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REGULAR ARTICLE

Elevated CO_2 and phosphorus deficiency interactively enhance root exudation in *Lupinus albus* L.

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Abstract

Purpose Rising atmospheric CO_2 levels associated with climate change increase plant nutrient demands. However, few studies have examined the interactions of atmospheric CO_2 and P supply on root exudation which plays a crucial role in mobilising non-labile P in soil. This study aimed to examine the interactive effects of elevated CO_2 and P deficiency on root exudation of organic acid anions and sugars over time.

Methods White lupin (*Lupinus albus* L.) was grown at 1, 5 and 50 μM P in solution culture under ambient (380 ppm) and elevated (700 ppm) CO_2 levels. Root exudates were collected after 3, 4, 5 and 6 weeks of treatment, and organic acid anions and sugars were quantified using liquid chromatography-mass spectrometry.

Results Elevated CO_2 and P deficiency positively interacted to enhance citrate exudation between 3 and 5 weeks of growth, while malate was only sporadically affected by elevated CO_2 and fumarate remained unaffected. Elevated CO_2 also increased exudation of glucose and fructose with larger increases being observed in P-deficient plants, which was largely constrained after 4 weeks. Elevated CO_2 had no effect on exudation rates as plants matured.

Conclusion The positive interaction between CO_2 and P deficiency led to increases in organic acid anion and sugar exudation, indicating that rising atmospheric CO_2 levels could allow plants to access greater amounts of non-labile P when faced with P deficiency thereby reducing their reliance on non-renewable fertiliser inputs.

Keywords Carboxylate · Citric acid · Climate change · Organic anions · Rhizodeposition · Root exudation

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Introduction

Atmospheric CO_2 concentrations are expected to rise to 700–800 ppm by the year 2100, which will strongly influence the photosynthetic rate of plants (IPCC 2013). Photosynthesis is upregulated under elevated

CO_2 conditions, leading to greater fixation of C and hence, greater photosynthate availability which can be used for plant growth (Campbell and Sage 2006). A large proportion of photosynthates is allocated belowground used largely for root growth but a large amount of C is deposited into the soil-root interface in a process called rhizodeposition. Rhizodeposits consist of sloughed root cells, root mucilage, senesced root hairs and root exudates (Jones et al. 2004). As a major component of rhizodeposits, root exudates are important as they regulate biogeochemical processes within the rhizosphere such as nutrient acquisition and soil C cycling (Canarini et al. 2019). Root exudates are low-molecular-weight organic compounds largely composed of amino acids, organic acid anions (referred to as organic anions hereafter) and sugars, and can represent up to 30% of photosynthetically-derived C (Badri and Vivanco 2009). As elevated CO_2 enhances photosynthetic rates, this can lead to greater belowground C allocation in C3 plants, and hence increases root exudation (Cotrufo and Gorissen 1997; Haase et al. 2007; Dong et al. 2021).

Root exudates serve as important organic compounds for nutrient acquisition. In particular, organic anions such as citrate and malate are exuded by a range of crop species as an adaptation to P deficiency (Nuruzzaman et al. 2005; Vranova et al. 2013). These compounds can mobilise non-labile forms of P through desorption from clay minerals or by solubilising precipitated forms of P, thus enhancing P availability in the rhizosphere (Hoffland et al. 1989; Barrow et al. 2018). Crop species such as white lupin have a strong organic anion efflux in response to P deficiency, making these crop species highly efficient at mobilising P (Neumann et al. 1999). This occurs by the low-P environment reducing shoot P concentration which induces organic acid exudation (Li et al. 2008; Shane et al. 2003). Some evidence to suggests that elevated CO_2 can enhance organic anion exudation in some legumes due to greater photosynthetic activity. However, few studies consider the importance of P status when exploring root exudation rates under elevated CO_2. As elevated CO_2 enhances photosynthate availability, increases P demand and decreases shoot P concentration, exudation of organic anions could increase to satisfy plant P demand, particularly under P deficiency (Jiang et al. 2020). Upregulation of root exudation under elevated CO_2, particularly in P-deficient conditions, could allow plants to access P associated with precipitated or sorbed P pools thereby reducing the plants reliance on fertilisers.

Similar to organic anions, sugar exudation is upregulated under both P deficiency and elevated CO_2. Unlike organic anion exudation, which is a tightly regulated process where root concentrations do not correspond to exudation rates (Neumann et al. 2000), sugar exudation can occur through diffusion from roots along a concentration gradient into the rhizosphere via membrane channels (Canarini et al. 2019; Jones et al. 2009). The dominant sugars present in root exudates are glucose, fructose and sucrose which have been shown to be enhanced under P deficiency (Carvalhais et al. 2011). There are several reasons that explain the increase in sugar exudation under P deficiency. First, P deficiency increases translocation of sucrose to the roots because sugars are utilised for organic acid production via glycolysis and the tricarboxylic acid (TCA) cycle (Muller et al. 2015). Second, primary root growth is inhibited due to P deficiency but transport of sucrose to the root tips is maintained, thus leading to an accumulation of sugars within roots (Hammond and White 2008). Third, exudation of sugars may be important in recruiting beneficial soil microbes to aid nutrient acquisition (Sasse et al. 2018). Elevated CO_2-induced increases in sugar exudation are largely explained by enhanced photosynthetic rates and belowground C allocation. Although as P deficiency curtails primary root growth and elevated CO_2 can increase sugar supply to roots, elevated CO_2 could promote greater accumulation of sugars in P-deficient plants, leading to increases in sugar exudation. Given that elevated CO_2 and P deficiency can both enhance sugar exudation from roots, this may lead to interactive effects between CO_2 and P deficiency on sugar exudation.

Whilst plant P status plays a significant role in root exudation, growth stage and sampling method also strongly influence exudation rates which could dictate the interactive effects of elevated CO_2 and P deficiency on root exudation. Exudation rates are highly dynamic and depend on plant maturity (Sas et al. 2001). Therefore, it is important to consider the effect of elevated CO_2 on exudation rates over multiple sampling dates as elevated CO_2 may not have a prolonged effect on root exudation. For example, in *Phaseolus vulgaris*, Haase et al. (2007) demonstrated that elevated CO_2 only increased sugar exudation at early growth stages whereas organic anion exudation was only increased

in later growth stages. Plant growth stage or the time of sampling can influence exudation rates in several ways. First, as plant development progresses, shoot P concentration can be reduced, intensifying P deficiency leading to increased organic anion exudation. Second, exudation rates can increase during the early stages of plant growth but as plants mature and reach reproductive phases, exudation rates decline as photosynthates are directed to plant growth (Aulakh et al. 2001; Haase et al. 2007) and grain development. Sampling method should also be considered when assessing the impact of elevated CO_2 on root exudation. Some previous studies have focused their investigation on cluster roots which are regions of dense rootlets on lateral roots in white lupin (e.g. Watt and Evans 1999). However, whole root systems should also be considered as cluster roots and non-cluster root regions both exude organic anions (Neumann et al. 1999; Watt and Evans 1999). Furthermore, exudation rates from individual cluster roots are also highly dynamic with changes in exudation rates occurring in a matter of days, which outlines the importance of considering root exudation over time (Sas et al. 2001) when assessing the effects of elevated CO_2. Shoot biomass and root-to-shoot ratio also strongly influence exudation rates as plants with greater shoot biomass also allocate greater C belowground as exudates. As elevated CO_2 commonly enhances shoot biomass, accelerates plant development whilst decreasing root-to-shoot ratio, this could affect the duration and the magnitude of CO_2 effects on root exudation.

We aimed to investigate the interactive effects of elevated CO_2 and P deficiency on exudation dynamics of organic anions and sugars in white lupin. White lupin is highly adapted to low-P environments partly due to its high rate of organic anion exudation (Nuruzzaman et al. 2005). It was hypothesised that elevated CO_2 and P deficiency would positively interact to enhance exudation of organic anions and sugars due to increased P demand and plant growth.

Materials and methods

Plant cultivation

Uniformly-sized seeds of white lupin (*Lupinus albus* L. cv. Kiev) were germinated in the dark in 5-L of aerated 1 mM $CaCl_2$ and 5 μM H_3BO_3 solution at 20 °C for 7 days. *Bradyrhizobium sp.* strain WU425 was also added to the solution at the germination stage to ensure early nodulation and N_2 fixation. Plants were then transferred into 5-L buckets of hydroponic solution with each bucket initially containing 12 plants. The nutrient solution was based on Sas et al. (2001) and had the following composition (μM): 200 $MgSO_4$; 600 K_2SO_4; 600 $CaCl_2$; 10 FeNaEDTA; 5 H_3BO_3; 1 $MnSO_4$; 0.2 $CuSO_4$; 0.03 Na_2MoO_4; 1 $ZnSO_4$; 0.2 $CoCl_2$. No N was supplied in the nutrient solution therefore N_2 fixation was the sole source of N. Shoot N was analysed and was within the adequate range (unpublished data). Phosphorus was added as KH_2PO_4 at 1, 5 and 50 μM P which represents severely P deficient, moderately P deficient and P sufficient treatments, respectively. Each treatment was replicated 4 times. To induce early P deficiency and minimise P remobilisation from the cotyledons to the shoots, cotyledons were removed 10 d after transplanting to nutrient solution. Solution pH was adjusted to 6.0 daily using 0.1 M KOH to prevent H^+ toxicity. Nutrient solution was continuously aerated and renewed every second day. Plants were grown under 20 ºC days for 16 h and 18 ºC nights in two ambient and two elevated CO_2 controlled growth cabinets (Fitotron SGC 120, Weiss Technik, Loughborough, UK). The ambient and elevated CO_2 concentrations were 380 ppm and 700 ppm, respectively. The elevated CO_2 concentration is within range of the estimated atmospheric CO_2 by the end of the century (IPCC 2013). The ambient CO_2 concentration was maintained in the growth cabinets using a NaOH CO_2 scrubber. Light intensity at the canopy level was 400 μmol m^{-2} s^{-1}. Buckets were rearranged within the cabinets every two days and rotated between cabinets weekly.

Collection and analysis of root exudates

After 3 weeks of P and CO_2 treatments, the roots of all plants from each bucket, after 2 h of light exposure, were washed 4 times in hydroponic solution made from Milli-Q water. The plant roots were then immersed in 250 mL of C-free hydroponic solution for 2 h within the growth cabinets to collect root exudates. Fe-EDTA was excluded from the

washing and exudate capturing solutions to avoid organic C contamination. Plants were left in the growth cabinet during exudate collection to ensure the conditions remained consistent. After 2 h, plant roots were removed from exudate-capturing solution and the exudate solutions were immediately passed through a sterile 0.2 µm polyethersulfone syringe filter (Uniflo, GE Healthcare, UK) and snap frozen in liquid nitrogen. Three plants from each bucket were then harvested at each sampling time. The remaining plants were placed back in the nutrient solution under the previously described growing conditions and used for root exudate collection following the same procedure at 4, 5 and 6 weeks after treatment, respectively.

Exudates were stored at -20 °C until analysis. To estimate exudate recovery, subsamples of root washing solution was either immediately filtered and frozen or placed in exudate collection trays and incubated in the growth cabinets for 2 h. This recovery experiment indicated that about 5% of citrate was degraded during the 2 h collection process (data not shown). Sas et al. (2001) had lower recovery of exudates; however, our recovery experiment did not account for re-uptake of exudates by plants and therefore recovery may be underestimated.

Quantification of organic anions and sugars in the exudate solutions was achieved through liquid chromatography mass-spectrometry, using a Waters Alliance HT 2795 HPLC (Waters, Milford, MA, USA) interfaced to a QuattroMicro tandem mass spectrometer operating in the positive ion electrospray ionization mode (Waters Micromass, Manchester, UK). Simultaneous separation and detection of sugars and organic acids were achieved using a Rezex ROA-Organic acid column (300×7.8 mm, Phenomenex) with a Carbo H guard column (Phenomenex, Torrance, CA, USA). The autosampler and column compartment were maintained at 15 °C and 20 °C, respectively. The mobile phase was 0.5% acetic acid in water at a flow rate of 0.4 mL min^{-1}. Initial screening of root exudates indicated that acetic acid was not detected in the samples (data not shown) therefore this would not interfere with organic acid quantification. The total run time for each sample was 17 min with [U-^{13}C]glucose (389374; Sigma-Aldrich, St Louis, MO, USA) being used as an internal standard. The mass spectrometer capillary voltage was set at 3 kV, the source temperature at 120 °C, desolvation temperature at 380 °C. The N_2 flow rates were 30 L h^{-1} for cone gas and 500 L h^{-1} for desolvation gas. The dwell time for each multiple reaction monitoring transition (MRM) was 0.1 s. Quantification of individual compounds was achieved using Masslynx V4.1 software. Mass spectrometer parameters are displayed in Table S1.

Total organic C of filtered exudate solutions was determined using a total organic C analyser (GE Sievers InnovOx TOC, CO, USA).

Plant analysis

At each sampling time, the 3 plants were divided into shoots, roots and nodules. Roots were scanned and images were analysed using WinRhizo 2017 (Regent Instruments, Quebec, Canada) to determine root length. Specific root length was calculated by dividing root length by root dry weight. Plant materials were oven-dried at 70 °C for 3 d when a constant weight was achieved and then weights recorded. Shoot and root materials were then ball-milled and subsamples were digested using HNO_3:$HClO_4$ (4:1) in an open block digester. Unmilled dry nodule samples were digested using the same method. Phosphorus concentrations in the digests were analysed using inductively coupled plasma-optical emission spectrometry (Perkin Elmer Optima 8000, MA, USA). Nitrogen concentration in the shoots was determined using CHNS/O analyser (Perkin Elmer EA2400, Shelton, CT, USA). As no N was supplied and only small amounts of N were mobilised from the cotyledons (data not shown), most of the N content was derived from N_2 fixation. Nitrogen fixation rates at each sampling time were calculated by subtracting the shoot N content from the previous sampling period from the current sampling time point. The proportion of total P allocated to roots was calculated by dividing root P content by total P content.

Statistical analysis

A three-way analysis of variance (ANOVA) was carried out to determine the effect of CO_2, P level and the time of exudate collection on the exudation of citrate, malate, fumarate, glucose, fructose, sucrose and total organic C. A two-way ANOVA was used to test the effect of CO_2 and P levels and their interactions

on plant measurements. The data of citrate, glucose, fructose, sucrose and total organic C required square-root-transformation while the data of shoot P, root P and specific P uptake were log-transformed before ANOVA to ensure a normal distribution of residuals. Normality of residuals was assessed using the Shapiro–Wilk test and Q-Q plots. Tukey's honest significant difference (HSD) was used to determine differences between means. Pearson correlation coefficients were used to determine the association between root exudates and shoot dry weight, N_2 fixation and the proportion of P in roots. Correlations were carried out within each P level given that the response between the two variables depended on P supply. A two-phase linear regression model was used to estimate the critical shoot P concentration for citrate exudation. All statistical analyses were performed in R version 3.6.3 (R Core Team 2020) except for regression analysis which was carried out in GENSTAT (version 19; VSN International, Hemel Hempstead, UK).

Results

Plant growth and P uptake

Elevated CO_2 significantly increased shoot dry weight across all P levels by 18%, 13% and 14% after 3, 4 and 5 weeks of P and CO_2 treatments, respectively (Table 1). Root dry weight was not affected by CO_2 or P level after 3 and 4 weeks of treatment but P level influenced root dry weight after 5 and 6 weeks of treatment with the plants grown at 5 µM P having 16% and 27% greater biomass than those at 1 µM P. Root length was not significantly affected by the CO_2 treatment but was affected by the P level. The plants grown at 5 µM P had 18% and 30% greater root length than those at 50 µM P after 5 and 6 weeks of growth, respectively. Root-to-shoot ratio was consistently decreased by elevated CO_2 regardless of P level. Across all P levels elevated CO_2 decreased root-to-shoot ratio by 9% and 6% after 3 and 5 weeks of growth. Nodule dry weight was affected by P supply but not CO_2 treatment between 4 and 6 weeks of growth. Plants supplied with 50 µM P showed 53%, 92% and 250% greater nodule dry weight compared to plants receiving 1 µM P at 4, 5 and 6 weeks of growth. Regardless of P level, elevated CO_2 reduced specific root length after 3, 4 and 5 weeks of treatment. Elevated CO_2 reduced specific root length by 14%, 9% and 9% after 3, 4 and 5 weeks of growth, respectively (Table 1).

Shoot P concentration was significantly affected by the CO_2 and P level. Overall, elevated CO_2 decreased shoot P by 18%, 15%, 20% and 22% after 3, 4, 5 and 6 weeks of treatment, respectively. Root P was only affected by P supply, with plants supplied with 50 µM P having root P 10 times greater than plants supplied with 1 µM P across all sampling times. The concentration of P in nodules was approximately 2–5 times greater than that in shoots across all sampling times. At Week 6, the nodule P concentration in plants supplied with 50 µM P was 3.4-fold higher compared to that at 1 µM P. CO_2 and P level interactively affected total plant P content at 4 and 6 weeks after treatment. Elevated CO_2 decreased total P content at 50 µM P by 13% at 4 weeks but increased it by 18% at 6 weeks whilst elevated CO_2 did not affect the P content at 1 and 5 µM P in any sampling time. Elevated CO_2 significantly increased specific P uptake by 15% at 3 weeks after treatment (Table 2).

Root exudation

Citrate was the dominant organic anion exuded by white lupin representing up to 40% of C in exudates (Fig. S1). Citrate exudation was significantly affected by CO_2 concentration, P level and time of exudate collection. Overall, elevated CO_2 enhanced citrate exudation by 60% across all P levels and sampling times ($P<0.001$) (Table 3). Specifically, elevated CO_2 enhanced citrate exudation by 21, 15 and 9.4 nmol h^{-1} m^{-1} root at 1, 5 and 50 µM P, respectively, across all sampling times. The largest elevated CO_2-induced increase in citrate exudation occurred at the 5th week of treatment where citrate exudation was enhanced by 82% at 1 µM P (Fig. 1a). Across all harvests and CO_2 treatments, citrate exudation at 1 µM P was 8 times greater than at 50 µM P. After 6 weeks of treatment, there were no significant CO_2 effects on citrate exudation. There was a significant CO_2 effect on malate exudation with elevated CO_2 enhancing malate exudation overall by 21% ($P<0.001$) (Table 1). However, elevated CO_2 did not consistently affect malate exudation. The only instance of enhanced malate exudation at 1 µM P was at 4 weeks after treatment, where elevated CO_2 enhanced exudation by 54%. Elevated CO_2 increased

Table 1 Shoot and root dry weights (DW), root-to-shoot ratio, nodule dry weight, root length and specific root length of white lupin (*Lupinus albus* L.) grown after 3, 4, 5 and 6 weeks of exposure (Week) to ambient and elevated CO_2 concentrations at 1, 5 and 50 µM P

Week	P level (µM)	CO_2 level	Shoot DW (g plant^{-1})	Root DW (g plant^{-1})	Root-to-shoot ratio	Nodule DW (mg plant^{-1})	Root length (m plant^{-1})	Specific root length (m g^{-1})
3	1	Ambient	0.37b	0.17	0.44ab	27.4	8.65	52.6a
		Elevated	0.46a	0.18	0.39ab	24.4	8.06	44.6bc
	5	Ambient	0.38b	0.17	0.45a	30.4	8.59	50.6ab
		Elevated	0.46a	0.19	0.41b	29.7	7.89	42.0c
	50	Ambient	0.42ab	0.16	0.39ab	26.5	7.60	46.4abc
		Elevated	0.47a	0.17	0.37b	26.8	7.11	41.2c
Significance level								
CO_2			< 0.001	0.086	0.011	0.470	0.100	< 0.001
P level			0.235	0.553	0.013	0.101	0.055	0.032
CO_2 × P level			0.395	0.911	0.531	0.686	0.969	0.572
4	1	Ambient	0.52b	0.25	0.49a	30.5c	13.80	56.0a
		Elevated	0.61ab	0.27	0.44bc	32.6bc	12.77	46.8abc
	5	Ambient	0.55ab	0.26	0.48ab	41.9abc	13.71	52.0ab
		Elevated	0.65a	0.29	0.44bc	44.5ab	15.71	49.2abc
	50	Ambient	0.63ab	0.29	0.46abc	46.9a	13.44	46.4bc
		Elevated	0.65a	0.28	0.43c	48.6a	11.72	42.2c
Significance level								
CO_2			0.004	0.283	< 0.001	0.364	0.546	0.006
P level			0.042	0.224	0.047	< 0.001	0.066	0.004
CO_2 × P level			0.272	0.269	0.923	0.988	0.175	0.266
5	1	Ambient	0.74c	0.37b	0.51a	41.1b	19.3ab	51.5a
		Elevated	0.81bc	0.38b	0.47abc	34.8b	18.0ab	47.8ab
	5	Ambient	0.83abc	0.42a	0.5ab	59.4ab	21.1a	50.4ab
		Elevated	1.02a	0.48a	0.47abc	58.8ab	21.7a	45.7bc
	50	Ambient	0.87abc	0.38ab	0.44bc	76.4a	17.6ab	46.6ab
		Elevated	0.95ab	0.41ab	0.43c	67.1a	15.3a	40.8c
Significance level								
CO_2			0.005	0.134	0.033	0.265	0.410	< 0.001
P level			0.005	0.024	0.003	< 0.001	0.001	< 0.001
CO_2 × P level			0.329	0.550	0.558	0.749	0.384	0.708
6	1	Ambient	0.90c	0.5b	0.57a	51.5 cd	24.6c	48.8a
		Elevated	0.81c	0.47b	0.59a	40.0d	22.5c	48.2a
	5	Ambient	1.24b	0.68a	0.55a	93.4b	31.1a	45.8ab
		Elevated	1.31b	0.66a	0.50ab	86.0bc	29.8ab	45.3ab
	50	Ambient	1.45ab	0.64a	0.44b	179.4a	25.6bc	40.3bc
		Elevated	1.69a	0.75a	0.44b	156.2a	26.1bc	34.9c
Significance level								
CO_2			0.179	0.134	0.673	0.054	0.296	0.087
P level			< 0.001	0.024	< 0.001	< 0.001	< 0.001	< 0.001
CO_2 × P level			0.064	0.550	0.312	0.625	0.528	0.198

Means followed by a common letter are not significantly different from each other (Tukey HSD, $P < 0.05$)

Table 2 The concentrations of P in shoots, roots and nodules, total P content per plant, and specific P uptake of white lupin (*Lupinus albus* L.) grown after 3, 4, 5 and 6 weeks of exposure (Week) to ambient (380 ppm) and elevated (700 ppm) CO_2 concentrations at 1, 5 and 50 µM P

Week	P level (µM)	CO_2 level	Shoot P ($mg\ g^{-1}$)	Root P ($mg\ g^{-1}$)	Nodule P ($mg\ g^{-1}$)	P content (mg^{-1})	Specific P uptake ($\mu g\ m^{-1}$)
3	1	Ambient	1.54cd	1.95b	4.88c	0.89b	103c
		Elevated	1.29d	2.01b	5.88c	0.96b	120c
	5	Ambient	2.67b	2.63b	7.33bc	1.45b	168b
		Elevated	1.97c	2.75b	7.60abc	1.43b	179b
	50	Ambient	6.01a	18.03a	10.29ab	5.48a	722a
		Elevated	5.12a	20.72a	12.26a	5.97a	840a
Significance level							
CO_2			< 0.001	0.687	0.097	0.001	< 0.001
P level			< 0.001	< 0.001	< 0.001	< 0.001	< 0.001
$CO_2 \times$ P level			0.313	0.397	0.639	0.221	0.591
4	1	Ambient	1.21d	1.97c	3.54b	1.13c	92 cd
		Elevated	1.00d	1.93c	4.12b	1.14c	89d
	5	Ambient	1.88c	2.46b	4.84b	1.69c	122bc
		Elevated	1.65c	2.51b	4.87b	1.80c	127b
	50	Ambient	6.60a	17.55a	11.10a	9.22a	687a
		Elevated	4.95b	17.45a	10.61a	8.07b	691a
Significance level							
CO_2			< 0.001	0.897	0.465	0.025	0.883
P level			< 0.001	< 0.001	< 0.001	< 0.001	< 0.001
$CO_2 \times$ P level			0.455	0.966	0.278	0.002	0.965
5	1	Ambient	0.84e	1.95b	3.09b	1.04b	82b
		Elevated	0.79e	1.78b	3.26b	1.31b	73b
	5	Ambient	1.56c	2.32b	4.26b	2.26b	108b
		Elevated	1.18d	2.27b	4.20b	2.27b	104b
	50	Ambient	6.33a	17.52a	10.89a	12.08a	687a
		Elevated	5.01b	16.29a	12.35a	11.32a	725a
Significance level							
CO_2			< 0.001	0.461	0.448	0.071	0.506
P level			< 0.001	< 0.001	< 0.001	< 0.001	< 0.001
$CO_2 \times$ P level			0.017	0.920	0.760	0.691	0.694
6	1	Ambient	0.95d	1.46b	1.90d	1.62c	64c
		Elevated	0.73e	1.38b	2.54 cd	1.23c	54c
	5	Ambient	1.45c	2.00b	3.80bc	3.15c	101b
		Elevated	1.11d	1.93b	5.13b	2.44c	111b
	50	Ambient	5.67a	14.23a	9.06a	16.38b	640a
		Elevated	4.45b	15.81a	10.66a	19.25a	741a
Significance level							
CO_2			< 0.001	0.509	0.026	0.040	0.720
P level			< 0.001	< 0.001	< 0.001	< 0.001	< 0.001
$CO_2 \times$ P level			0.961	0.330	0.783	0.009	0.120

Means followed by a common letter are not significantly different from each other (Tukey HSD, $P < 0.05$). The data were log-transformed before ANOVA

Table 3 Significance levels (*P* values) of the effects of CO_2 level, P supply, sampling time and their interactions on specific exudation rates of citrate, malate, fumarate, glucose, fructose, sucrose and total organic C (TOC) at 3, 4, 5 and 6 weeks after treatment

Factor	Citrate	Malate	Fumarate	Glucose	Fructose	Sucrose	TOC
CO_2	< 0.001	0.002	0.268	< 0.001	< 0.001	0.231	< 0.001
P level	< 0.001	< 0.001	0.022	< 0.001	< 0.001	0.007	< 0.001
Sampling time	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001
CO_2 × P level	0.617	0.924	0.820	0.027	0.059	0.694	0.512
CO_2 × Time	< 0.001	0.345	0.792	0.005	0.001	0.747	0.204
P level × Time	0.001	< 0.001	0.008	< 0.001	< 0.001	0.001	< 0.001
CO_2 × P level × Time	0.030	0.224	0.084	0.036	0.269	0.001	0.179

Citrate, glucose, fructose, sucrose and TOC were square-root transformed before statistical analysis

malate exudation by 106% in plants supplied with 50 µM P after 5 weeks of P and CO_2 treatment. It did not affect malate exudation in plants provided with 5 µM P. Malate exudation peaked at 4 weeks of treatment at 1 and 50 µM P and decreased by 54% and 87%, respectively, after further 2 weeks of treatment (Fig. 1b). Fumarate exudation was only significantly affected by P level and sampling time. The plants receiving 1 µM P exuded 20% more fumarate compared to those at 50 µM P (Fig. 1c).

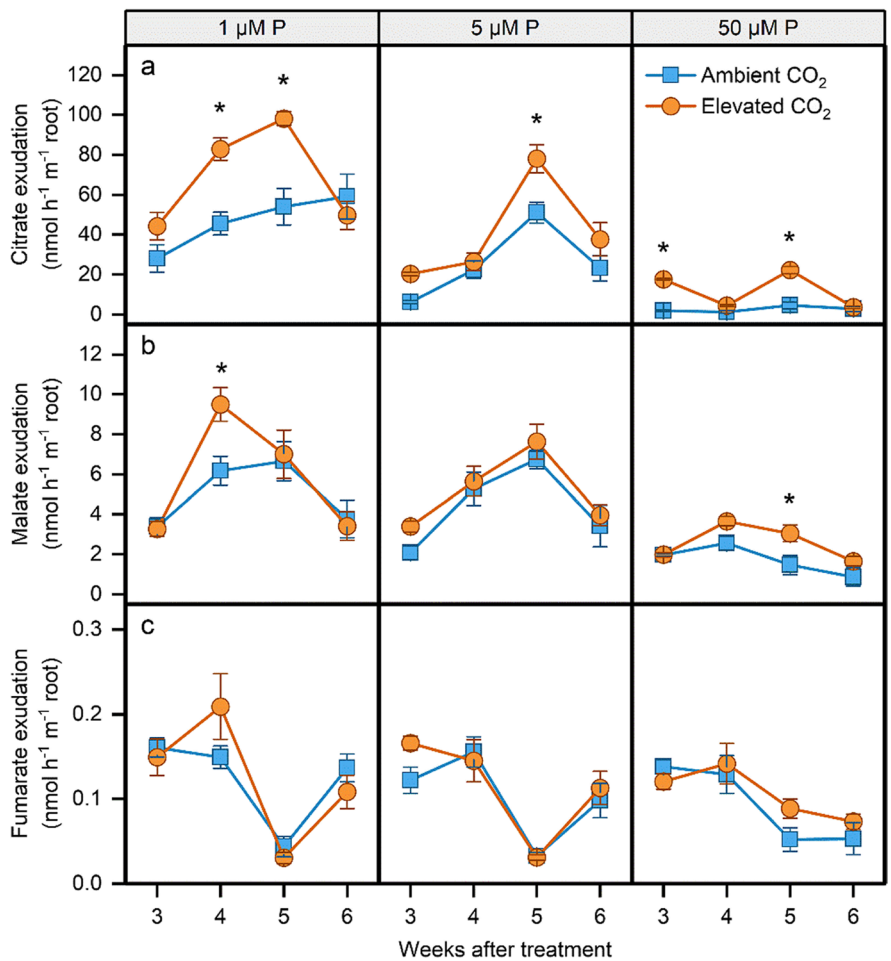

Fig. 1 Specific exudation rates of citrate (**a**), malate (**b**) and fumarate (**c**) from roots of white lupin (*Lupinus albus* L.) grown for 3, 4, 5 and 6 weeks under ambient (380 ppm) and elevated (700 ppm) CO_2 concentrations at 1, 5 and 50 µM P in hydroponic solution. Error bars represent ± standard error of the mean. Asterisks indicate that means between CO_2 levels are significantly different (Tukey HSD, $P < 0.05$). Note the difference in scale on the y-axis between organic anions

Glucose exudation was affected by CO_2 level, P level and sampling time. Overall, elevated CO_2 enhanced glucose exudation by 50% ($P<0.001$) (Table 1). After 4 weeks, elevated CO_2 increased glucose exudation by 103%, 59% and 98% in the 1, 5 and 50 µM P treatments, respectively (Fig. 2a). It also doubled glucose exudation at 5 µM P after 5 weeks of P treatment. Glucose exudation rates increased by 241% and 504% at 1 and 5 µM P, respectively, between 3 and 4 weeks of treatment, and peaked at 4 weeks of growth. These declined by 38% and 30% at 1 and 5 µM P, respectively, between 4 and 5 weeks of treatment (Fig. 2a).

Fructose was the most abundant sugar and represented up to 15% of C in exudates (Fig. S1). The effect of elevated CO_2 on fructose exudation was limited to 4 weeks of P treatment; elevated CO_2 increased fructose exudation by 69%, 40% and 75% at 1, 5 and 50 µM P, respectively (Fig. 2b). Sucrose exudation was affected by CO_2 level, P level and sampling time. Across all P levels and sampling times, elevated CO_2 increased sucrose exudation by 18% ($P=0.004$) (Table 1; Fig. 2c). Primarily, it increased sucrose exudation by 75% after 4 weeks of treatment at 1 µM P and 40% at 5 weeks of treatment at 5 µM P. At 1 µM P, sucrose exudation peaked at 4 weeks of treatment in plants exposed to elevated CO_2, increasing by 117% between 3 and 4 weeks of treatment. Sucrose exudation then declined by 40% at 6 weeks of treatment. Phosphorus rate also influenced sucrose exudation ($P<0.001$) with the 1 and 5 µM P levels displaying 37% and 48% higher sucrose exudation than 50 µM P (Table 1; Fig. 2c).

Elevated CO_2 enhanced total organic C exudation at 4 and 5 weeks after treatment. Elevated CO_2 did not interact with P level, and increased organic C exudation by 20% and 38% after 4 and 5 weeks of treatment, respectively. After 5 weeks of P treatment,

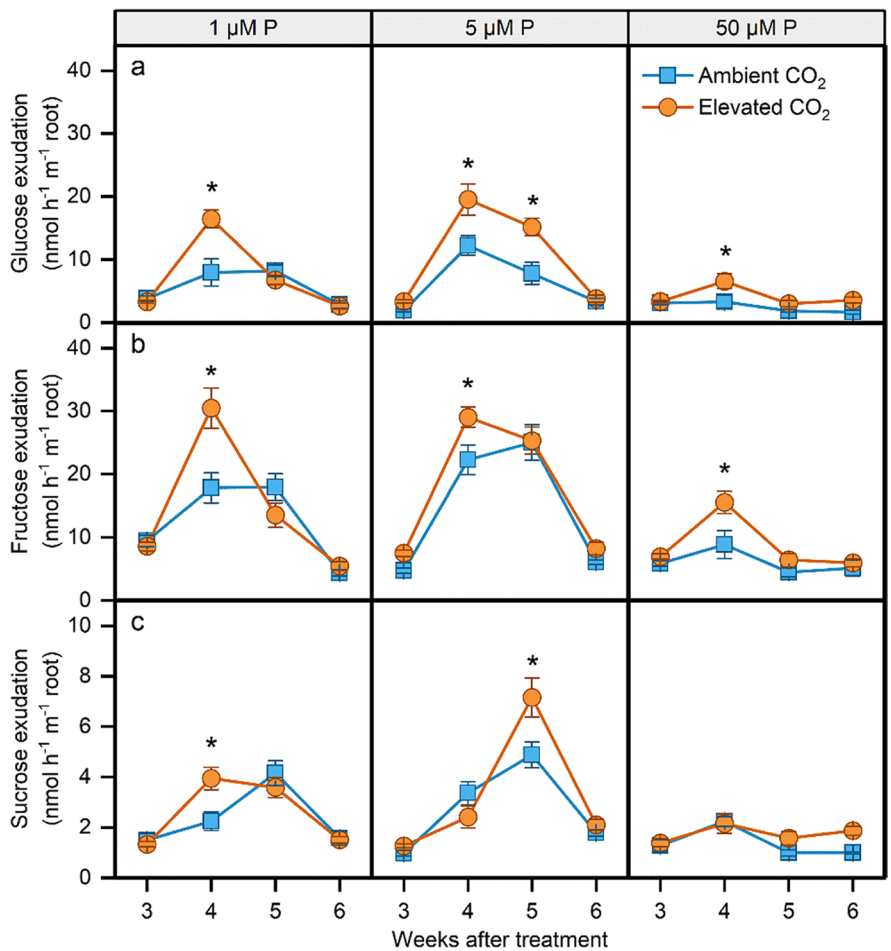

Fig. 2 Specific exudation rates of glucose (**a**), fructose (**b**) and sucrose (**c**) from roots of white lupin (*Lupinus albus* L.) grown for 3, 4, 5 and 6 weeks under ambient (380 ppm) and elevated (700 ppm) CO_2 concentrations at 1, 5 and 50 µM P in hydroponic solution. Error bars represent ± standard error of the mean. Asterisks indicate that means between CO_2 levels are significantly different (Tukey HSD, $P<0.05$). Note the difference in scale on the y-axis between sugars

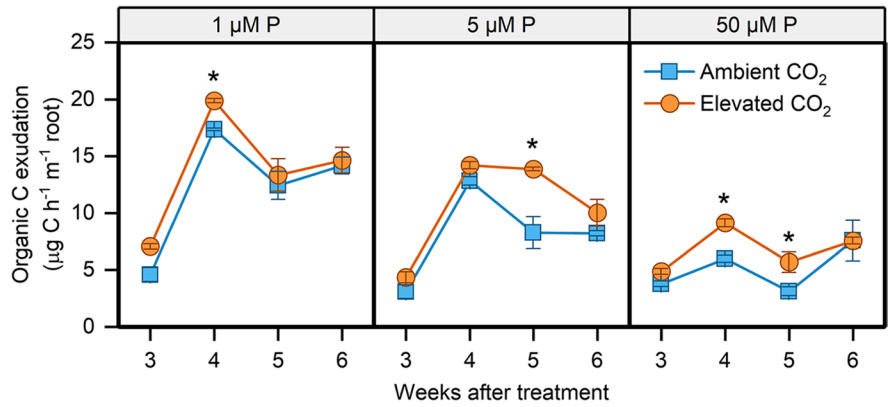

Fig. 3 Specific exudation rates of organic C from roots of white lupin (*Lupinus albus* L.) grown for 3, 4, 5 and 6 weeks under ambient (380 ppm) and elevated (700 ppm) CO_2 concentrations at 1, 5 and 50 µM P in hydroponic solution. Error bars represent ± standard error of the mean. Asterisks indicate that means between CO_2 levels are significantly different (Tukey HSD, $P < 0.05$)

elevated CO_2 enhanced organic C exudation by 113% and 81% for the plants supplied with 5 and 50 µM P, respectively. Phosphorus supply also influenced organic C exudation with the plants at 1 µM P exuding 2.2 times more C than at 50 µM across all sampling times. Exudation of organic C increased by 186% between 3 and 4 weeks after treatment but then declined by 21% between 4 and 6 weeks of treatment (Fig. 3).

Similar effects of CO_2 and P level on organic anion and sugar exudation were observed when exudation rates were calculated based on per unit of root length, root dry weight (Figs S2, S3 and S4) and on a whole plant basis (Figs S5, S6 and S7).

Relationships with citrate exudation

Shoot P concentrations dictated citrate exudation rates between 3 and 6 weeks of P and CO_2 treatment, with the two-phase linear model explaining 75% and 70% of the variance for the ambient and elevated CO_2 treatments. The critical shoot P concentration for citrate exudation was 2.53 ± 0.23 and 1.85 ± 0.18 mg g^{-1} for ambient and elevated CO_2, respectively. Based on regression analysis, citrate exudation from P-sufficient plants was 3.30 ± 2.53 nmol h^{-1} m^{-1} root and 13.4. ± 2.99 nmol h^{-1} m^{-1} root under ambient and elevated CO_2, respectively. The slope of the linear phase was -30 ± 6 and -63 ± 14 for ambient and elevated CO_2, respectively (Fig. 4; Table S2). Shoot dry weight and citrate exudation were positively correlated at 1 µM P ($r = 0.72$, $P < 0.001$) and 5 µM P ($r = 0.93$, $P < 0.001$) but not 50 µM P level

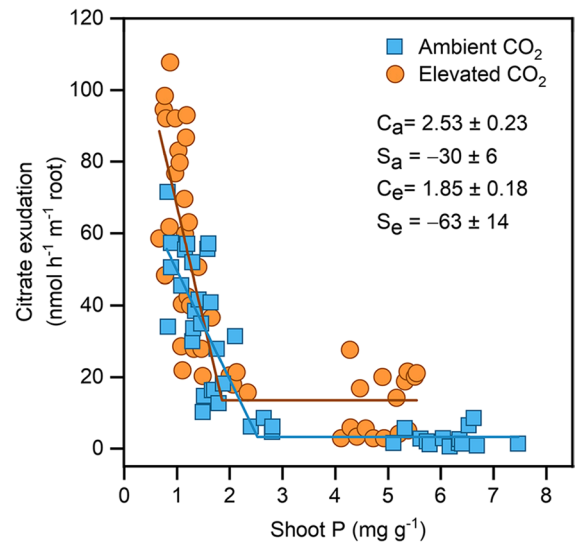

Fig. 4 Relationship between citate exudation and shoot P concentration for white lupin (*Lupinus albus* L.) exposed to ambient (380 ppm) and elevated (700 ppm) CO_2 concentrations at supply of 1, 5 and 50 µM P in hydroponic solution. Exudates were collected after 3, 4, 5 and 6 weeks of treatment. Fitted lines represent the line of best fit for a two-phase linear model. C_a and C_e represent the critical shoot P concentration (± standard error) for citrate exudation under ambient and elevated CO_2, respectively. S_a and S_e are the slope of the linear phase (± standard error) under ambient and elevated CO_2, respectively

($P = 0.165$) (Fig. S8). The proportion of P allocated to roots and citrate exudation were also positively correlated at 1 µM P ($r = 0.80$, $P < 0.001$) and 5 µM P ($r = 0.78$, $P < 0.001$) but not at 50 µM P ($P = 0.073$) (Fig. 5). There was a significant correlation between the shoot N accumulation rate and citrate exudation ($r = -0.57, P < 0.001$) (Fig. S9).

Discussion

The present study explored the interactive effects of elevated CO_2 and P deficiency on root exudation dynamics. We found that elevated CO_2 and P deficiency had a positive interaction in the exudation of citrate, the dominant organic anion exuded by white lupin, leading to elevated CO_2-induced increases in citrate exudation being larger in P-deficient than P-sufficient plants. It was further indicated that elevated CO_2 likely facilitated P deficiency and led to the preferential allocation of resources into citrate exudation. Similarly, elevated CO_2 increased glucose, fructose and sucrose exudation with elevated CO_2 inducing larger increases in sugar exudation in P-deficient than P-sufficient plants. The effect of elevated CO_2 on root exudation was limited to the earlier stages of plant growth. This work suggests that elevated CO_2 may improve the ability for plants to acquire non-labile forms of P. Future work is needed to examine these processes in plant-soil systems as hydroponic culture does not reflect the suite of plant-soil feedback mechanisms.

Citrate was the dominant organic compound detected in root exudates and was consistently increased by elevated CO_2 from Week 3 to Week 5 (Fig. 1a). The largest elevated CO_2-induced increases in citrate exudation occurred in extremely P-deficient plants, particularly between 4 and 5 weeks after treatment. When shoot P and citrate exudation were fitted with a two-phase linear model, the slope of the linear phase was more negative under elevated CO_2 compared to ambient CO_2 (Fig. 4; Table S2). This demonstrates that every mg g^{-1} decrease in shoot P for P-deficient plants would induce 30 and 63 nmol h^{-1} m^{-1} root increase in citrate exudation in plants exposed to ambient and elevated CO_2, respectively. Therefore, plants exposed to elevated CO_2 exuded an additional 33 nmol h^{-1} m^{-1} root of citrate for every mg g^{-1} decrease in shoot P compared to ambient CO_2, thus contributing to larger CO_2-induced increases in citrate exudation in P-deficient plants. Furthermore, a synergistic effect was observed in Week 5 where the combined effect of P deficiency and elevated CO_2 led to greater increase in citrate exudation compared to each treatment alone. This may have occurred because elevated CO_2 can increase carbohydrate concentrations in shoots and roots, particularly in P-deficient plants (Aranjuelo et al. 2013; Campbell and Sage 2002). Given that many enzymes related to organic acid synthesis and exudation are upregulated under P deficiency (such as phosphoenolpyruvate carboxylase), the enhanced substrate availability under elevated CO_2 could lead to greater citrate synthesis and exudation (Venuti et al. 2019; Wang et al. 2014). The smaller

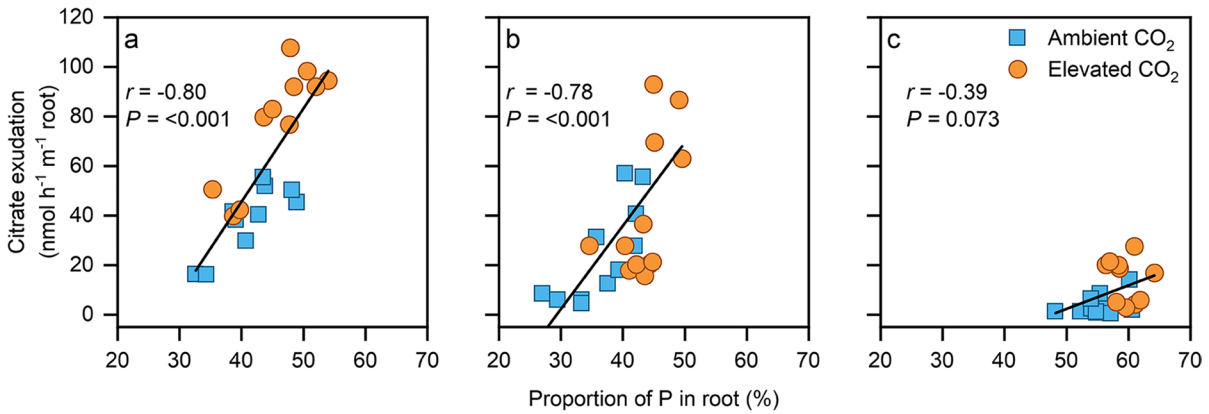

Fig. 5 Relationships between citrate exudation and the proportion of plant P content allocated to roots for white lupin (*Lupinus albus* L.) exposed to ambient (380 ppm) and elevated (700 ppm) CO_2 concentrations at 1 (**a**), 5 (**b**) and 50 (**c**) µM P in hydroponic solution. Exudates were collected after 3, 4 and 5 weeks of treatment

response to elevated CO_2 in P-sufficient plants was likely because the expression of key enzymes and transporters related to citrate synthesis and exudation was low compared to P-deficient plants; hence the response to enhanced carbohydrate supply was not of the same magnitude. Elevated CO_2 also decreased shoot P concentration which could further contribute to greater citrate exudation through intensifying P deficiency. With elevated CO_2 increasing citrate exudation, particularly under P-deficient conditions, this could lead to greater mobilisation of precipitated and sorbed P under elevated CO_2, thereby alleviating P deficiency.

The impact of elevated CO_2 was less prominent on malate compared to citrate. Overall, elevated CO_2 only increased malate exudation by 22% compared to 60% for citrate. As elevated CO_2 decreased shoot P concentrations, this intensified P deficiency and led to preferential allocation of resources into citrate production over malate. When P deficiency is intensified by elevated CO_2, additional C allocated belowground was likely to be diverted into citrate exudation rather than malate exudation as malate is less effective at promoting dissolution reactions due to possessing only two carboxyl groups compared to citrates three (Jones 1998). Under P deficiency, malate within root tissues can be transported into the mitochondria for citrate synthesis (Neumann et al. 1999). This process could be favoured under elevated CO_2, thus diverting resources away from malate exudation (Venuti et al. 2019). These results indicate that elevated CO_2 favours the exudation of citrate over malate, hence further enhancing the plant's ability to access non-labile P.

Elevated CO_2 increased exudation rates of glucose, fructose and sucrose, particularly in P-deficient plants, which could be due to greater sugar concentrations in roots. Elevated CO_2 has previously been shown to increase sugar concentration within roots, which coincides with enhanced sugar exudation likely due to sugar exudation occurring through facilitated diffusion (Canarini et al. 2019; Li et al. 2018). For glucose and sucrose exudation, we also observed larger CO_2-induced increases in P-deficient plants compared to P-sufficient plants. This positive interaction could be driven by P deficiency inhibiting primary root growth and promoting organic anion exudation whilst elevated CO_2 increases C supply to roots in the form of sucrose. Sucrose is then hydrolysed within the roots leading to the accumulation of glucose and fructose within root tissues contributing to the increase in exudation rates (Durand et al. 2018; Neumann et al. 2000). The elevated CO_2-induced increases in sugar exudation, particularly in P-deficient plants, could stimulate microbial activity in the rhizosphere and lead to the mobilisation of non-labile P forms (Hennion et al. 2019; Spohn et al. 2013). The lack of a consistent CO_2 effect over time could reflect the highly dynamic nature of sugar concentrations within plant tissues as sugars are constantly being used for organic acid synthesis and root growth (Durand et al. 2018). Nevertheless, enhanced sugar exudation induced by elevated CO_2 could further stimulate the utilisation of non-labile P, particularly in P-deficient plants.

Elevated CO_2 increased the exudation of total organic C but did not interact with P supply. The consistent increases in organic C exudation, between 20 and 42% across all P supply levels under elevated CO_2 in Weeks 4 and 5, are consistent with other studies (Dong et al. 2021). Enhanced organic C exudation has been widely attributed to greater photosynthetic rates and belowground C allocation commonly observed under elevated CO_2. The increases in exudation of sugars and organic anions did not fully account for the elevated-CO_2-induced increase of organic C exudation across all P levels. Elevated CO_2 might enhance rhizodeposition of other organic compounds such as root mucilage, proteins or amino acids, which could mask the contribution of enhanced exudation of organic anions and sugars to exudation of total organic C (Fig. S4). However, there are few studies that have explicitly examined the effect of elevated CO_2 and its interaction with P status on rhizodeposition beyond root exudates.

Exudation rates of organic compounds decreased, and the CO_2 effects diminished at the late stage of the experimental period, probably due to down-regulation of root exudation in favour of shoot and root growth. For example, Sas et al. (2001) demonstrated that rates of citrate and malate exudation were reduced after 41 days of P treatment. We observed the largest increase in shoot and root growth, particularly at 5 and 50 µM P in Week 6 which corresponds to periods of reduced root exudation. In earlier growth stages, high exudation rates enable plants to acclimatise to low-P conditions and assimilate non-labile P. During these periods, elevated CO_2 can further enhance root exudation which improves plant establishment. Periods

of rapid plant growth and greater plant maturity not only reduce exudation but also minimises the impact of elevated CO_2 on root exudation. It was evident that there are no significant CO_2 effects on exudation rates after 6 weeks of treatment during this peroid of rapid plant growth. While plants exposed to 1 μM P displayed slow growth after 5 weeks, decreased exudation was still observed, which was likely due to large down-regulation of photosynthetic activity as a result of severe P deficiency (Campbell and Sage 2006). These results demonstrate that the effect of elevated CO_2 on root exudation might be limited to the initial growth stages in annual crop species.

Elevated CO_2 preferentially promoted accumulation of P in roots in comparison to shoot, which was linked to greater citrate exudation. Greater P allocation to roots under elevated CO_2 has been observed in previous studies but has not been linked to citrate exudation (Almeida et al. 1999; Campbell and Sage 2002). Phosphorus deficiency induces gene expression for many pathways involving organic anion exudation and P assimilation. As elevated CO_2 may exacerbate P deficiency and increase P demand, gene and protein expressions would be further upregulated (Venuti et al. 2019). Given that production of RNA and proteins requires large amounts of P in roots, translocation of P to the shoots may be limited and hence leads to a decrease in P concentration within the shoots. The greater allocation of P to the roots is not only linked to citrate exudation but P acquisition mechanisms more generally such as increased phosphatase activity (O'Sullivan et al. 2020). Decreases in shoot P concentration, which are often observed in elevated CO_2 studies, are often linked to a dilution effect due to stimulation in plant growth (Jiang et al. 2020). However, the present study suggests that elevated CO_2 enhanced the need for P belowground thus limiting its translocation. The ratio of P allocated to roots and shoots under elevated CO_2 could be used as an indication for stimulation in citrate exudation or upregulation of other P acquisition mechanisms although this hypothesis requires further investigation.

More research is needed to elucidate the interaction between elevated CO_2 and P deficiency on C allocation in N_2-fixing legumes. It has been demonstrated that under elevated CO_2, root nodules that fix atmospheric N_2 represent a stronger C sink whilst rhizodeposition is simultaneously increased (Parvin et al. 2020). In the current experiment, P deficiency limited nodule formation which would likely decrease the sink strength of root nodules (Valentine et al. 2017). Given that elevated CO_2 did not enhance nodule formation, C not utilised for N_2 fixation can then be allocated towards P acquisition in the form of root exudates and cluster roots. As P deficiency decreases nodule sink strength and elevated CO_2 enhances photosynthetic rates, greater amounts of C can be allocated for root exudation. Under P sufficiency, the opposite occurs as exudation rates are curtailed, cluster root formation is reduced, nodule size increases, and N_2 fixation is stimulated (Sas et al. 2002; Schulze et al. 2006). Therefore, when C availability is enhanced by elevated CO_2, C can be directed into N_2 fixation to meet increased N-demand induced by greater plant growth and released as exudates. This could contribute to the greater frequency of enhanced exudation rates in P-deficient plants compared to P-sufficient plants and partially explain the negative correlation between citrate exudation and N_2 fixation (Fig. S9). Currently, this topic remains largely unexplored but requires investigations due to the interactions of photosynthesis, N_2 fixation and P acquisition.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s11104-021-04991-0.

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

Declarations

Conflict of interest The authors declare that they have no competing interests.

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