf K [mg g^{-1} DW]	61.3 ± 3.5	68 ± 3	n.s.	55.8 ± 4.2	n.s.	56.3 ± 2.7	n.s.	54.9 ± 4.5	n.s.
Leaf Mg [mg g^{-1} DW]	11.2 ± 0.7	11.0 ± 0.7	n.s.	10.0 ± 1.1	n.s.	14.2 ± 1.5	*	7.92 ± 0.69	*
Leaf Na [mg g^{-1} DW]	59.3 ± 3.7	59.9 ± 2.3	n.s.	66.1 ± 6.3	n.s.	56.5 ± 2.1	n.s.	59.6 ± 3.3	n.s.
Leaf P [mg g^{-1} DW]	5.46 ± 0.46	5.23 ± 0.40	n.s.	5.11 ± 0.61	n.s.	5.29 ± 0.79	n.s.	4.75 ± 0.30	n.s.
Leaf S [mg g^{-1} DW]	2.48 ± 0.17	2.55 ± 0.37	n.s.	2.23 ± 0.29	n.s.	2.28 ± 0.28	n.s.	1.75 ± 0.05	*

For further details see legend Table 1

NaCl-treated plants, Na was more than sixfold higher (Table 2). Since only one of nine available plants without N supply produced root pressure saps, no data of this treatment are presented.

Isotopic composition, amino acids, sugars, elements and elemental increments in leaves

Most changes in the isotopic composition in leaves were observed due to N-withdrawal: δ^{13}C of the water-soluble fraction became more negative, δ^{15}N (44% of the control) and total N concentrations (63%) were significantly decreased (Table 3). In leaves of plants without P-supply, δ^{15}N was lowered (90% of the control). The withdrawal of N or K significantly lowered total N in leaf dry matter (65% or 83%). The salt treatment did not result in any changes to isotopic composition. The withdrawal of N resulted in a strong statistically significant increase of amino acid concentration in leaves (183%), whilst in response to other treatments amino acid concentrations were reduced (95–66%, n.s.).

N-withdrawal reduced sugar concentration in leaves: glucose (76%), myo-inositol (75%) and raffinose (60%, Table 3). In eucalyptus without P-supply only myo-inositol was lowered (82%). Following K-withdrawal, myo-inositol was once again decreased (85%); however, sucrose increased (141%). Sucrose was also increased (149%) due to salt treatment.

The elemental composition of leaves was mostly affected by the salt treatment: Mg (70%) and S (70%) were decreased (Table 3). Surprisingly, Na of leaves in the Na treatment was not affected (101%). The effects on elemental composition of leaves due to nutrient withdrawal were relatively low, and only in K-limited plants Mg was increased (126%).

The elemental increments in leaves were apparently affected only by N-withdrawal and salt application for N, P and K (Fig. 1 b, c and d). However, only the loss of N from leaves after N-withdrawal (minus 35% of the initial content) or the lower N increment (48% of the control) following salt application was statistically significant, although N increment was reduced to 48–78% in other treatments compared to the controls. Similarly, salt treatment resulted in a loss of P from leaves (− 104%) and only 34% K was accumulated compared to the control without to be statistically significant. Remarkably, while 100 mM Na was applied in the treatments, the increment of Na in leaves was not affected (93% of the controls) and remained relatively unchanged across all plants.

Phloem sap composition

The most abundant sugar in phloem sap was sucrose; however, this sugar, raffinose and δ^{13}C were not affected in phloem sap by the applied treatments (Table 4). Nutrient withdrawal affected the elemental composition of phloem sap in some cases. These included: Na from N-limited plants (69%) and Ca in P-limited plants (62%) significantly decreased, as well as P in plants following withdrawal of K was significantly increased (119%). In phloem sap of salt-treated plants, several effects were observed, most prominent being the 262% increase of Na. P was also increased (124%) but S was decreased (57%, Table 4).

Correlations

Concentrations of sucrose and raffinose in phloem sap were very well correlated (Table 5). Additionally, the bivalent cations Mg and Ca, which are less mobile in phloem sap, were well correlated across all treatments. Interestingly, a correlation of P and Na was found (Table 5), pointing to a co-transport of both elements in the phloem.

To test the potential effects of soluble compounds in the leaves (sources) on the composition of phloem sap (the conduit to the "sinks"), regression analysis was performed. A clear relation between the δ^{13}C of leaf extract and that in phloem sap was observed (Fig. 2a). Additionally, the amino acid concentrations in leaves (soluble N) were well correlated with those in phloem sap (Fig. 2c). In both cases, the intercept of the linear regression model was not significant and, therefore, omitted (see Supplementary Material, Table A.6). All other relationships in this context—sucrose, P, K, and Na− were statistically not significant (see Supplementary Data, Table A.6). Similarly, no growth or gas exchange parameters could be correlated to relevant concentrations of compounds obtained from the phloem sap (see Supplementary Material, Table A.7).

Discussion

Effects of nutrient withdrawal on growth and gas exchange

In the present study, only withdrawal of N from nutrient supply ("−N") resulted in a statistically significant reduction of leaf and stem growth whilst the shoot to root ratio was decreased (n.s.). Our study, therefore, successfully applied single nutrient withdrawals that elicit changes in the abundance of the remaining nutrients (P, K and Na) prior to thresholds that impart growth responses. Observed changes in nutrient concentrations in the tissues (outlined below), therefore, represent potential tools that can inform managers of nutrient deficiencies *in advance* of growth impacts, offering significant advantages over current visual and leaf chemistry-based methods.

For the nitrogen treatment, significantly lower apical stem growth was attributable to lower height increment and less to secondary thickening. Enhanced relative root growth

Fig. 1 Elemental increment in leaves of Tasmanian blue gum (*Eucalyptus globulus* LABILL.) saplings after 6 weeks of treatment, control (full strength nutrient solution), "−N" (NH_4NO_3 was omitted from the nutrient solution), "− P" (NaH_2PO_4 was omitted), "−K" (K_2SO_4 was omitted), and "+ NaCl" (plus 100 mM NaCl). **a** Carbon [g], **b** nitrogen [g], **c** phosphorus [mg], **d** potassium [mg], and **e** sodium [mg] in the total amount of leaf mass per plant. The values indicate the average of 12 (control) or 7 (treatments) replicates ± SE
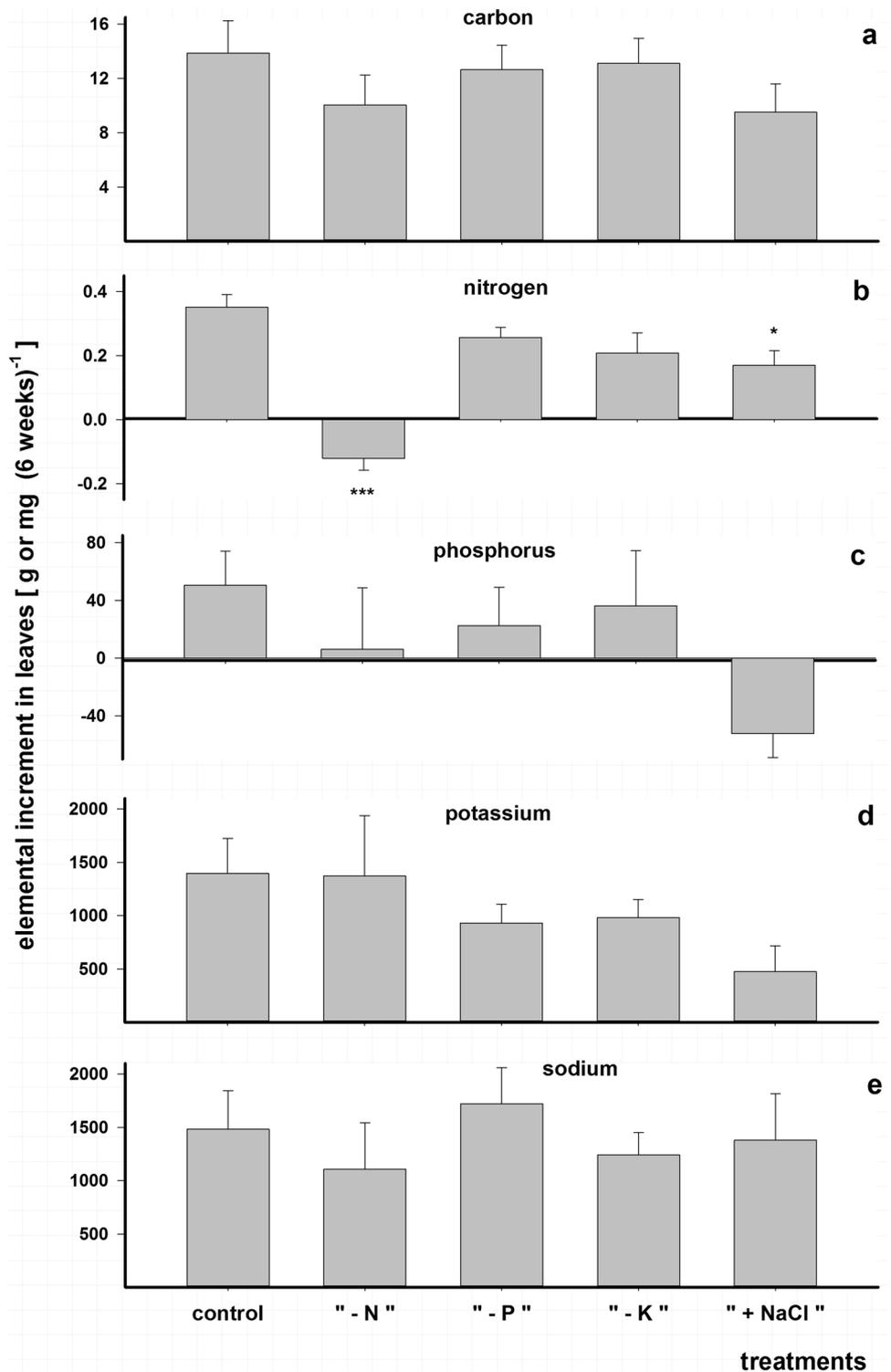
is a common response to reduced availability of nutrients (Hermans et al. 2006; Peuke 2010), which was observed previously in *E. globulus* (Macfarlane et al. 2005). The lack of growth responses attributable to withdrawal of the other nutrients, P or K (Table 1), may be attributed to a former 'excess' supply from the initial growth medium such that the 'in planta' reserves were adequate to maintain supply during the experimental period.

Significant biomass effects may become obvious over a longer experimental period. In field trials with *E. grandis*, a closely related *Eucalyptus* species in Brazil, N- and K-fertilisation increased shoot net primary production (Laclau

Table 4 Solute concentrations in phloem saps of 5-month-old Tasmanian blue gum (*Eucalyptus globulus* Labill.) saplings after 6 weeks of nutrient withdrawal ("−N", "−P", "−K") or salt application

Treatment	Control	"−N"		"−P"		"−K"		"+NaCl"	
	Mean ± SE	Mean ± SE	Sig	Mean ± SE	Sig	Mean ± SE	Sig	Mean ± SE	Sig
Phloem $\delta^{13}C$ [$^0/_{00}$]	−27.9 ± 0.3	−27.49 ± 0.46	n.s.	−27.8 ± 0.3	n.s.	−28.1 ± 0.5	n.s.	−27.3 ± 0.3	n.s.
Phloem ABA [nM]	7.61 ± 0.81	13.02 ± 1.56	*	7.99 ± 1.24	n.s.	9.43 ± 1.65	n.s.	9.67 ± 1.32	n.s.
Phloem sucrose [mM]	239 ± 20	250 ± 43	n.s.	203 ± 8	n.s.	227 ± 12	n.s.	217 ± 7	n.s.
Phloem raffinose [mM]	48.2 ± 6.6	58.1 ± 6.4	n.s.	44.3 ± 3.4	n.s.	45.4 ± 3.8	n.s.	44.7 ± 3.4	n.s.
Phloem amino acids [µg mL^{-1}]	170 ± 69	279 ± 81	n.s.	99 ± 20	n.s.	144 ± 53	n.s.	188 ± 37	n.s.
Phloem Ca [mg L^{-1}]	26.5 ± 3.0	19.7 ± 3.6	n.s.	16.5 ± 3.3	*	35.8 ± 10.7	n.s.	16.1 ± 4.5	n.s.
Phloem K [mg L^{-1}]	1287 ± 42	1358 ± 89	n.s.	1341 ± 52	n.s.	1223 ± 108	n.s.	1182 ± 151	n.s.
Phloem Mg [mg L^{-1}]	29.6 ± 2.5	22.4 ± 3.1	n.s.	23.2 ± 2.9	n.s.	33.3 ± 5.2	n.s.	25.5 ± 5.1	n.s.
Phloem Na [mg L^{-1}]	542 ± 41	376 ± 51	*	552 ± 50	n.s.	622 ± 109	n.s.	1257 ± 123	***
Phloem P [mg L^{-1}]	128 ± 6	124 ± 12	n.s.	125 ± 9	n.s.	152 ± 10	*	158 ± 17	*
Phloem S [mg L^{-1}]	36.6 ± 2.8	39.4 ± 4.1	n.s.	32.6 ± 4.1	n.s.	37.5 ± 2.9	n.s.	21.0 ± 4.5	*

For further details see legend Table 1

Table 5 Correlation analysis of compounds in phloem saps of 5-month-old Tasmanian blue gum (*Eucalyptus globulus* Labill.) saplings after 6 weeks of nutrient withdrawal ("−N", "−P", "−K") or salt application

	$\delta^{13}C$	C	Sucrose	Raffinose	Suc/raf ratio	Ca	K	Mg	Na	P	S
$\delta^{13}C$	###	0.003	−0.025	−0.085	0.037	−0.189	−0.051	−0.332	0.032	−0.165	−0.070
C	n.s.	###	0.237	0.244	−0.083	−0.194	−0.020	−0.133	0.193	0.009	−0.178
Sucrose	n.s.	n.s.	###	**0.866**	−0.221	−0.032	−0.086	−0.119	−0.145	−0.306	0.121
Raffinose	n.s.	n.s.	<0.0001	###	**−0.627**	−0.157	−0.140	−0.221	−0.213	−0.409	0.060
Suc/raf	n.s.	n.s.	n.s.	<0.0001	###	0.199	0.171	0.199	0.125	0.291	−0.017
Ca	n.s.	n.s.	n.s.	n.s.	n.s.	###	−0.262	**0.893**	0.023	0.271	0.393
K	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	###	−0.047	−0.054	0.450	0.296
Mg	0.048	n.s.	n.s.	n.s.	n.s.	<0.0001	n.s.	###	0.176	0.459	0.423
Na	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	###	**0.560**	−0.366
P	n.s.	n.s.	n.s.	0.018	n.s.	n.s.	0.005	0.004	0.000	###	0.199
S	n.s.	n.s.	n.s.	n.s.	n.s.	0.016	n.s.	0.009	0.026	n.s.	###

The Pearson correlation coefficient (K_p) and *p* value for H_0: $K_p = 0$ are given; significant $K_p > 0.5$ are shown bold. For further details see legend Table 1

et al. 2009). The effect for N was observed in the first 2 years whilst K-fertilisation lasted 3 years. Similarly, annual NPK-fertilisation increased the growth of 4-year-old *E. globulus* in a field trial plantation growing on uniform leached sands (Turnbull et al. 2007). Leaf N and P concentrations in leaf bulk material were increased in NPK-fertilised trees compared to unfertilized trees, respectively.

Effects of single nutrient withdrawal on leaf, phloem and xylem soluble constituents

We detected decreasing total N upon N-withdrawal; however, amino acid concentration in leaves increased. This suggests that N was remobilized from storage pools to soluble fractions and loaded into phloem for export from leaves

where concentrations also increased (but were not significant in the present study). As a consequence, we observed a significant correlation between leaf and phloem amino acids, and a loss of N from leaves (−35% of initial content) during the experimental period. Increasing amino acid concentrations in phloem sap under conditions of relatively low N availability presents a counterintuitive phenomenon for evaluating tree nutrient status. Nevertheless, this correlative relationship, if characterised over a broader range of conditions (particularly across a scope of water availability), may present an opportunity to develop in-field tests for N status similar to that of previously developed protocols for water status based on refractive index. Further work, such as stratified phloem sampling at the canopy scale, may help to determine the mechanistic basis for this phenomenon.

Fig. 2 Regression analysis for the effects on phloem concentrations by leaf concentrations in Tasmanian blue gum (*Eucalyptus globulus* LABILL.) saplings after 6 weeks of treatment (control, "−N", "−P", "−K", and "+ NaCl"). **a** $\delta^{13}C$ in phloem sap by that of hot water extract from leaves, **b** sucrose, **c** amino acids, **d** phosphorus, e: potassium, and **f** sodium. Significant relationships (H_0: estimate = 0; $p < 0.05$) are indicated by solid bold ($r^2 > 0.50$). For further details please see legend to Fig. 1
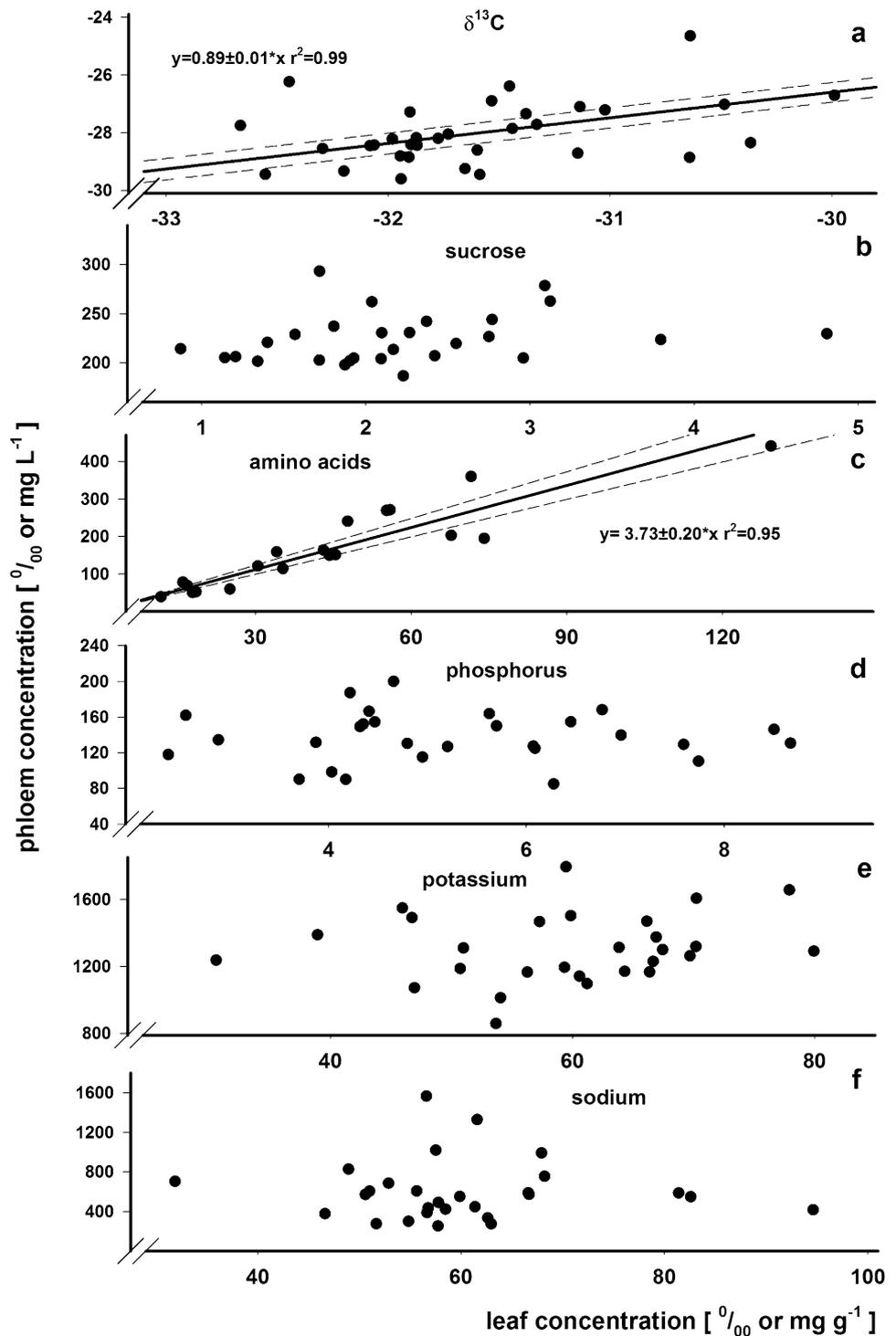
For phosphorus, the concentration of P in xylem sap of "−P" plants was only 12% of the controls representing a significant decline indicating a viable detection method for P-deficiency. However, plants can cope with low P in the rhizosphere often requiring a reduction in P of around 90% of the initial concentration before perturbations to chemical and physiological processes occur (Rychter and Rao 2005). For potassium, height and diameter increments were not affected by withdrawal of K from nutrient supply. Both growth parameters should be viewed in the context of wood formation, which is known to be affected by K-nutrition (Fromm 2010). Epron et al. (2016) showed the growth promoting effect of K application in young *Eucalyptus grandis* L. trees grown in a field trial and suggested reductions in

photosynthesis and C demand of sinks due "−K". Under K (and Mg) limitation, plants are less able to transport sugars in the phloem to the roots (Hermans et al. 2006) and the low effect of reduced K supply on total biomass increment in an experiment with potted Ricinus has been reported earlier (Peuke et al. 2002). In the present study, *E. globulus* also demonstrated a change in xylem sap composition (increase of Ca and Na) and phloem sap composition (P) after withdrawal of K. The effect of these changes on the composition of phloem sap agrees with those results observed previously in Ricinus (Peuke et al. 2002) suggesting a common, plant response. It is assumed that the charge imbalance associated with the reduction in K was compensated by increased uptake and concentration of other cations. Ca and Na (as well as Mg, but n.s.) increased in xylem sap after K-withdrawal regardless of the fact that K also increased (n.s.).

Leaf concentrations of the target elements in this study existed within the ranges detected among other similar studies. White and Brown (2010) suggested the "sufficient leaf concentrations" of elements are within the scope of 1.5–4% for N, 2–5 mg g^{-1} DM for P and 5–40 mg g^{-1} DM for K. In the present study, only the N-limitation resulted in concentrations lower than these critical values. Similarly, the observed concentrations of elements, sugars, ABA and amino acids obtained from the phloem and xylem saps in the present pot experiment were comparable with those from plantation trees (Pate et al. 1998). The changes compared to the control demonstrate that nutritional imbalance is reflected also in transport fluids.

Photosynthesis was reduced by "−N"; however, neither stomatal conductance nor δ^{13}C were affected by the applied treatments. Stomatal conductance (g_s) at the maximum photosynthetic rate (A_{max}) was between 0.2 and 0.4 mol m^{-2} s^{-1} higher in NPK-fertilised trees (Turnbull et al. 2007). P-limitation commonly inhibits photosynthesis in Eucalyptus due to unbalanced P$_i$ concentration in the cytosol (Warren 2011). Macfarlane et al. (2005) observed in leaves of N-limited eucalyptus lower intrinsic water-use efficiencies than leaves of plants supplied with adequate nutrition. For the present study, the combination of treatment severity and longevity appears to have achieved an impact on plant chemistry, without corresponding reductions in growth and/or physiology compared to control plants. Whilst some of these effects are somewhat counterintuitive, for example, in contrast to former studies (Peuke 2016), ABA in leaves of treated/stressed eucalyptus decreased due to N-withdrawal or salt application, further interrogation of plant chemistry through the analysis of phloem sap yielded interpretable results (in phloem sap ABA concentrations increased due to N-limitation and salt application).

The accumulation of sugars in leaves and phloem sap due to nutrient limitation are a common observation since photosynthesis is usually less affected than growth (Hermans et al. 2006; Peuke 2010; Kudoyarova et al. 2015; Körner 2015). In this study, sucrose increased in leaves, although this effect was only significant after withdrawal of K or salt application. Thomas et al. (2005) observed increased amounts of photoassimilates likely available for secondary wall thickening of fibre cells under reduced P-supply in *E. grandis* seedlings, and thus increased wood density associated with inhibited stem cambial activity. The phloem stream is subject to both supply and demand dynamics, and changes in hydration, all of which influence the concentration of solute pools within the system.

All treatments imparted a reduction in δ^{15}N in leaves in the present study. Generally, differences in δ^{15}N are due to fractionation during N-uptake and discrimination of ^{15}NO$_3$ during nitrate reduction (Tcherkez 2011). Therefore, the nitrate molecules which were not reduced and assimilated in roots are ^{15}N-enriched and translocated to shoots, while proteins, chlorophylls or alkaloids are ^{15}N-depleted. Consequently, lower δ^{15}N in leaves may point to relatively higher nitrate reduction in roots. Indeed, we have observed previously relatively higher nitrate reduction in roots following N-limitation, salt application (Peuke et al. 1996) or K-limitation (Peuke et al. 2002) in Ricinus, but not under P-deficiency (Jeschke et al. 1997). For the site of nitrate reduction in plants, the amount of nitrate supply as well as the transport in the xylem including the availability of counter ions is regulatory.

Effects of salt application

Salt application had a clear statistical effect on above-ground biomass increment and total plant increment (a similar observation was found in roots but was not significant). The height to diameter ratio also increased indicating lower height or higher diameter in salt-treated eucalyptus. However, both weekly increments (height as well as diameter) were not affected. The multifaceted effects of excess salt in the plant system can give rise to growth restrictions in a number of ways. For example, Escalante-Pérez et al. (2009) reported reduced xylem differentiation zone and reduced xylem cell luminae in salt-treated Populus. Gas exchange is commonly reduced by salt stress (Munns and Tester 2008); however, in the present study, photosynthesis and stomatal conductance were not significantly affected by salt (although both tended lower). To avoid the toxic effects of Na, plants adapt using a combination of three mechanisms: osmotic tolerance, Na exclusion from the leaves, and the tolerance of tissue to the accumulated Na (Munns and Tester 2008). In the present study, we have less evidence for point osmotic tolerance within leaves as the analysis of leaves demonstrated no changes of Na concentration (for any treatment). However, Na in xylem sap was increased more than sixfold and in phloem sap it was more than doubled. These

observations point at a very efficient recirculation of Na via the phloem restricting movement into the leaf. This may have occurred via a Na–P association, since P also increased in phloem sap of salt-treated eucalyptus and both elements are well correlated. This co-transport of Na and P following salt application likely resulted in the observed loss of P from leaves (n.s.). Additionally, N increment in leaves was lowered, which mostly can be related to the lower leaf increment and less to lower N concentration. Pate et al. (1998) reported previously regarding high amounts of sodium and concentrations of chloride and sulphate relative to phosphate in phloem sap of *E. globulus*. In the present study, the average Na concentration in leaves was 60.5 ± 2.0 mg g^{-1} DM. In contrast, White and Brown (2010) indicated 2–5 mg g^{-1} DM as toxic Na concentration in non-tolerant crop plants. A high cytosolic K/Na ratio appears to be critical to plant salt tolerance (Shabala and Cuin 2008; Luan et al. 2009). In the present study, the K/Na ratio in the entire biomass of leaves was 1.05 ± 0.06 g g^{-1} DM in controls and varied between 0.87 (salt application) and 1.15 g g^{-1} DM in the treatments (data not shown), indicating no significant changes of the K/Na ratio. This demonstrates also the enormous potential of *E. globulus* for homeostatic regulation since in the nutrient solution this ratio decreased from 2.8/0.5 (control) to 2.8/100.5 [mM/mM] (salt application). These results combined, specifically that of the magnitude of Na accumulation in phloem sap and its observed exclusion from leaf tissues suggests that phloem offers a viable method for detecting excess Na within the plant system and is of a considerable advantage over that of leaf-based analysis.

Correlations

A statistically significant correlation of chemical compounds in the fully expanded leaves (source) on the concentration in phloem at the shoot to root interface (transport to the sinks) was observed for amino acids and δ^{13}C (hot water extracts) between leaves and phloem sap (calculated by regression analysis, Fig. 2). This is in contrast to findings in Ricinus, where a number of nutrients—N, K, Mg, Ca, and Cl—were correlated (Peuke 2010). This observation may be attributable to the relative size and ontology of these species, and the ability to remobilise resources throughout the plant under nutrient limitations. Similarly, the correlation between sugar concentration and δ^{13}C in phloem saps of eucalyptus in plantations (Pate et al. 1998) was not found as expected. This latter observation is likely explained by the relatively narrow scope of water availably that was experienced in this study and the relative effects on the major steps responsible from fractionation as carbon diffuses into leaf tissues. In xylem sap, Mg and Ca correlated, Na with both bivalent cations, but, K with P and S (data not shown, Supplemental Table 3). Similarly in leaves, the bivalent cations,

K with S, and S with P, were correlated. These observations can be explained by activity of the transport system into xylem or leaf tissue (Peuke 2010). Additionally in leaves, different carbohydrates were correlated, potentially due to photosynthesis rates and/or phloem export. Overall, the absence of correlations between leaf-, phloem- and xylem-derived nutrients and metabolites highlights the advantages of diagnostic methods deployed in this study. Phloem and xylem sap-derived nutrient deficiencies were detected in the absence of similar patterns observed in leaf materials.
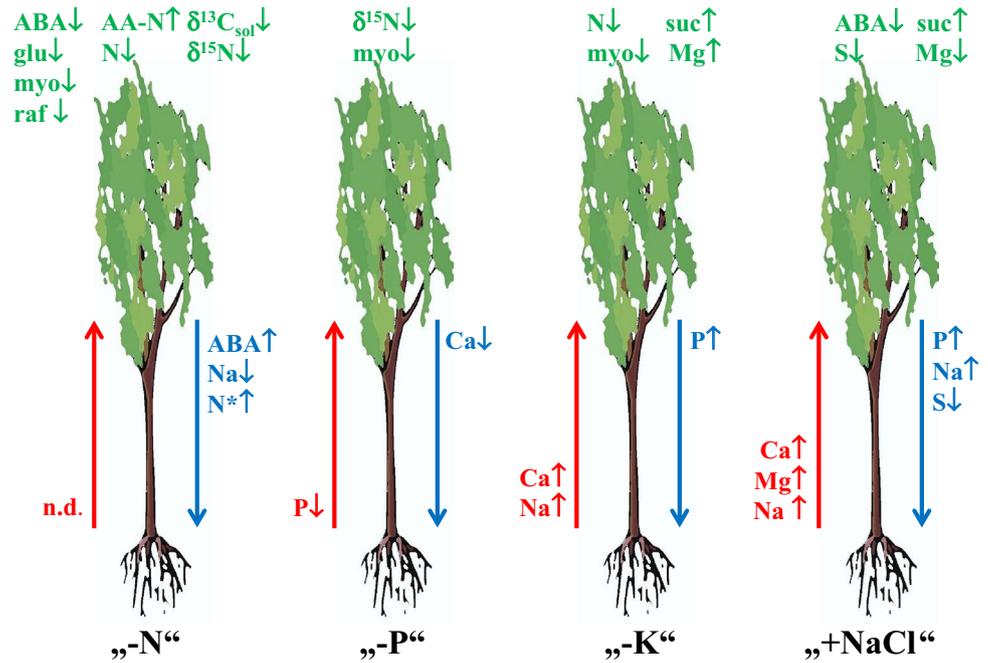
Conclusions

Under the conditions of this study, the only statistically significant effect of nutrient withdrawal on growth was that of "−N"; however, a series of chemical analyses obtained from leaf tissues, but also from transport fluids—phloem as well as xylem sap—successfully detected with withdrawal of the nutrients under investigation (Fig. 3). This methodology, therefore, enables the detection of nutrient imbalances prior to having an impact on growth. This offers significant advantages as one of few tissue-level changes in nutrient abundance indicating a deficiency prior to reductions in growth. Xylem sap successfully detected P-withdrawal from the nutrient solution, phloem sap successfully detected the addition of Na to the solution and whilst leaf analysis was the best detector of N deficiency by measuring total N. In addition, phloem amino acid concentrations were significantly correlated with leaf amino acid concentrations both of which increased under N-withdrawal suggesting the process of remobilization had been enacted.

The development of phloem-based diagnostic tools for the detection of plant nutrient status has a great potential to save time and resources both on- and offsite. The sampling method for phloem sap is also applicable in the field. Determination of nutrient status by leaf analysis is difficult due to spatial and temporal heterogeneity/ontogenies of leaves. It is likely that the composition of phloem sap may be a better proxy of nutritional status, since it will contain the "integrative signal" from the entire canopy to the roots. During the relative slow transport, compounds can be mixed and exchange so that a relatively integrated 'status' of the plant can be obtained. The results presented here demonstrate the capacity for phloem and xylem saps as a diagnostic tool for monitoring the nutritional status of plants.

Author contribution statement ADP designed the experiments, performed the greenhouse experiment, harvested the plants, analysed data and wrote the manuscript. AM participated in the greenhouse experiment and harvest, performed the chemical analysis and determination, and assisted in analysis of data and in writing and improving the

Fig. 3 Summary of significant effects on compounds in leaves (green), xylem (red) or phloem (blue) sap in Tasmanian blue gum (*Eucalyptus globulus* LABILL.) saplings after 6 weeks of treatment ("−N", "−P", "−K", and "+ NaCl"). Arrows up or down indicate increases or decreases compared to the control. *n.d.* not detected, *N** just below the significance level, *myo* myo-inositol, *suc* sucrose, *raf* raffinose, *glu* glucose, *AA-N* amino acid nitrogen, $\delta^{13}C_{sol}$: $\delta^{13}C$ of water extract. For further details please see legend to Fig. 1

"-N"
- ABA↓ AA-N↑ $\delta^{13}C_{sol}$↓
- glu↓ N↓ $\delta^{15}N$↓
- myo↓
- raf↓
- ABA↑ Na↓ N*↑
- n.d.

"-P"
- $\delta^{15}N$↓
- myo↓
- Ca↓
- P↓

"-K"
- N↓ suc↑
- myo↓ Mg↑
- P↑
- Ca↑ Na↑

"+NaCl"
- ABA↓ suc↑
- S↓ Mg↓
- P↑ Na↑ S↓
- Ca↑ Mg↑ Na↑

manuscript. All authors have read and approved the final manuscript.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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