White lupin (*Lupinus albus* L.) exposed to elevated atmospheric CO₂ requires additional phosphorus for N₂ fixation

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Abstract

Purpose The steady rise of atmospheric CO₂ concentrations enhances symbiotic N₂ fixation and plant growth. However, it is largely unknown whether more P is required to enhance N₂ fixation under elevated CO₂. We examined if plants under elevated CO₂ require more P to support enhanced N₂ fixation.

Methods White lupin (*Lupinus albus* L. cv. Kiev) was grown in nutrient solution under ambient (380 ppm) and elevated (700 ppm) CO₂ levels at 0, 2, 5, 12, 25 and 50 µM P. Nodulation, nodule function, N₂ fixation and plant growth were assessed at 4 and 6 weeks post inoculation. Excess cation uptake and proton release were also analysed due to its importance in P acquisition.

Results Elevated CO₂ increased the critical P concentration in nutrient solution for N₂ fixation, indicating plants under elevated CO₂ need to acquire additional P to support greater N₂ fixation. Elevated CO₂ improved N₂ fixation in P-deficient plants by

increasing specific nitrogenase activity. Elevated CO₂ also enhanced excess cation uptake which increased proton release by roots.

Conclusions As elevated CO₂ increased the plant demand for P, it increased the critical P concentration in nutrient solution for N₂ fixation, thereby plants require more P to mitigate the effect of P deficiency on N₂ fixation. Despite this, elevated CO₂ could enhance N₂ fixation in P-limiting environments.

Keywords Climate change · Nitrogen fixation · Nitrogenase · Nodulation · Phosphate · Rhizosphere acidification

Introduction

Atmospheric CO₂ concentrations are projected to reach 700–800 ppm by the year 2100 (IPCC 2021). Rising CO₂ concentrations stimulates photosynthetic activity in many C₃ crop species and induces significant increases in plant production (Ainsworth and Long 2005). The elevated CO₂-induced increase in plant growth requires plants to enhance N uptake to support this process (Lam et al. 2012). Symbiotic N₂ fixation is a process where plants form a symbiotic relationship with N₂-fixing bacteria such as *Frankia* or *Rhizobium* species. These bacteria are supplied C from the host plant and fix atmospheric N₂ into NH₃ using nitrogenase enzyme complex, which is then supplied to plants

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and utilised for plant growth (Sachs et al. 2018). To meet the increase in N demand under elevated CO₂, plants commonly increase N₂ fixation, particularly in agricultural leguminous (Aranjuelo et al. 2014; Lam et al. 2012) and actinorhizal species (Chen and Markham 2021; Tobita et al. 2010).

Elevated CO₂ increases N₂ fixation via two main mechanisms. First, elevated CO₂ increases nodule formation and nodule development. Li et al. (2017) demonstrated that elevated CO₂ could drastically increase nodule number and fresh weight which played a critical role in supporting increased N₂ fixation. Improved nodulation under elevated CO₂ can be facilitated by enhancing flavonoid exudation which could promote infection of plant roots by rhizobia and hence increase nodule formation (Haase et al. 2007). Second, elevated CO₂ enhances photosynthesis, leading to more primary metabolites (malate and sucrose) being allocated to nodules, to increase nitrogenase activity (Haase et al. 2007; Parvin et al. 2020). Therefore, in some plant species elevated CO₂ can increase N₂ fixation without an increase in nodule number or nodule biomass as found by Butterly et al. (2015). The mechanism by which elevated CO₂ enhances N₂ fixation will likely depend on plant nutritional status and plant species (Jin et al. 2012; Palit et al. 2020).

Nodules have significantly higher P concentration compared to host plants, which outlines the importance of P for N₂ fixation (Schulze et al. 2006). Phosphorus is essential for nodule formation, nodule development and nodule function with P deficiency directly inhibiting N₂ fixation in some crop species (Le Roux et al. 2008; Tang et al. 2001). Phosphorus deficiency also decreases host plant growth which reduces plant demand for N and induces a down-regulation in nitrogenase activity (Suliman et al. 2013). Given that elevated CO₂ increases N₂ fixation but P deficiency decreases N₂ fixation, these two factors play two opposing roles in N₂ fixation. The response of N₂ fixation to elevated CO₂ in P-deficient plants will depend on whether P deficiency directly limits N₂ fixation (Almeida et al. 2000; Tang et al. 2001). If nodule function is directly impaired by P deficiency, elevated CO₂ may not enhance N₂ fixation in P-deficient plants (Sa and Israel 1998). However, Nguyen et al. (2006) found that elevated CO₂ could enhance N₂ fixation in P-deficient *Acacia* trees, suggesting that elevated CO₂ enhances biomass production in P-deficient plants, and may increase N₂ fixation.

Given that elevated CO₂ enhances plant biomass and promotes production of P-rich nodules, plants may require more P to support enhanced N₂ fixation under elevated CO₂ (Rogers et al. 1993). To determine the amount of P required to achieve maximum N₂ fixation, P response curves can be generated under different CO₂ treatments (Pang et al. 2011). As elevated CO₂ can enhance nodulation, greater P supply may be necessary to support greater nodule growth and N₂ fixation under elevated CO₂ (Lam et al. 2012).

Proton release, excess-cation uptake and N₂ fixation are closely linked. When N₂ fixation is the main source of N, plants absorb more cation nutrients than anion nutrients which results in net release of protons, thus promoting rhizosphere acidification (Tang et al. 1999). In addition to N₂ fixation, P deficiency promotes proton release in many leguminous plant species (Sas et al. 2001). With greater N₂ fixation and elevated CO₂-induced increases in plant growth, this could lead to greater uptake of cations over anions, particularly in P-limiting environments and hence lead to greater rhizosphere acidification. For plants to access sorbed and precipitated forms of P in soil, transformation is required before root uptake. Enhanced rhizosphere acidification could promote the solubilisation and desorption of non-labile P sorbed to non-crystalline materials or precipitated with metals such as Ca (Barrow 2017; Hinsinger et al. 2003). In soils, optimal P mobilisation occurs at pH 6 which is also the optimal pH for nodulation in *Lupinus* species (Andersson et al. 2015; Tang and Robson 1993).

White lupin is a crop species highly adapted to P-deficient soils. In response to P deficiency, white lupin produces dense rootlets along lateral roots, termed cluster roots (Lambers et al. 2006; Wang et al. 2019). Not only do cluster roots increase the surface area of roots for P uptake, these are also the site of intense rhizosphere acidification and organic anion efflux (Neumann et al. 2000). These P acquisition mechanisms allow white lupin to mobilise significant quantities of non-labile P from inorganic sources, particularly under elevated CO₂ (Braum and Helmke 1995; O'Sullivan et al. 2020). Furthermore, N₂ fixation in white lupin is highly resilient to P-deficient conditions. For example, despite not being supplied with any P, N₂ fixation was uninhibited after 21 d of growth (Schulze et al. 2006). As white lupin can sustain N₂ fixation in low-P soils, it is unknown if elevated CO₂-induced increases in biomass production

will affect N_2 fixation in P-deficient white lupin (O'Sullivan et al. 2021).

This study aimed to investigate the effects of elevated CO_2 on the critical P concentration in nutrient solution, nodules and shoots for N_2 fixation in white lupin. The critical P concentration was defined as the concentration of P needed to achieve maximum N_2 fixation. We hypothesised that (i) elevated CO_2 would increase the N_2 fixation in P-deficient plants, (ii) elevated CO_2 would increase the P requirement for N_2 fixation, and (iii) enhanced plant growth and N_2 fixation under elevated CO_2 would promote excess cation uptake and proton release.

Materials and methods

Plants were grown in solution culture to facilitate the measurements of nodule formation, nodule function and proton release. Uniform-sized seeds of white lupin (*Lupinus albus* L. cv. Kiev) were germinated at 20 °C for 5 d in 30-L tubs containing aerated solution of 1 mM $CaCl_2$ and 5 μ M H_3BO_3 . After germination, 12 seedlings were transferred to each 5-L container of nutrition solution. The nutrient solution had the following composition (μ M): 600 K_2SO_4 ; 600 $CaCl_2$; 200 $MgSO_4$; 10 $FeNaEDTA$; 5 H_3BO_3 ; 1 $MnSO_4$; 1 $ZnSO_4$; 0.2 $CuSO_4$; 0.2 $CoCl_2$; 0.03 Na_2MoO_4 (based on Sas et al. 2001). To generate a P response curve, P was supplied as KH_2PO_4 at 6 levels: 0, 2, 5, 12, 25 and 50 μ M P. These P levels were in range of previous studies to induce P-deficiency and to cover adequate P supply in white lupin (O'Sullivan et al. 2021). After seedling transferring to nutrient solution, a dense water suspension of *Bradyrhizobium* sp. strain WU425 was applied at approximately 2×10^8 cells L^{-1} . The nutrient solution was renewed after 2 d, and the rhizobial inoculum was reapplied. Uniform seedlings were equally divided amongst 6 CO_2 -controlled growth chambers with 20 °C days for 16 h and 18 °C nights under 380 ppm and 700 ppm CO_2 concentrations for ambient and elevated CO_2 levels, respectively (Fitotron SGC 120, Weiss Technik, Loughborough, UK; Climatron 1100-SL-H, Thermoline, Australia). Each treatment was replicated 3 times. In total, there were 36 hydroponic containers distributed amongst 6 growth chambers. Light intensity at the canopy level was around 400 μ mol $m^{-2} s^{-1}$. Containers were randomised within cabinets every

2 d and were rotated weekly between cabinets. The ambient CO_2 concentration was maintained by using a NaOH CO_2 scrubber. The elevated CO_2 concentration is in range of the predicted CO_2 level by 2100 in addition to other CO_2 enrichment studies (Jiang et al. 2020; IPCC 2021). To minimize the effect of seed P and induce early P response and stimulate N_2 fixation, cotyledons were removed 10 d post inoculation. Nutrient solutions were continuously aerated and renewed every 2 d. Nutrient solution pH was adjusted daily to 6 using standardised 0.1 M KOH, which is the optimal pH for nodulation in *Lupinus* species (Tang and Robson 1993). The amount of KOH consumed for pH adjustment and the pH of the discarded nutrient solutions were recorded and used to determine proton release.

Nodules were counted after signs of initial emergence at 10, 12, 14, 16 and 20 d, and 4 and 6 weeks after inoculation. Plants were destructively sampled at 4 and 6 weeks after inoculation. These sampling times were chosen because elevated CO_2 -induced increases in plant growth and nitrogenase activity in soybean began at Week 4 (Sa and Israel 1998). Plant growth of white lupin at Week 6 was relatively rapid which coincided with high plant N-demand, intense nitrogenase activity under P sufficiency and inhibition of nitrogenase activity by P deficiency (O'Sullivan et al. 2021; Schulze et al. 2006). These sampling times represented early (6 leaves emerged) and mid (9 leaves emerged) vegetative growth stages, respectively. At each harvest, 6 plants were harvested from each container. Plants were immediately divided into shoots and roots. To measure nodule nitrogenase activity, roots with intact nodules were placed in sealed 1-L Mason jars and acetylene was injected through a septum. The final concentration of acetylene in the jar was 2.5% (v/v). Gas was sampled from each jar at 15, 30 and 60 min after acetylene addition and ethylene was quantified using a gas chromatograph fitted with a Varian GS-Q capillary column and flame ionisation detector (Varian CP-3800 CA USA). Nitrogenase activity was calculated from the slope of the linear accumulation of ethylene over the 3 sampling times.

After nitrogenase analysis, nodules were counted and then removed from roots. Shoots, roots and nodules were placed in a 70 °C oven for 3 days and their dry weights recorded. Nodule size was calculated by dividing nodule dry weight over nodule number.

Shoot and root materials were finely ground and then digested with HNO_3 in a microwave digester (Ethos up, Milestone, Milan, Italy). Nodule samples were digested using HNO_3 and HClO_4 (4:1) in an open block digester (Seal Analytical, USA). Plant digests were analysed for P, S, Ca, Mg, Mn, Zn, K, Na and Fe using inductively coupled plasma optical emission spectrometry (Perkin Elmer Optima 8000, MA, USA). Excess cation uptake ($\text{mmol}_{(+)} \text{plant}^{-1}$) was calculated as the number of moles of positive charges acquired as cations (K^+ , Ca^{2+} , Mg^{2+} , Na^+) minus the number of moles of negative charges acquired as anions (PO_4^- , SO_4^{2-}). Chloride (Cl^-) was not included in the analysis; however, chloride only accounts for small proportion (6%) of excess cation uptake (Tang and Yu 1999). Elements mobilised from seed were not accounted in the excess cation uptake calculations.

Ground shoot and root samples were also analysed for C and N using dry combustion method (PerkinElmer EA2400, CT, USA). As no N was supplied to plants in nutrient solution, the amount of N_2 fixed was the content of N in the entire plant minus the contribution of N from the seed before cotyledons were removed.

Statistical analysis

Analysis of variance was performed to determine the effects of CO_2 treatment, P supply and their interaction on all measurements except for early nodule formation. The concentrations of P in shoot, root and nodule, and total P content were $\log_{10}$ -transformed before analysis to ensure normality of residuals. Linear mixed-effect modelling was used to determine the effects of CO_2 , P supply, and time of nodule counting on early nodule formation with a random intercept used for each replicate. Normality of residuals for each test was assessed using histograms, Q-Q plots and the Shapiro-Wilk test. To compare means, the least significant difference (LSD) was calculated at the 5% significance level.

A two-phase linear model was used to determine the critical P concentrations in nutrient solution, nodules and shoots for N_2 fixation. The breakpoint on the x-axis was considered the critical P concentration which was defined as the P concentration where N_2 fixation was not increased by additional P. If the standard error for the critical P concentration did not

overlap between CO_2 treatments, the difference was considered significant.

Analysis of variance and correlations were performed in R version 4.0.5 (R Core Team 2021) and post-hoc tests and linear mixed effect modelling was carried out using the predictmeans (Luo et al. 2020) and nlme (Pinheiro et al. 2021) packages in R, respectively. The two-phase linear model was performed in GENSTAT (v19; VSN International, Hemel Hempstead, UK).

Results

Plant biomass

Elevated CO_2 significantly increased shoot dry weight at 4 and 6 weeks after transplanting by 13% and 18%, respectively ($P < 0.001$, Fig. 1). Increasing P supply from 0 to 50 μM P increased shoot dry weight by 49% and 157% at 4 and 6 weeks, respectively ($P < 0.001$, Fig. 1a, b). At Week 4, root dry weight was greatest in plants supplied 2 μM P whereas at Week 6, increasing P supply increased root dry weight between 0 and 5 μM P but then plateaued between 5 and 50 μM P. The root dry weight was 76% greater at 50 μM P compared to nil-P control ($P < 0.001$, Fig. 1d).

Nodulation and nitrogenase activity

Elevated CO_2 but not P supply influenced early nodule formation. Elevated CO_2 enhanced early nodule emergence by 20, 17 and 6% at 12, 14, and 16 days after inoculation ($P = 0.016$, Fig. S2). The effect of P supply on nodule number was only apparent at Week 6 with plants supplied 50 μM P having 14% more nodules compared to plants without P supply ($P = 0.024$, Table 1). Nodule dry weight was affected by P supply but not CO_2 level at Week 4 ($P < 0.001$) and Week 6 ($P < 0.001$) (Table 1). At Week 4, plants supplied 0 μM P displayed reduced nodule dry weight compared to all other P treatments (Fig. 2a). The effect of P supply on nodule dry weight was stronger at Week 6 compared to Week 4, with increased P supply increasing nodule dry weight up to 5 μM P. Nodule dry weight was over 2-fold greater at 50 μM P than 0 μM P (Fig. 2b). Nodule size was unaffected by CO_2 treatment but increased with increasing P supply at both Weeks 4 ($P < 0.001$) and 6 ($P < 0.001$) with

Fig. 1 Dry weights of shoots (a, b) and roots (c, d) of white lupin grown under ambient (380 ppm) and elevated (700 ppm) CO₂ concentrations. Plants were supplied with 0, 2, 5, 12, 25 and 50 μM P, and were harvested 4 (a, c) and 6 (b, d) weeks after transplanting. Vertical bar indicates the least significant difference ($P = 0.05$) for any two means where the main effects and/or interaction are significant. Error bars indicate the standard error of the mean ($n = 3$)

Table 1 Significance levels (P values) of the effects of CO₂ level, P supply and their interactions on dry weights of shoots, roots and nodules, nodule number and size, nitrogenase activity, specific nitrogenase activity, whole plant N concentration and whole plant N content after 4 and 6 weeks of plant growth

Measurement	CO ₂		P supply		CO ₂ × P supply	
	Week 4	Week 6	Week 4	Week 6	Week 4	Week 6
Shoot dry weight	<0.001	<0.001	<0.001	<0.001	0.301	0.279
Root dry weight	0.913	0.248	<0.001	<0.001	0.210	0.631
Nodule dry weight	0.485	0.323	<0.001	<0.001	0.845	0.784
Nodule number	0.664	0.900	0.124	0.024	0.532	0.967
Nodule size	0.851	0.277	<0.001	<0.001	0.461	0.784
Nitrogenase activity	0.015	0.729	<0.001	<0.001	0.084	0.041
Specific nitrogenase activity	<0.001	0.002	0.001	<0.001	0.381	0.506
Shoot N concentration	<0.001	0.484	0.149	<0.001	0.726	0.389
Root N concentration	0.351	0.623	0.247	0.002	0.999	0.578
Plant N content	0.201	<0.001	0.028	<0.001	0.056	0.891

nodule size being 68% and 180% larger at 50 μM P than 0 P at Weeks 4 and 6, respectively (Fig. 2e, f).

Elevated CO₂ increased total nitrogenase activity across all P treatments by 28% at Week 4, but only increased total nitrogenase activity in plants supplied 25 and 50 μM P by 9 and 11%, respectively, at Week 6 (Fig. 3a, b). Nitrogenase activity was enhanced with increasing P supply from 0 to 5 μM P at Week 4 ($P < 0.001$) and from 0 to 12 μM P at Week 6 ($P < 0.001$) before it was plateaued to 50 μM P. Elevated CO₂ increased specific nitrogenase activity by 47% and 9% at Week 4 ($P < 0.001$) and Week 6 ($P = 0.002$) (Fig. 3c, d; Table 1). Specific nitrogenase activity increased with increasing P supply from 0 to 5 μM P at Week 4 and from 0 to 12 μM P at Week 6 and then plateaued at higher P supplies (Fig. 3d).

Nitrogen concentration and content

Nitrogen mobilised from the seed to plant was approximately 8 mg and was not affected by CO₂ or P treatment ($P > 0.05$), this was determined by calculating the difference between seed N content and removed cotyledon N content (data not shown). Elevated CO₂ significantly reduced shoot N concentration by 14% after 4 weeks of growth ($P < 0.001$) but was unaffected by CO₂ level after 6 weeks of growth ($P = 0.484$, Table 1). The effect of P supply on shoot N was only observed at Week 6 ($P < 0.001$, Table 1). Shoot N concentration decreased by 45% with increasing P supply from 0 to 5 μM P (Fig. 4b). Root N concentration was not affected by CO₂, P supply or their interaction at Week 4, and was only affected by

Fig. 2 Dry weight (**a**, **b**), number (**c**, **d**) and size (**e**, **f**) of nodules in white lupin grown under ambient (380 ppm) and elevated (700 ppm) CO_2 concentrations. Plants were supplied with 0, 2, 5, 12, 25 and 50 μM P, and were harvested 4

(**a**, **c**, **e**) and 6 (**b**, **d**, **f**) weeks after transplanting. Vertical bar indicates the least significant difference ($P = 0.05$) for any two means where the main effect and/or interaction are significant. Error bars indicate the standard error of the mean ($n = 3$)

P supply at Week 6 where plants supplied 0 μM P had a root P concentration 12% higher than other P supplies (Fig. 4; Table 1).

Elevated CO_2 did not significantly affect the N content of plants at Week 4 ($P = 0.201$) but increased N content by 15% by Week 6 ($P < 0.001$; Fig. 4e, f). Increasing P supply also increased N content of plants. The 50 μM P resulted in 44% greater N content than those receiving no P.

Nutrient uptake, excess cation uptake and proton release

Elevated CO_2 decreased shoot P concentration by 15% at Week 4 ($P = 0.004$) and decreased it by 3% across all P rates at Week 6 ($P = 0.049$, Table 2). The shoot P concentration increased linearly with increasing P supply from 0 to 50 μM with plants grown at 50 μM having 9 and 5 times higher shoot P concentration, compared to the 0 μM , at Weeks 4 and 6, respectively (Table 2). Root P concentration was unaffected by elevated CO_2 . Nodule P was only affected by P supply with nodule P concentrations being 3 and 5 times larger at 50 μM P than 0 μM P at Weeks 4

and 6, respectively. Plant P content was not affected by elevated CO_2 at Week 4 ($P = 0.061$). By Week 6, elevated CO_2 increased plant P content only at 25 and 50 μM P, leading to a significant $\text{CO}_2 \times \text{P}$ interaction ($P = 0.004$). Plant P content was influenced by P supply with plants supplied with 50 μM P containing 9 and 14 times more P than plants without P supply at Weeks 4 and 6, respectively (Table 2).

At Week 4, elevated CO_2 enhanced excess cation uptake by 4% irrespective of P supply ($P = 0.002$). By Week 6, elevated CO_2 interacted with P supply to increase excess cation uptake with increases only occurring in plants supplied 0 (14%) and 12 μM P (18%) ($P = 0.007$; Table 3). Elevated CO_2 also increased specific excess cation uptake by 4% at Week 4 ($P = 0.020$) but did not affect it at Week 6 ($P = 0.332$). At both Weeks 4 and 6, excess cation uptake increased with increasing P supply from 0 to 5 μM but decreased when P supply increased from 5 to 50 μM (Table 3).

Elevated CO_2 enhanced proton release by 4% and 3% at Weeks 4 and 6, respectively (Table 3). Increasing P supply decreased proton release with the highest proton release at 2 μM P at both sampling times

Fig. 3 Whole plant nitrogenase activity (a, b) and specific nitrogenase activity (c, d) of white lupin grown for 4 (a, c) and 6 (b, d) weeks under ambient (380 ppm) and elevated (700 ppm) CO₂ concentrations. Plants were supplied with 0, 2, 5,

12, 25 and 50 μM P. Vertical bar indicates the least significant difference ($P = 0.05$) for any two means. Error bars indicate the standard error of the mean ($n = 3$)

($P < 0.001$). Elevated CO₂ increased specific proton release only in plants supplied 0 and 25 μM P at Week 4. By Week 6, specific proton release was only affected P supply ($P < 0.001$) with 35% decrease in specific proton release when P supply increased from 0 to 50 μM.

Elevated CO₂ increased the molar ratio of proton release to N₂ fixation (H^+/N) by 5% at Week 4 ($P = 0.023$) but decreased it by 11% at Week 6 ($P < 0.001$). The highest H^+/N ratio was observed at 5 μM P at Week 4, and at 0 μM P at Week 6 (Table 3). Irrespective of CO₂ and P treatments, proton release and excess cation uptake were positively correlated (Fig. S1).

Critical P concentration for N₂ fixation

The critical P concentration is the concentration of P in nutrient solution, nodules or shoots needed to achieve maximum N content. Elevated CO₂ increased

the critical P concentration in nutrient solution for N₂ fixation, with plants requiring 3.7 and 6.4 μM P supply under ambient and elevated CO₂, respectively, to reach maximum N content (Fig. 5a). It also increased the critical P concentration in shoots for N₂ fixation by 17%. However, elevated CO₂ did not affect the critical P concentration in nodules, being 4.95–5.17 mg g⁻¹ to achieve maximum N content (Fig. 5b, Table S1).

Discussion

P effect on N₂ fixation

Phosphorus deficiency inhibited N₂ fixation as demonstrated by the positive relationship between P supply and plant N content (Fig. 4f). As no N was supplied to the plants, most of the N was derived from N₂ fixation. The reduction in N₂ fixation induced by

Fig. 4 The concentrations of N in shoots (a, b) and roots (c, d) and total N content (e, f) of white lupin grown under ambient (380 ppm) and elevated (700 ppm) CO₂ concentrations. Plants were supplied with 0, 2, 5, 12, 25 and 50 μM P. Plants were harvested 4 (a, c) and 6 (b, d) weeks after transplanting.

Vertical bar indicates the least significant difference ($P = 0.05$) for any two means where the main effect and/or interaction are significant. Error bars indicate the standard error of the mean ($n = 3$)

P-deficiency at Week 6 was coupled to reduced host plant growth, impaired nodule function and decreased nodule size (Figs. 1b, 2f and 3b). Phosphorus deficiency may directly inhibit nodule development and function (Tang et al. 2001) or may signal N sufficiency and lead to a reduction in plant N demand (Sulieman et al. 2013). Zhu et al. (2021) proposed that P deficiency induced excessive malate accumulation in nodules which directly impaired nodule metabolism, while Sulieman et al. (2013) suggested that P deficiency decreased host-plant growth and hence plant N demand, leading to downregulation of nitrogenase activity. Given that P deficiency increased shoot and root N concentrations, this would suggest that P deficiency reduces plant demand for N leading to downregulation in nitrogenase for white lupin.

CO₂ effect on N₂ fixation and nodulation

In the current experiment, elevated CO₂ stimulated N₂ fixation. This is also supported by a wide range of CO₂ enrichment studies. In a meta-analysis, Lam et al. (2012) demonstrated that elevated CO₂ increased

N₂ fixation by 38% which is a similar magnitude to the current experiment (44%) (Fig. 4f). Elevated CO₂ increased plant growth, which intensified plant demand for N. Therefore, N₂ fixation was enhanced to support the increased plant growth and avoid elevated CO₂-induced N deficiency. The increase in N₂ fixation under elevated CO₂ was supported by the greater specific nitrogenase activity but not increased nodulation (Figs. 2b and 3c). Increased nitrogenase activity without an increase in nodulation indicates that individual nodule activity was enhanced by elevated CO₂, which could be achieved through increased allocation of carbohydrates towards nodules (Parvin et al. 2020; Rogers et al. 2006). Elevated CO₂ was also shown to increase N₂ fixation in field pea (Butterly et al. 2015) and *Medicago truncatula* (Qiao et al. 2021) without affecting nodulation. Therefore, elevated CO₂ enhanced N₂ fixation by increasing plant growth and enhancing nitrogenase activity within nodules.

Elevated CO₂ but not P supply influenced early nodule formation. In the current study, elevated CO₂ enhanced early nodulation between 12 and 16 days after inoculation (Fig. S2). While enhanced nodulation

Table 2 The concentrations of P in shoots, roots and nodules, and whole plant P content of white lupin grown at 0, 2, 5, 12, 25 and 50 μM P for 4 and 6 weeks (Week) under ambient (380 ppm) and elevated CO_2 (700 ppm). Means ($n = 3$) followed by a common letter are not significantly different from each other (LSD, $P = 0.05$)

Week	P supply (μM)	CO ₂	Shoot P (mg g ⁻¹)	Root P (mg g ⁻¹)	Nodule P (mg g ⁻¹)	P content (mg plant ⁻¹)
4	0	Ambient	0.84fg	1.55ef	3.59f	0.77g
		Elevated	0.73g	1.34f	3.66f	0.67g
	2	Ambient	1.33e	1.77e	4.67e	1.24f
		Elevated	1.05f	1.81e	4.56e	1.18f
	5	Ambient	1.85d	2.57d	6.54d	1.67e
		Elevated	1.64d	2.38d	6.02d	1.54e
	12	Ambient	2.60c	5.17c	9.67c	2.60d
		Elevated	2.40c	5.06c	9.58c	2.72d
	25	Ambient	3.50b	11.43b	12.36b	5.26b
		Elevated	2.84bc	10.59b	12.08b	4.42c
	50	Ambient	6.38a	17.00a	14.48a	6.68a
		Elevated	5.63a	17.53a	14.88a	7.45a
Significance level						
CO ₂			<0.001	<0.001	0.073	0.063
P supply			<0.001	<0.001	<0.001	<0.001
CO ₂ × P supply			0.881	0.478	0.939	0.051
6	0	Ambient	1.14e	1.61f	2.46f	1.12h
		Elevated	0.82f	1.64ef	2.01g	1.15h
	2	Ambient	1.43d	1.86ef	2.66f	2.38g
		Elevated	1.43d	1.80ef	2.84f	2.55 fg
	5	Ambient	1.64d	2.10e	4.46e	3.65f
		Elevated	1.53d	2.06ef	4.43e	3.65f
	12	Ambient	2.20c	4.24d	8.65d	5.75e
		Elevated	2.14c	4.93d	8.22d	6.53e
	25	Ambient	2.74b	9.57c	10.97c	9.71d
		Elevated	2.66b	12.75b	11.25bc	11.67c
	50	Ambient	5.30a	18.65a	13.78ab	17.87b
		Elevated	5.20a	19.22a	13.52a	20.86a
Significance level						
CO ₂			0.049	0.241	0.404	<0.001
P supply			<0.001	<0.001	<0.001	<0.001
CO ₂ × P supply			0.066	0.547	0.448	0.004

is widely reported in plants grown under elevated CO_2 , few studies report the effect of elevated CO_2 on early nodule formation (Lam et al. 2012; Li et al. 2017). Root infection by rhizobia is driven by flavonoid exudation to attract the free-living rhizobia and to ultimately initiate infection of plant roots. Given that elevated CO_2 increased early nodulation, elevated CO_2 could have increased flavonoid exudation and aid in plant's attraction of rhizobia, inducing root infection and early nodule formation (Haase et al. 2007; Wang et al. 2018). The lack of CO_2 effect on nodule number at Weeks 4 and 6 after treatment (Fig. 2c, d) could be linked to

the final number of nodules being regulated through Autoregulation of Nodulation (Ferguson et al. 2019; Mortier et al. 2012). These results show that elevated CO_2 could aid in the attraction and recruitment of beneficial rhizobia during plant establishment even if the plants were grown in low-P conditions.

CO_2 effect on the critical P concentration for N_2 fixation

Plants grown under elevated CO_2 required additional P supply to support increased N_2 fixation.

Table 3 Excess cation uptake (eCat), specific excess cation uptake, proton (H⁺) release, specific proton release and the molar ratio of proton release to N₂ fixed (H⁺/N) of white lupin grown at 0, 2, 5, 12, 25 and 50 μM P for 4 and 6 weeks underambient (380 ppm) and elevated CO₂ (700 ppm). Means ($n = 3$) followed by a common letter are not significantly different from each other (LSD, $P = 0.05$)

Week	P supply (μM)	CO ₂	eCat uptake (mmol _c plant ⁻¹)	Specific eCat uptake (mmol _c g ⁻¹ root dry weight)	H ⁺ release (mmol plant ⁻¹)	Specific H ⁺ release (mmol g ⁻¹ root dry weight)	H ⁺ /N
4	0	Ambient	0.80d-g	3.95bc	1.06ef	5.10c-e	0.95bc
		Elevated	0.79fg	4.13ab	1.11b-d	5.80a	0.99a-c
	2	Ambient	0.90ab	3.58ef	1.19a	4.84ef	0.94bc
		Elevated	0.93a	3.69de	1.23a	5.02c-f	1.02ab
	5	Ambient	0.84cd	4.10ab	1.15b	5.57ab	1.02a-c
		Elevated	0.86bc	4.06a-c	1.14bc	5.41a-d	1.07a
	12	Ambient	0.78fg	3.86 cd	1.05f	5.16b-e	0.93bc
		Elevated	0.84c-e	4.25a	1.10cd	5.42bc	0.88cd
	25	Ambient	0.79e-g	3.45f	1.05f	4.61f	0.77d
		Elevated	0.83c-f	3.65d-f	1.09de	5.05de	0.98a-c
	50	Ambient	0.69h	3.63ef	1.00g	5.21b-e	0.92bc
		Elevated	0.77g	3.65d-f	1.05f	5.05d-e	0.90b-d
Significance level							
CO ₂			0.002	0.020	0.025	0.038	0.023
P supply			<0.001	<0.001	<0.001	<0.001	0.003
CO ₂ × P supply			0.163	0.150	0.369	0.030	0.089
6	0	Ambient	0.96e	3.07	3.13 h	9.99a	2.09a
		Elevated	1.09d	3.01	3.25g	9.06a	1.83bc
	2	Ambient	1.57bc	3.35	3.80ab	7.42b	2.07a
		Elevated	1.64bc	3.13	3.89a	7.37b	1.88b
	5	Ambient	1.88a	3.00	3.77bc	6.22bc	1.82bc
		Elevated	1.91a	3.28	3.87a	6.66bc	1.67de
	12	Ambient	1.60bc	3.13	3.57e	7.03bc	1.71 cd
		Elevated	1.88a	3.15	3.70cd	6.56bc	1.53f
	25	Ambient	1.53c	2.85	3.37f	6.27bc	1.55ef
		Elevated	1.67b	3.20	3.62de	6.96bc	1.47f
	50	Ambient	1.56bc	2.96	3.40f	6.44bc	1.57d-f
		Elevated	1.52c	2.81	3.34fg	5.79c	1.32 g
Significance level							
CO ₂			<0.001	0.332	<0.001	0.520	<0.001
P supply			<0.001	0.231	<0.001	<0.001	<0.001
CO ₂ × P supply			0.007	0.158	0.076	0.417	0.501

Elevated CO₂ doubled the critical P concentration in nutrient solution for N₂ fixation (Fig. 5a; Table 4). Because elevated CO₂ did not enhance nodulation or alter the critical P concentration in nodules, the additional P supply was allocated towards shoot biomass production rather than producing nodules. The additional P was likely utilised

to support higher photosynthetic rates and to supply nodules with additional C to enhance nitrogenase activity, and hence N₂ fixation (Liu et al. 2018). In a previous study, elevated CO₂ increased the critical P concentration for plant growth in cotton (Rogers et al. 1993). The increase in critical P concentration in nutrient solution for N₂ fixation

Fig. 5 Critical P concentrations in nutrient solution (a), nodules (b) and shoots (c) for N content of white lupin grown for 6 weeks under ambient (380 ppm) and elevated CO₂ (700 ppm). Solid and dashed lines represent the line of best fit using a two-

phase linear model for ambient and elevated CO₂ respectively. C_a and C_e represent the critical P concentration (± standard error) for maximum nitrogen fixation. See Table S1 for regression analysis summary

is likely to be larger in leguminous species which display enhanced nodulation under elevated CO₂. Therefore, to maximise the benefit of rising atmospheric CO₂ concentrations on N₂ fixation, plants will have to acquire more P. Although it is unclear if improved P acquisition mechanisms or managing soil P through cover cropping will negate the need for additional P inputs under elevated CO₂ (Hallama et al. 2019; O'Sullivan et al. 2020, 2021).

Although elevated CO₂ increased the critical P concentration in nutrient solution for N₂ fixation, it also partially alleviated the negative effect of P deficiency on N₂ fixation. In the current experiment, P deficiency reduced N₂ fixation, but elevated CO₂ counteracted this effect by stimulating plant growth, nitrogenase activity and N₂ fixation under P-limiting conditions (Fig. 4f). Furthermore, the critical P concentration in nodules for N₂ fixation was unaffected by CO₂ treatment despite enhanced N₂ fixation under elevated CO₂ (Table S1, Fig. 5). It appears that plants exposed to elevated CO₂ could fix the same amount of N₂ as that under ambient CO₂ but with lower nodule P status. To achieve higher N₂ fixation in P-deficient nodules, elevated CO₂ could favour the use of carbohydrates in the plant and carboxylates that accumulate in nodules under P deficiency to drive nitrogenase activity (Le Roux et al. 2008; Sa and Israel 1998). Elevated CO₂ partially reversed the negative

effect of P deficiency on N₂ fixation by enhancing nodule function via increased specific nitrogenase activity.

Elevated CO₂ or P deficiency did not influence N₂ fixation at 4 weeks of growth, and thus the critical P concentration could not be defined. Despite plant growth being limited by P supply, nodule P concentration was significantly higher than shoot P which enabled nodule function and N₂ fixation in the low-P environment (Table 3). For example, Shulze et al. (2006) found that N₂ fixation in white lupin was unaffected by P deficiency after 3 weeks of plant growth. However, elevated CO₂ enhanced shoot dry weight at Week 4 across all P rates but this did not result in enhanced N₂ fixation. The results indicate that whilst nodules formed during an early nodulation process could support N₂ fixation under ambient CO₂, they are unable to enhance N₂ fixation under elevated CO₂ despite increased shoot dry weight, resulting in a decrease in shoot N concentration (Haase et al. 2007; Sa and Israel 1998). This could be due to nodule dry weight and nodule size being too small, particularly when compared to nodule size at Week 6. A similar effect was observed by Sa and Israel (1998) who found that elevated CO₂ did not increase N₂ fixation of soybean after 4 weeks of growth. This indicates that elevated CO₂ is likely to affect the critical

P concentration for N_2 fixation when nodules are mature and P deficiency is intensified.

Excess cation uptake and proton release

Elevated CO_2 promoted excess cation uptake, resulting in enhanced proton release (Table 2, Fig. S1), which could aid in acquiring non-labile P forms when plants are grown in P-deficient soils. Legumes that rely on N_2 fixation for N take up more cations than anions, which results in net proton release into the rhizosphere (Tang et al. 1999). Phosphorus deficiency also leads to rhizosphere acidification to mobilise non-labile P forms (Pueyo et al. 2021). Elevated CO_2 might promote excess cation uptake due to enhanced biomass production of N_2 -fixing species, favouring the uptake of cations over anions (McLay et al. 1997). It also increased specific proton release at Week 4 under low P supply, which would intensify rhizosphere acidification, contributing to mobilisation of non-labile P. Specific excess cation uptake and specific proton release are closely related, indicating that excess cation uptake contributed to the enhanced acidification. However, elevated CO_2 can also enhance exudation of organic acid anions under low P, which could further promote proton release (O'Sullivan et al. 2021). The increase in rhizosphere acidification under P deficiency may aid in acquiring non-labile P which could contribute to the increased P demands needed for N_2 fixation under elevated CO_2 .

Conclusions

Elevated CO_2 increased N_2 fixation rates whereas P deficiency decreased it. Under elevated CO_2 , plants require greater P supply to support enhanced N_2 fixation. To maximise the effect of elevated CO_2 on enhancing N_2 fixation, plants may have to acquire more P than what is currently required under ambient CO_2 concentrations. Therefore, as atmospheric CO_2 concentrations rise, there will be greater emphasis on managing soil P levels. Enhanced N_2 fixation of P-deficient plants under elevated CO_2 was achieved through promoting specific nitrogenase activity and not increasing nodule mass. Elevated CO_2 also promoted excess cation uptake and proton release, which would promote rhizosphere acidification and mobilisation

of non-labile P in the soil when plants are grown in P-deficient soils. Future research should examine the underlying physiological mechanism of enhanced N_2 fixation of P-deficient plants under elevated CO_2 .

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Declarations

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References

- Ainsworth EA, Long SP (2005) What have we learned from 15 years of free-air CO_2 enrichment (FACE)? A meta-analytic review of the responses of photosynthesis, canopy properties and plant production to rising CO_2 . *New Phytol* 165:351–371. <https://doi.org/10.1111/j.1469-8137.2004.01224.x>

- Almeida JPF, Hartwig UA, Frehner M, Nosberger J, Lüscher A (2000) Evidence that P deficiency induces N feedback regulation of symbiotic N₂ fixation in white clover (*Trifolium repens* L.). J Exp Bot 51:1289–1297. <https://doi.org/10.1093/jexbot/51.348.1289>
- Andersson KO, Tighe MK, Guppy CN, Milham PJ, McLaren TI (2015) Incremental acidification reveals phosphorus release dynamics in alkaline vertic soils. Geoderma 259–260:35–44. <https://doi.org/10.1016/j.geoderma.2015.05.001>
- Aranjuelo I, Cabrerizo PM, Aparicio-Tejo PM, Arrese-Igor C (2014) Unravelling the mechanisms that improve photosynthetic performance of N₂-fixing pea plants exposed to elevated [CO₂]. Environ Exp Bot 99:167–174. <https://doi.org/10.1016/j.envexpbot.2013.10.020>
- Barrow NJ (2017) The effects of pH on phosphate uptake from the soil. Plant Soil 410:401–410. <https://doi.org/10.1007/s11104-016-3008-9>
- Braum SM, Helmke PA (1995) White lupin utilizes soil phosphorus that is unavailable to soybean. Plant Soil 176:95–100. <https://doi.org/10.1007/BF00017679>
- Butterly CR, Tang C, Armstrong R, Chen D (2015) Free-air CO₂ enrichment (FACE) reduces the inhibitory effect of soil nitrate on N₂ fixation of *Pisum sativum*. Ann Bot 117:177–185. <https://doi.org/10.1093/aob/mcv140>
- Chen H, Markham J (2021) The interactive effect of elevated CO₂ and herbivores on the nitrogen-fixing plant *Alnus incana* ssp. *rugosa*. Plants 10:440. <https://doi.org/10.3390/plants10030440>
- Ferguson BJ, Mens C, Hastwell AH, Zhang M, Su H, Jones CH, Chu X, Gresshoff PM (2019) Legume nodulation: The host controls the party. Plant Cell Environ 42:41–51. <https://doi.org/10.1111/pce.13348>
- Haase S, Neumann G, Kania A, Kuzyakov Y, Römhild V, Kandeler E (2007) Elevation of atmospheric CO₂ and N-nutritional status modify nodulation, nodule-carbon supply, and root exudation of *Phaseolus vulgaris* L. Soil Biol Biochem 39:2208–2221. <https://doi.org/10.1016/j.soilbio.2007.03.014>
- Hallama M, Pekrun C, Lambers H, Kandeler E (2019) Hidden miners – the roles of cover crops and soil microorganisms in phosphorus cycling through agroecosystems. Plant Soil 434:7–45. <https://doi.org/10.1007/s11104-018-3810-7>
- Hinsinger P, Plassard C, Tang C, Jaillard B (2003) Origins of root-mediated pH changes in the rhizosphere and their responses to environmental constraints: A review. Plant Soil 248:43–59. <https://doi.org/10.1023/A:1022371130939>
- IPCC (2021) Climate Change 2021: The physical science basis. Contribution of working group I to the sixth assessment report of the Intergovernmental Panel on Climate
- Jiang M, Caldararu S, Zhang H, Fleischer K, Crous KY, Yang J, De Kauwe MG, Ellsworth DS, Reich PB, Tissue DT, Zaehle S, Medlyn BE (2020) Low phosphorus supply constrains plant responses to elevated CO₂: A meta-analysis. Global Change Biol 26:5856–5873. <https://doi.org/10.1111/gcb.15277>
- Jin J, Tang C, Armstrong R, Sale P (2012) Phosphorus supply enhances the response of legumes to elevated CO₂ (FACE) in a phosphorus-deficient vertisol. Plant Soil 358:91–104. <https://doi.org/10.1007/s11104-012-1270-z>
- Lam SK, Chen D, Norton R, Armstrong R, Mosier AR (2012) Nitrogen dynamics in grain crop and legume pasture systems under elevated atmospheric carbon dioxide concentration: A meta-analysis. Global Change Biol 18:2853–2859. <https://doi.org/10.1111/j.1365-2486.2012.02758.x>
- Lambers H, Shane MW, Cramer MD, Pearse SJ, Veneklaas EJ (2006) Root structure and functioning for efficient acquisition of phosphorus: Matching morphological and physiological traits. Ann Bot 98:693–713. <https://doi.org/10.1093/aob/mcl114>
- Le Roux MR, Khan S, Valentine AJ (2008) Organic acid accumulation may inhibit N₂ fixation in phosphorus-stressed lupin nodules. New Phytol 177:956–964. <https://doi.org/10.1111/j.1469-8137.2007.02305.x>
- Li Y, Yu Z, Liu X, Mathesius U, Wang G, Tang C, Wu J, Liu J, Zhang S, Jin J (2017) Elevated CO₂ increases nitrogen fixation at the reproductive phase contributing to various yield responses of soybean cultivars. Front Plant Sci 8. <https://doi.org/10.3389/fpls.2017.01546>
- Liu A, Contador CA, Fan K, Lam H-M (2018) Interaction and regulation of carbon, nitrogen, and phosphorus metabolisms in root nodules of legumes. Front Plant Sci 9. <https://doi.org/10.3389/fpls.2018.01860>
- Luo D, Ganesh S, Koolaard J (2020) predictmeans: Calculate predicted means for linear models. R package version 1.0.4 <https://CRAN.R-project.org/package=predictmeans>
- McLay CDA, Barton L, Tang C (1997) Acidification potential of ten grain legume species grown in nutrient solution. Aust J Agric Res 48:1025–1032. <https://doi.org/10.1071/A96174>
- Mortier V, Holsters M, Goormachtig S (2012) Never too many? How legumes control nodule numbers. Plant Cell Environ 35:245. <https://doi.org/10.1111/j.1365-3040.2011.02406.x>
- Neumann G, Massonneau A, Langlade N, Dinkelaker B, Hengeler C, Römhild V, Martinoia E (2000) Physiological aspects of cluster root function and development in phosphorus-deficient white lupin (*Lupinus albus* L.). Ann Bot 85:909–919. <https://doi.org/10.1006/anbo.2000.1135>
- Nguyen NT, Mohapatra PK, Fujita K (2006) Elevated CO₂ alleviates the effects of low P on the growth of N₂-fixing *Acacia auriculiformis* and *Acacia mangium*. Plant Soil 285:369–379
- O'Sullivan JB, Jin J, Tang C (2020) Elevated CO₂ promotes the acquisition of phosphorus in crop species differing in physiological phosphorus-acquiring mechanisms. Plant Soil 455:397–408. <https://doi.org/10.1007/s11104-020-04698-8>
- O'Sullivan JB, Plozza T, Stefanelli D, Jin J, Tang C (2021) Elevated CO₂ and phosphorus deficiency interactively enhance root exudation in *Lupinus albus* L. Plant Soil 465:229–243. <https://doi.org/10.1007/s11104-021-04991-0>
- Qiao Y, Miao S, Jin J, Mathesius U, Tang C (2021) Differential responses of the *sun4* and *rdn1-1* super-nodulation mutants of *Medicago truncatula* to elevated atmospheric CO₂. Ann Bot 128:441–452. <https://doi.org/10.1093/aob/mcab098>
- Palit P, Ghosh R, Tolani P, Tarafdar A, Chitkineni A, Bajaj P, Sharma M, Kudapa H, Varshney RK (2020) Molecular and physiological alterations in chickpea under elevated

- CO₂ concentrations. *Plant Cell Physiol* 61:1449–1463. <https://doi.org/10.1093/pcp/pcaa077>
- Pang J, Tibbett M, Denton MD, Lambers H, Siddique KHM, Ryan MH (2011) Soil phosphorus supply affects nodulation and N: P ratio in 11 perennial legume seedlings. *Crop Pasture Sci* 62:992–1001. <https://doi.org/10.1071/CP11229>
- Parvin S, Uddin S, Tausz-Posch S, Armstrong R, Tausz M (2020) Carbon sink strength of nodules but not other organs modulates photosynthesis of faba bean (*Vicia faba*) grown under elevated [CO₂] and different water supply. *New Phytol* 227:132–145. <https://doi.org/10.1111/nph.16520>
- Pinheiro J, Bates D, DebRoy S, Sarkar D, Core Team R (2021) nlme: Linear and nonlinear mixed effects models. R package version 3.1-152 <https://CRAN.R-project.org/package=nlme>
- Pueyo JJ, Quiñones MA, Coba de la Peña T, Fedorova EE, Lucas MM (2021) Nitrogen and phosphorus interplay in lupin root nodules and cluster roots. *Front Plant Sci* 12. <https://doi.org/10.3389/fpls.2021.644218>
- R Core Team (2021) R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna. <https://www.R-project.org/>
- Rogers A, Gibon Y, Stitt M, Morgan PB, Bernacchi CJ, Ort DR, Long SP (2006) Increased C availability at elevated carbon dioxide concentration improves N assimilation in a legume. *Plant Cell Environ* 29:1651–1658. <https://doi.org/10.1111/j.1365-3040.2006.01549.x>
- Rogers GS, Payne L, Milham P, Conroy J (1993) Nitrogen and phosphorus requirements of cotton and wheat under changing atmospheric CO₂ concentrations. *Plant Soil* 155:231–234. <https://doi.org/10.1007/bf00025026>
- Sa TN, Israel DW (1998) Phosphorus-deficiency effects on response of symbiotic N₂ fixation and carbohydrate status in soybean to atmospheric CO₂ enrichment. *J Plant Nutr* 21:2207–2218. <https://doi.org/10.1080/01904169809365555>
- Sachs JL, Quides KW, Wendlandt CE (2018) Legumes versus rhizobia: a model for ongoing conflict in symbiosis. *New Phytol* 219:1199–1206. <https://doi.org/10.1111/nph.15222>
- Sas L, Rengel Z, Tang C (2001) Excess cation uptake, and extrusion of protons and organic acid anions by *Lupinus albus* under phosphorus deficiency. *Plant Sci* 160:1191–1198. [https://doi.org/10.1016/S0168-9452\(01\)00373-9](https://doi.org/10.1016/S0168-9452(01)00373-9)
- Schulze J, Temple G, Temple SJ, Beschow H, Vance CP (2006) Nitrogen fixation by white lupin under phosphorus deficiency. *Ann Bot* 98:731–740. <https://doi.org/10.1093/aob/mcl154>
- Suliman S, Ha CV, Schulze J, Tran L-SP (2013) Growth and nodulation of symbiotic *Medicago truncatula* at different levels of phosphorus availability. *J Exp Bot* 64:2701–2712. <https://doi.org/10.1093/jxb/ert122>
- Tang C, Unkovich MJ, Bowden JW (1999) Factors affecting soil acidification under legumes. III. Acid production by N₂-fixing legumes as influenced by nitrate supply. *New Phytol* 143:513–521. <https://doi.org/10.1046/j.1469-8137.1999.00475.x>
- Tang C, Robson AD (1993) pH above 6.0 reduces nodulation in *Lupinus* species. *Plant Soil* 152:269–276. <https://doi.org/10.1007/BF00029097>
- Tang C, Yu Q (1999) Impact of chemical composition of legume residues and initial soil pH on pH change of a soil after residue incorporation. *Plant Soil* 215:29–38. <https://doi.org/10.1023/A:1004704018912>
- Tang C, Hinsinger P, Drevon JJ, Jaillard B (2001) Phosphorus deficiency impairs early nodule functioning and enhances proton release in roots of *Medicago truncatula* L. *Ann Bot* 88:131–138. <https://doi.org/10.1006/anbo.2001.1440>
- Tobita H, Uemura A, Kitao M, Kitaoka S, Utsugi H (2010) Interactive effects of elevated CO₂, phosphorus deficiency, and soil drought on nodulation and nitrogenase activity in *Alnus hirsuta* and *Alnus maximowiczii*. *Symbiosis* 50:59–69. <https://doi.org/10.1007/s13199-009-0037-7>
- Wang Q, Liu J, Zhu H (2018) Genetic and molecular mechanisms underlying symbiotic specificity in legume-rhizobium interactions. *Front Plant Sci* 9. <https://doi.org/10.3389/fpls.2018.00313>
- Wang X, Ding W, Lambers H (2019) Nodulation promotes cluster-root formation in *Lupinus albus* under low phosphorus conditions. *Plant Soil* 439:233–242. <https://doi.org/10.1007/s11104-018-3638-1>
- Zhu S, Chen Z, Xie B, Guo Q, Chen M, Liang C, Bai Z, Wang X, Wang H, Liao H, Tian J (2021) A phosphate starvation responsive *malate dehydrogenase*, *GmMDH12* mediates malate synthesis and nodule size in soybean (*Glycine max*). *Environ Exp Bot* 189:104560. <https://doi.org/10.1016/j.envexpbot.2021.104560>

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