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REGULAR ARTICLE

Is pH the key reason why some *Lupinus* species are sensitive to calcareous soil?

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Abstract
Aims Previous studies have shown that pH, rather than calcium (Ca), is the main reason why some *Lupinus* species are sensitive to nutrient solutions mimicking calcareous soils; however, a hydroponic system is quite different from soil systems, and plants may respond differently to these two growing conditions. Thus, studies with *Lupinus* species grown in calcareous soils are needed.
Methods Two calcicole and two calcifuge species were grown in river sand with different Ca forms and amounts, pH levels, and [bicarbonate (HCO_3^-)] (HCO_3^- concentration, which is produced by calcium carbonate ($CaCO_3$)). Leaf symptoms, leaf area, gas exchange, biomass, and root morphology were recorded; whole leaf and root nutrient concentrations were analysed.
Results We observed leaf chlorosis of the youngest leaves under high pH (adjusted by KOH) and high pH + high Ca (representing high [HCO_3^-], high pH and high Ca) treatments for all *Lupinus* species. However, after 2 weeks, leaf chlorosis of all *Lupinus* species under high pH started to disappear, with calcicole species fully, and calcifuge species only partly recovering. Leaf chlorosis symptoms of calcicole species under high pH + high Ca partly disappeared as well, while those of calcifuge species did not disappear at all.
Conclusions High pH (resulting from either KOH or HCO_3^-) inhibited root growth, and subsequently uptake of some nutrients and shoot growth of *Lupinus* species. However, the strong buffering capacity of HCO_3^- is the key factor determining if *Lupinus* species can survive in calcareous soils. Among all studied *Lupinus* species, *L. pilosus* was the most tolerant to high [HCO_3^-] and/

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or high pH, followed by *L. cosentinii* and *L. angustifolius*, while *L. hispanicus* was the most sensitive.

Keywords Calcicole · Calcifuge · High pH · High bicarbonate concentration · *Lupinus*

Introduction

Calcareous soils are alkaline and contain free $CaCO_3$, and high concentrations of, calcium (Ca) and bicarbonate (HCO_3^-). The availability of phosphorus (P) and some micronutrients, including iron (Fe), zinc (Zn), manganese (Mn) and copper (Cu) in calcareous soils is very low (Tyler 2003). Most *Lupinus* species grow poorly on calcareous soils, and this has been attributed to various factors, including Ca toxicity, sensitivity to high [HCO_3^-] (HCO_3^- concentration) and high pH and Fe deficiency, and poor nodulation (Abd-Alla 1999; Ding et al. 2018b; Jessop et al. 1990; Kerley 2000; Tang et al. 1995b; Tang and Thomson 1996; White 1990).

Calcium toxicity has been considered one of the major causes for why some *Lupinus* species are sensitive to calcareous soils (De Silva and Mansfield 1994; Jessop et al. 1990; Kerley et al. 2001). For example, a high Ca supply may disturb the lamellar structure of chloroplasts, and consequently negatively affect net photosynthetic rates (Chevalier and Paris 1980; Chevalier and Paris-Pireyre 1984; De Silva et al. 1994). Excessive Ca may precipitate with P as $Ca_3(PO_4)_2$ in plant tissues which makes both Ca and P unavailable (Ding et al. 2018b; McLaughlin and Wimmer 1999; Zohlen and Tyler 2004). We found that Ca-tolerant *Lupinus* species tended to have tight control over Ca uptake and/or translocation from roots to leaves, or better Ca compartmentation at the cellular level (Ding et al. 2018a). These abilities likely play an important role in the tolerance of some *Lupinus* species to calcareous soils, as suggested for other calcicole species (He et al. 2014; Jefferies and Willis 1964; Raza et al. 2000; Valentinuzzi et al. 2015; Webb 1999; Wu et al. 2011).

In addition, high [HCO_3^-] and high pH in calcareous soils are considered the main factors affecting the sensitivity of some *Lupinus* species to calcareous soils (Coulombe et al. 1984; Mengel et al. 1984; Romera et al. 1992; Tang and Thomson 1996; Waters and Troupe 2012). For example, a high rhizosphere pH caused by a high [HCO_3^-] in calcareous soils reduces nutrient availability, especially that of P, Fe, Mn, Zn, Cu and boron (B) for root uptake, and thus limits plant growth (George et al. 2012; Neumann and Römheld 2012; Parker et al. 1999; Tyler and Ström 1995; Yue Ao et al. 1987). In addition, a high [HCO_3^-] and high pH can also inhibit root growth of some *Lupinus* species (Peiter et al. 2001). Tang and Thomson (1996) found that root elongation of *L. angustifolius* is inhibited by high [HCO_3^-] and high pH in nutrient solution, and this was even observed in nutrient solution with pH $\geq$ 6 (Tang et al. 1992). Our comparative study showed that there is a correlation between the decreased lateral root growth and nutrient availability of some *Lupinus* species grown in nutrient solution with high [HCO_3^-] and/or high pH (Ding et al., personal observations).

In a series of hydroponic experiments, we found that a high Ca supply caused P deficiency in some *Lupinus* species, but its effects on the growth, especially the leaf symptoms of *Lupinus* species were inconsistent with what has been reported for those grown in calcareous soils (Ding et al. 2018b). In contrast, the effects of high pH (buffered by MES/TES or caused by high [HCO_3^-]) on different *Lupinus* species are generally consistent with those of plants grown in calcareous soils, for example, leaf chlorosis were developed in the calcifuge *Lupinus* species under high pH (Ding et al., personal observations). Considering the results of additional studies (Kerley and Huyghe 2002; White and Robson 1990), we concluded that pH is the main reason why some *Lupinus* species are sensitive to nutrient solutions mimicking calcareous soils. However, a hydroponic system is quite different from soil systems, and plants may respond differently to these two growing conditions. Therefore, in this study, we aimed to test if pH is the main factor determining why some *Lupinus* species are sensitive to calcareous soils, by growing different *Lupinus* species in soils with different forms of Ca, pH levels, and [HCO_3^-]. We compared leaf symptoms, root nodulation, root and shoot biomass, and root and shoot nutrient status. We hypothesised that pH, rather than Ca, is the main reason why *Lupinus* species respond differently to calcareous soils. In addition, we hypothesised that root surface area, root length, and fine root growth of calcifuge species will be inhibited significantly in calcareous soils, while those of calcicole species will be slightly inhibited or not inhibited at all.

Materials and methods

Plant growth

Four *Lupinus* species were selected (two calcifuge species: *L. angustifolius* L. cv. Mandelup and *L. hispanicus* ssp. *bicolor* Boiss. and Reut. P22999; two calcicole species: *L. pilosus* Murr. P27440 and *L. cosentinii* Guss. P27225) (Table 1). All seeds were obtained from the Australian Lupin Collection (Department of Primary Industries and Regional Development, WA, Australia). Except for *L. angustifolius*, seeds were scarified. Seeds were then sterilised in 5% (*v*/*v*) sodium hypochlorite for 20 min and rinsed thoroughly with deionised (DI) water. All seeds were pre-germinated in sterilised river sand for 4 days, and then three or four seeds were inoculated with Group G® (*Bradyrhizobium* sp. (*Lupinus*) WU425), which is effective for the inoculation of *Lupinus* species (Tang and Robson 1993), sown in prepared pots, and then later thinned to one plant. All sands were washed, sterilised and then dried first. After drying, $CaCO_3$ or $CaSO_4$ was mixed evenly with sand, as shown in Table 2. Each pot was then filled with 1.2 kg dry river sand.

All essential nutrients other than Ca were provided as: 189.5 mg kg^{-1} $Ca(NO_3)_2.4H_2O$, 28.6 mg kg^{-1} NH_4Cl, 65.9 mg kg^{-1} KH_2PO_4, 101.2 mg kg^{-1} $MgSO_4.7H_2O$, 12.3 mg kg^{-1} $MnSO_4.H_2O$, 8.8 mg kg^{-1} $ZnSO_4.7H_2O$, 0.7 mg kg^{-1} H_3BO_3, 1.0 mg kg^{-1} Na_2MoO_4, 2.0 mg kg^{-1} $CuSO_4.5H_2O$ and 32.9 mg kg^{-1} FeNaEDTA. This nutrient composition has been used in our lab and proved to be suitable for the growth of legumes (Pang et al. 2010, 2011). The N was 80 mg kg^{-1}, and this was to supply some N for young plants; when they grow bigger, they will need to rely on biological N_2 fixation. Besides, and the amount of $Ca(NO_3)_2$ added to all the treatments was very small compared with the amount of $CaSO_4$ or $CaCO_3$ added, so it hardly affected the experiment design. All these nutrients were added as a nutrient solution with DI water, and KOH was used to adjust solution pH to 6 and 8 according to different treatments shown in Table 2. K_2SO_4 was used to balance [K], and the final [K] was 80 mg kg^{-1} for all the treatments, the final [S] was 133.4 mg kg^{-1} for control and pH 6, the final [S] was 40 mg kg^{-1} for high Ca and high pH + high Ca treatment. After adding basic nutrition solution for 1 week, all pots were ready to use, and sands from three plain pots of each treatment were air-dried and passed through a 2-mm sieve to measure the actual pH (10 g dry sand in 50 ml $CaCl_2$ as shown in Table 2) and exchangeable Ca^{2+} of each treatment. Exchangeable Ca^{2+} was then extracted by 1 M ammonium acetate at pH 7 (Rayment and Lyons 2011) and analysed by inductively coupled plasma optical emission spectrometry (ICP-OES; School of Agriculture and Environment, University of Western Australia, Perth, Australia).

The experiment was run in a glasshouse (20 °C day/15 °C night). All pots were watered with DI water to weight, three times per week, to 75% field capacity.

Gas exchange and chlorophyll fluorescence measurements

The day before harvest, the youngest fully-expanded leaves were chosen to measure net photosynthesis rate (A_{max}), stomatal conductance (g_s) and intercellular carbon dioxide concentration (C_i) with a LI-6400 portable gas exchange system (Li-Cor, Lincoln, NE, USA) at 1500 μmol quanta m^{-2} s^{-1}. The same leaf used to measure photosynthesis was dark-adjusted in a dark room for 1 h, and then the maximum photochemical quantum yield of PSII (F_v/F_m) was measured using a LI-6400 portable gas exchange system as described above.

Table 1 *Lupinus* species and genotypes used in this study, and the collection site soil pH

Species	Genotype	Breeding status	Collection site soil pH	Country of origin	Calcicole/calcifuge
L. angustifolius	Mandelup	Cultivar	unknown	Australia	Calcifuge
L. hispanicus ssp. *bicolor*	P22999	Naturalised	5.7	Portugal	Calcifuge
L. pilosus	P27440	Naturalised	9.0	Syria	Calcicole
L. cosentinii	P27225	Naturalised	9.0	Morocco	Calcicole

Calcicole or calcifuge was classified according to the literature (Gladstones 1970; Tang et al. 1995b; White 1990). "naturalised" means seeds of this species were collected from the nature where they originally grow

Table 2 Calcium (Ca), exchangeable Ca^{2+}, and pH of the different treatments used in the experiment

Treatment	$CaSO_4$ (g kg^{-1})	$CaCO_3$ (g kg^{-1})	Bicarbonate concentration	Exchangeable Ca^{2+} (g kg^{-1})	pH (0.01 M $CaCl_2$)	pH (0.2 mM $CaCl_2$)
Control	–	–	–	0.06	6–6.2	6.4–6.7
High Ca	20	–	–	4.8	6–6.2	6.4–6.7
High pH	–	6	Low	1.9	8–8.2	8.6–9.0
High pH + High Ca	–	50	High	3.4	8–8.2	8.6–9.0

The relative high or low concentration of bicarbonate was decided based on the amount of $CaCO_3$ added

Plant harvest and carboxylate extraction

All the pots were watered before harvest to make sure the roots were easily removed and the root damage was minimal. The intact plants were removed from the pots, and the bulk sand around the roots was removed gently. The roots were then placed into a 100- or 150-ml beaker, and a certain amount of $CaCl_2$ (0.2 mM), ranging from 25 to 100 ml, depending on root size, was added to the beaker to remove the rhizosphere sand. After removing the roots, the beaker was shaken by hand, and the pH of the extract was measured by a portable pH meter (pH 300/310, Eutech Instruments Pte Ltd., Singapore). A subsample of the extract was filtered through a 0.2 μm syringe filter into a 1-ml HPLC vial and acidified with one drop of concentrated phosphoric acid. The sample was then stored at −20 °C until HPLC analysis. The whole process for each plant was about 5 min.

Nodulation was assessed according to the British Columbia Ministry of Forests field guide (British Columbia Ministry of Forests 1991), where a ranking of 0–5 is given to each of five categories, including plant growth and vigour (1 for very chlorotic plants, 2 for slightly chlorotic plants, 3 for green and relatively small plants and 5 for green and vigorous plants); nodule number (0 for no nodules, 2 for few nodules (<5) with slight pigmentation, 3 for few nodules (<5) with pink pigmentation, 3 also for many nodules (>50) mainly white or smaller, 5 for many nodules (>50) with pink pigmentation, 5 for 5–50 nodules as well); nodule position (1 for nodules on lateral roots only, 3 for nodules on both crown and lateral roots, 5 for nodules predominantly on the crown); nodule colour (0 for white or green colour, 2 for some pink or whitish with green areas, 5 for predominantly pink); and nodule appearance (0 for ineffective, 3 for intermediate, 5 for effective). A total score was summed to evaluate the effectiveness of nodulation, and a score between 20 and 25 is considered very effective, a score between 15 and 20 less effective, while a score between 0 and 14 is considered unsatisfactory nodulation.

The whole roots were then thoroughly washed to remove the remaining sand. After that, plants were separated into mature leaves, immature leaves, stems (including petioles), and roots. Leaf area was measured and fresh biomass was recorded. Leaf symptoms were recorded by taking photos prior to harvest. Roots of all plants were scanned by an Epson Perfection V700 Photo Scanner and analysed by WinRHIZO 2009a, b (Regents Instruments Canada lnc, Quebec City, Quebec, QC, Canada). After that, all the plant parts were dried in an oven at 70 °C for 7 days, and dry biomass was recorded.

Carboxylate analysis

The pre-frozen extracts were brought back to room temperature, and a 100-μl sample of each extract was analysed by HPLC with a 600E pump, 717 plus autosampler and a 996 photodiode array detector (Waters, Milford, MA, USA). A reversed-phase column liquid chromatography (RPLC) was used to separate and quantify organic acids as described by Cawthray (2003). The carboxylic acid working standards included acetic, citric, *cis*-aconitic, fumaric, lactic, malic, malonic, maleic, succinic and *trans*-aconitic acids.

Determination of whole leaf and root nutrient concentrations

To determine whole leaf and root nutrient concentrations, all the dry mature leaves and roots were ground to a fine powder with a GenoGrinder (SPEX SamplePrep

LLC, Metuchen, New Jersey, USA), and 0.1 g of ground material was digested with concentrated HNO_3 and $HClO_4$ (3:1) and analysed by inductively coupled plasma optical emission spectrometry (ICP-OES). Nitrogen was determined by a CNS analyser (Elementar Vario Macro Elemental Analyzer, Elementar Analysensytem GmbH, Hanau, Germany).

Statistics

Data and statistical analyses were performed with the R software platform (R Core Team 2017). As data of *L. hispanicus* grown under high pH + high Ca treatment were not included, data structure of the whole experiment was unbalanced. General linear mixed-effect models were used to test the differences in whole leaf and root nutrient concentrations, root surface area, average root diameter, total root length, root nodulation, carboxylates, leaf area, shoot biomass, root biomass and total biomass among different treatments within each species. We tested the differences of gas exchange parameters and chlorophyll fluorescence with general linear mixed-effect models among different treatments within each species; individual plant was considered as the random effect.

The residuals of each model were inspected for heteroscedasticity, and in the presence of heteroscedasticity, models with different variance structures were compared, and a suitable variance structure significantly improved the model were specified based on Akaike Information Criterion (AIC) (Burnham and Anderson 2003; Zuur et al. 2009). The effects package was used to determine the mean values and 95% confidence intervals (Cl) (Fox 2003). Significant differences were defined based on Tukey's post-hoc analysis ($P < 0.05$).

Results

Leaf symptoms

Leaf chlorosis was observed in the youngest leaves under high pH and high pH + high Ca for all *Lupinus* species from the appearance of seedlings. However, after 2 weeks, the leaf chlorosis symptoms of all *Lupinus* species under high pH started to disappear, with calcicole species fully recovering, and calcifuge species partly recovering (Fig. 1). The leaf chlorosis symptoms of calcicole species under high pH + high Ca partly decreased as well, while those of calcifuge species did not decline at all (Fig. 1).

Leaf nutrients

Compared with control plants, leaf N concentrations of *L. hispanicus* under either high Ca or high pH, and those of *L. pilosus* and *L. cosentinii* under high pH and high pH + high Ca were significantly lower. However, leaf N concentration of *L. angustifolius* under high Ca was the lowest among all treatments, and there were no significant differences among the other three treatments (Fig. 2a).

Leaf P concentrations of *L. angustifolius* and *L. pilosus* under high Ca, high pH, and high pH + high Ca treatments and those of *L. hispanicus* under either high Ca or high pH were significantly lower than those of control plants. For *L. cosentinii*, only the leaf P concentration under the high pH + high Ca treatment was significantly lower than that under other treatments (Fig. 2b).

Leaf Ca concentrations of all *Lupinus* species under high Ca, high pH, and high pH + high Ca treatments were significantly higher than those of control plants. There were no significant differences for leaf Ca concentrations of *L. angustifolius*, *L. cosentinii*, and *L. pilosus* between these three treatments. However, the leaf Ca concentration of *L. hispanicus* under high pH was significantly lower than that under high Ca (Fig. 2c).

Leaf Mg concentrations of *L. angustifolius* and *L. pilosus* were significantly lower under high pH and high pH + high Ca treatments than those of control plants and plants of high Ca treatment. However, leaf Mg concentrations of *L. hispanicus* under high Ca were the lowest among all the treatments. There was no significant difference for *L. cosentinii* between different treatments (Fig. 2d).

Leaf Mn and Fe concentrations of all *Lupinus* species were significantly lower under high pH and/or high pH + high Ca treatments than those of control plants and plants of high Ca treatment (Fig 2e and f).

Leaf potassium (K), sulfur (S), zinc and copper concentration are shown in Fig. S1.

Root nutrients

Compared with control plants, root N concentrations of *L. angustifolius*, *L. hispanicus* and *L. pilosus* at high Ca supply were significantly lower. Root N concentrations

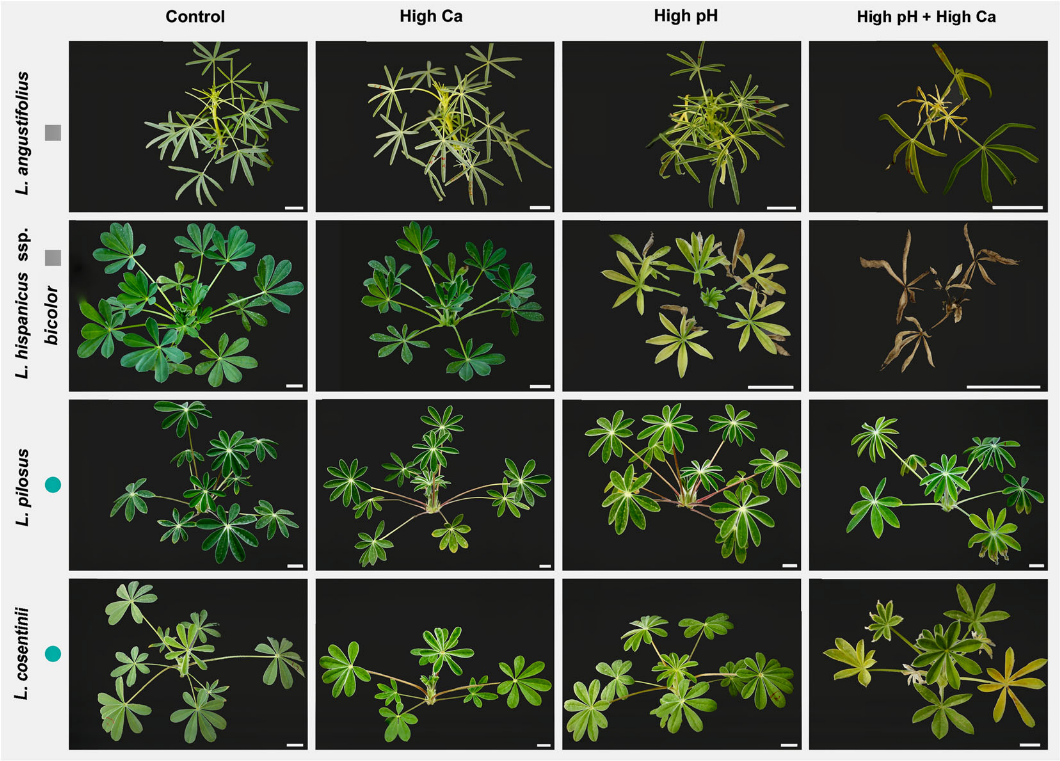

Fig. 1 Leaf symptoms of four *Lupinus* species 1 day prior to harvest when grown under different pH and calcium (Ca) treatments. Scale bar at the bottom of each photo is 1 cm. ■ marks calcifuge species, ● marks calcicole species

of *L. hispanicus*, *L. pilosus* and *L. cosentinii* under high pH and high pH + high Ca treatments were significantly lower than those of control plants (Fig. 3a).

Root P concentration of *L. hispanicus* under high Ca supply was the lowest among all treatments, whereas the P concentration of *L. pilosus* under high pH was the lowest among all treatments. There were no significant differences for the root P concentrations of *L. angustifolius* and *L. cosentinii* among all treatments (Fig. 3b).

Compared with control plants, root Ca concentrations of all *Lupinus* species in the treatments were significantly higher, except for *L. hispanicus* under a high Ca supply. There were no significant differences for either *L. angustifolius* or *L. pilosus* between these three treatments. However, the root Ca concentrations of *L. cosentinii* under high pH + high Ca treatment was the highest among all treatments (Fig. 3c).

Compared with control plants, root Mg concentrations of all *Lupinus* species at high pH and/or high pH + high Ca treatments were significantly higher. The root Mg concentration of *L. angustifolius* under high Ca supply was also significantly higher than that of control plants. However, the root Mg concentration of *L. cosentinii* under high Ca supply was significantly lower than that of the control. There were no significant differences in root Mg concentrations of *L. hispanicus* and *L. pilosus* between control and the high-Ca treatments (Fig. 3d).

Root Mn concentration of *L. angustifolius* at a high Ca supply was the highest among all treatments, while that of *L. cosentinii* under a high Ca supply was the lowest among all treatments. Root Mn concentrations of *L. hispanicus* under high pH were significantly higher than those of the control and the high-Ca treatments, whereas root Mn concentrations of *L. pilosus* under high pH and high pH + high Ca treatments were significantly lower than those of the control and the high-Ca treatments (Fig. 3e).

Root Fe concentrations of *L. angustifolius* and *L. cosentinii* under high pH and high pH + high Ca treatments were significantly lower than those of the control and plants of the high-Ca treatments. Root Fe concentration of *L. hispanicus* under high pH was significantly lower than that of the control. Root Fe

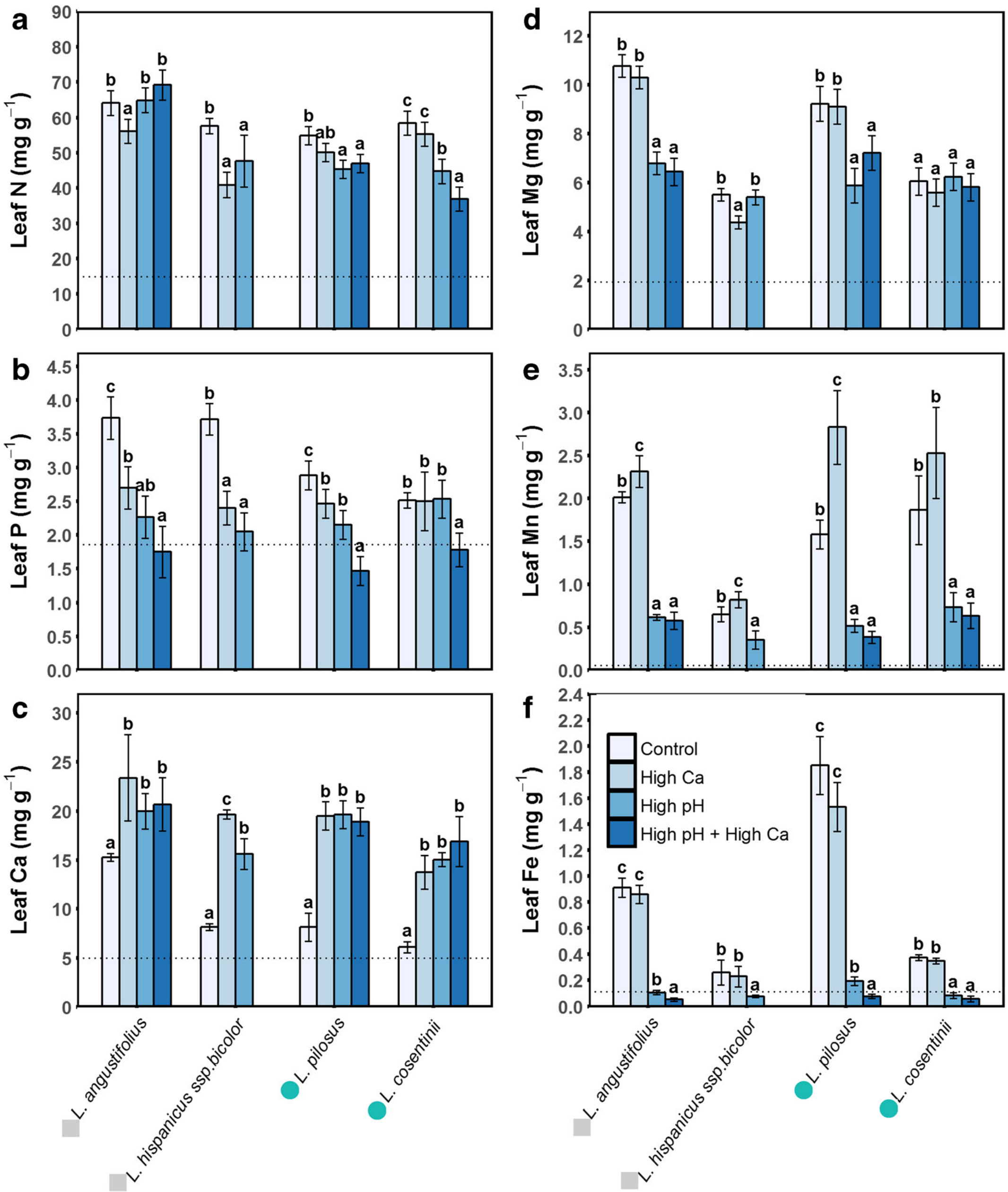

Fig. 2 Concentrations of a range of nutrients in leaves of four *Lupinus* species when grown under different pH and calcium (Ca) treatments. No data are shown for *L. hispanicus* grown under high pH + high Ca, as they died in this treatment. Error bars represent 95% confidence intervals (Cl). Letters show significant differences of different treatments within each species (based on Tukey's post-hoc analysis, $P < 0.05$). The grey dashed line represents the corresponding nutrient concentration in plant shoot dry matter considered adequate for crop growth (Kirkby 2012). ■ marks calcifuge species, ● marks calcicole species

concentration of *L. pilosus* under high Ca was the highest, while that under high pH + high Ca was the lowest among all treatments (Fig. 3f).

Root potassium (K), sulfur (S), zinc and copper concentration are shown in Fig. S2.

Gas exchange parameters

Net photosynthetic rate (A_{max}), stomatal conductance (g_s), and the maximum photochemical quantum yield of PSII (*Fv/Fm*) of *L. angustifolius* under

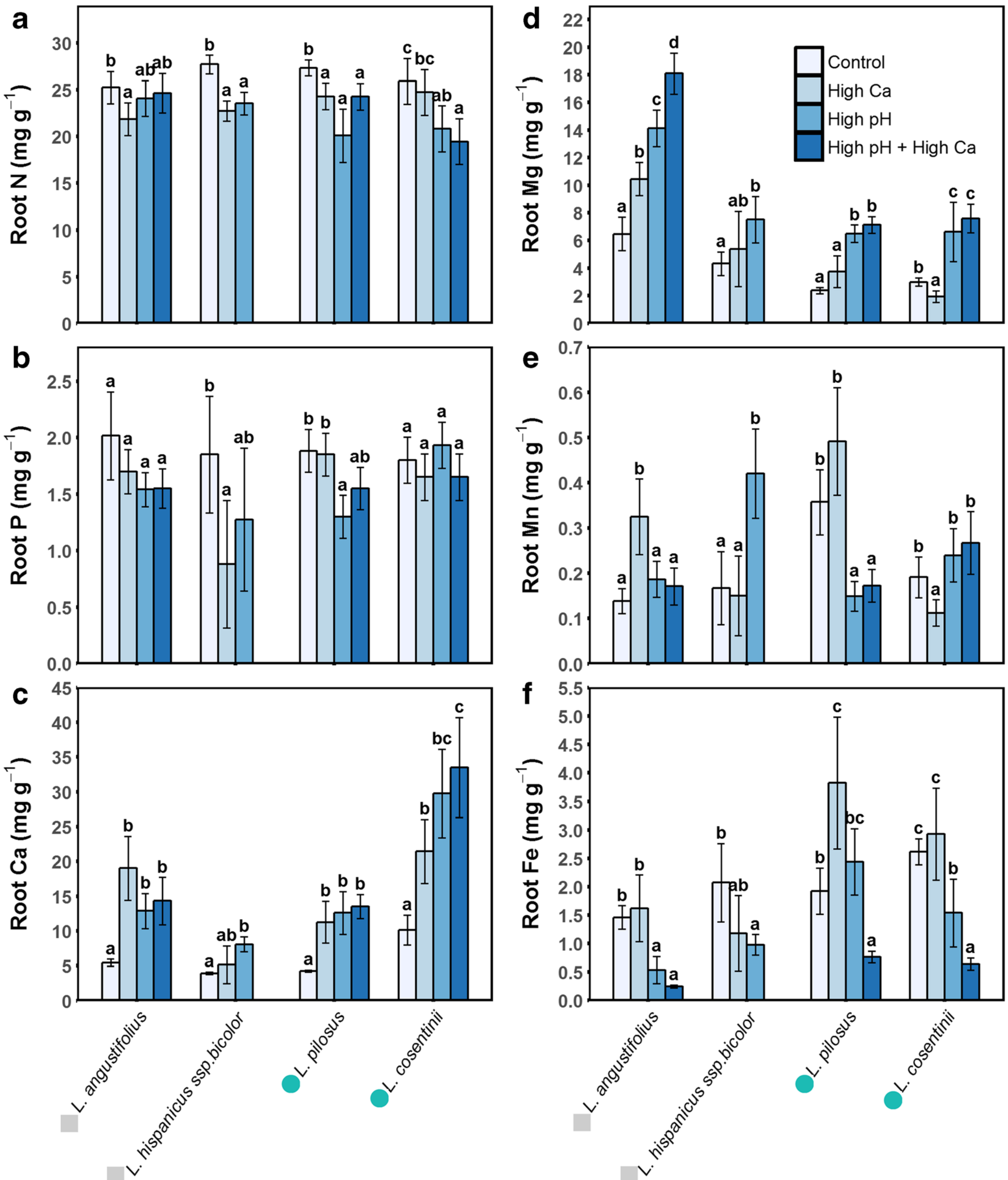

Fig. 3 Concentrations of a range of nutrients in roots of four *Lupinus* species when grown under different pH and calcium (Ca) treatments. No data are shown for *L. hispanicus* grown under high pH + high Ca, as they died in this treatment. Error bars represent 95% confidence intervals (Cl). Letters show significant differences of different treatments within each species (based on Tukey's post-hoc analysis, $P < 0.05$). ■ marks calcifuge species, ● marks calcicole species

high pH + high Ca were the smallest among all treatments, while the intercellular carbon dioxide concentration (C_i) was the largest among all treatments. There were no significant differences in these values among the other three treatments (Fig. 4).

Lupinus hispanicus had the smallest A_{max}, g_s and C_i under high pH, while the largest *Fv/Fm* was under high pH. There were no significant differences in these values between control and high-Ca treatments (Fig. 4).

The A_{max}, g_s and C_i of *L. pilosus* under high pH + high Ca were the smallest among all treatments, while the A_{max} under high pH was the largest. There were no significant differences for *Fv/Fm* between different treatments (Fig. 4).

The A_{max}, g_s and *Fv/Fm* of *L. cosentinii* under high pH and high pH + high Ca were significantly smaller than those under control or high Ca, while there were no significant differences for C_i of *L. cosentinii* between different treatments (Fig. 4).

Root morphology

Root surface area of *L. angustifolius* under high pH and high pH + high Ca was significantly less than that of control plants and plants in the high-Ca treatment. Root surface area of *L. pilosus* and *L. cosentinii* under high pH + high Ca was the smallest among all treatments. There was no significant difference for the root surface area of *L. hispanicus* among treatments (Fig. 5a).

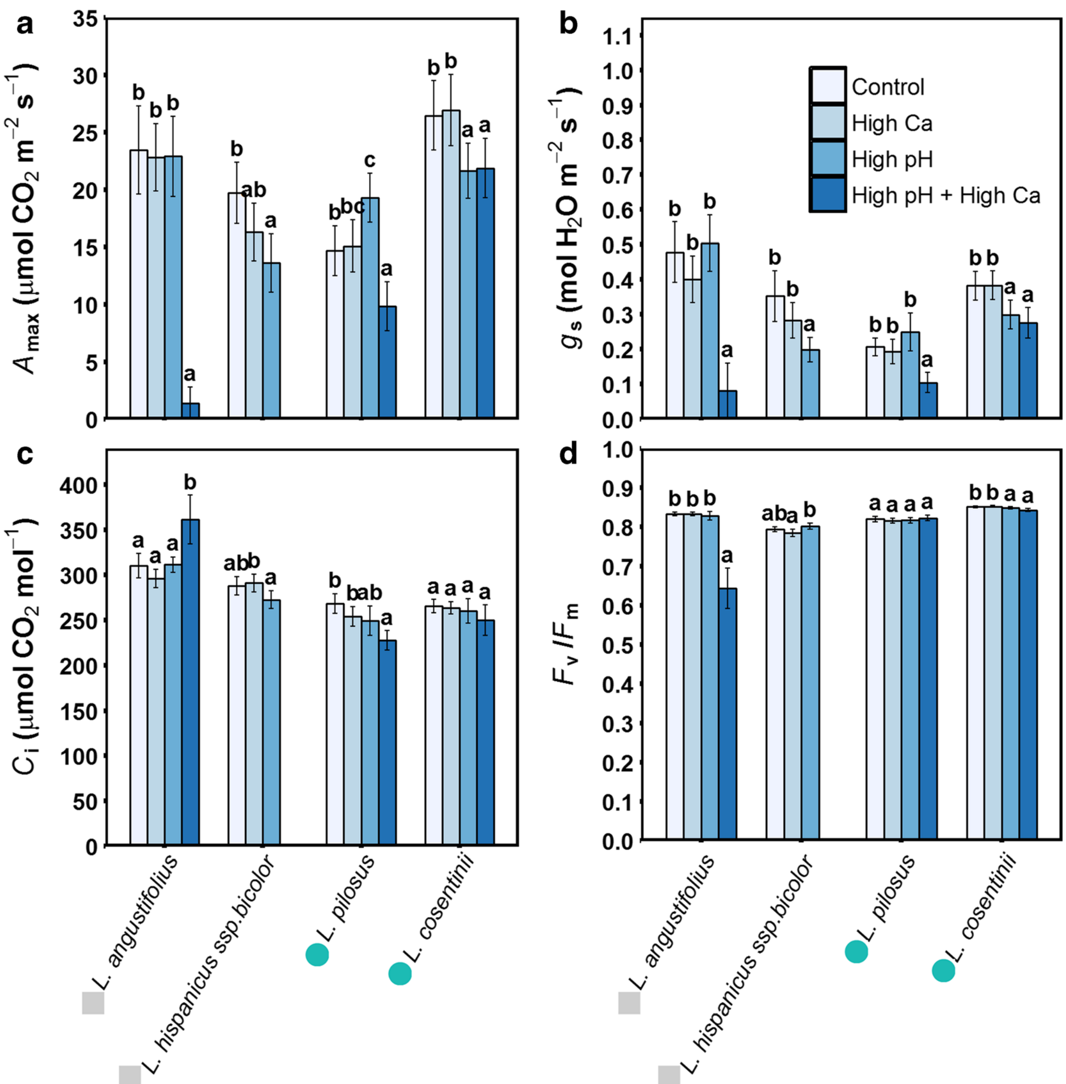

Fig. 4 Net photosynthetic rate (A_{max}) (**a**), stomatal conductance (g_s) (**b**), intercellular carbon dioxide concentration (C_i) (**c**) and the maximum photochemical quantum yield of PSII (*Fv/Fm*) (**d**) of four *Lupinus* species when grown under different pH and calcium (Ca) treatments. No data are shown for *L. hispanicus* grown under high pH + high Ca, as they died in this treatment. Error bars represent 95% confidence intervals (CI). Letters show significant differences of different treatments within each species (based on Tukey's post-hoc analysis, $P < 0.05$). ■ marks calcifuge species, ● marks calcicole species

Average root diameter of all *Lupinus* species under high pH and high pH + high Ca treatments was significantly greater than that under control or high Ca. Average root diameter of *L. angustifolius* under a high Ca supply was significantly greater than that of control plants (Fig. 5b).

Root length of *L. angustifolius* and *L. cosentinii* under high pH and high pH + high Ca was significantly less than that of control and high-Ca plants. Root length of *L. pilosus* under high pH and high pH + high Ca was significantly less than that of high-Ca plants. Root length of *L. angustifolius*, *L. pilosus* and *L. cosentinii* under high pH + high Ca was even less than that under high pH. However, there were no significant differences in root length of *L. hispanicus* among treatments (Fig. 5c).

Root nodulation of *L. angustifolius* was only effective under control conditions. The root nodulation of *L. hispanicus* was effective under control conditions and at high pH, while that under high pH was less effective. Root nodulation of *L. pilosus* and *L. cosentinii* under high pH + high Ca was not effective. Root nodulation was more effective for *L. pilosus* under high Ca and high pH than under control conditions, while there was no significant difference for *L. cosentinii* under these three treatments (Fig. 5d).

Carboxylates

The amounts of rhizosphere citrate for all *Lupinus* species under high Ca, high pH, and high pH + high Ca were significantly greater than those of control plants (Fig. 6a). The rhizosphere malate amounts in *L. angustifolius* and *L. pilosus* under high pH were the greatest among all the treatments, while there were no significant differences for *L. hispanicus* and *L. cosentinii* among treatments (Fig. 6b). Rhizosphere fumarate amounts of all *Lupinus* species showed no significant difference among treatments (Fig. 6c). Rhizosphere *cis*-aconitate amounts in *L. angustifolius* and *L. pilosus* under high pH were the greatest among all treatments, while we found no significant difference for *L. cosentinii* among treatments. No *cis*-aconitate was detected for *L. hispanicus* under any treatment (Fig. 6d).

Biomass

The total, root and shoot biomass, and leaf area of *L. angustifolius* under high pH and high pH + high Ca were significantly less than those under control conditions and high Ca treatments. The total biomass, root biomass, and shoot biomass of *L. hispanicus* under high pH + high Ca were the lowest among all treatments, and there was no significant difference among control, high Ca, and high pH treatments. However, the leaf area of *L. hispanicus* under high pH was significantly less than that of control plants. The total biomass, shoot biomass and leaf area of *L. pilosus* were the greatest under high-Ca conditions among all treatments, while the root biomass was greatest under high pH. There were no significant differences for the total and root biomass of *L. cosentinii* among treatments. However, the shoot biomass and leaf area of *L. cosentinii* under high pH + high Ca were significantly less than under control conditions (Fig. 7).

A detailed shoot biomass, including mature leaf, immature leaf and stem biomass of different *Lupinus* species, are shown in Fig. S3.

Discussion

The present study demonstrates that *Lupinus* species responded very differently to high pH and high pH + high Ca treatments, with the calcicole species *L. pilosus* being the most tolerant, followed by the calcicole species *L. cosentinii*. The other two calcifuge species, *L. angustifolius* and *L. hispanicus*, were sensitive to high pH and high pH + high Ca treatments, and *L. hispanicus* even died in the high pH + high Ca treatment. "Tolerant" means the species can grow healthily or the growth is only slightly inhibited in calcareous soils, and "sensitive" means the growth of the species can be inhibited from an intermediate to the worst level. In this study, tolerance was mainly based on the biomass data. This is consistent with results on these species when grown in nutrient solution with high pH (buffered by MES/TES or caused by high [HCO_3^-]) (Ding et al., personal observations), some field and glasshouse studies, and their natural occurrence on acid or alkaline soils (Brand et al. 2002; Clements and Cowling 1990; Tang et al. 1993a, 1995b; White 1990). However, this result is inconsistent with the Ca sensitivity of different *Lupinus* species grown in a hydroponic system, where *L. pilosus* and *L. angustifolius* were tolerant of high Ca, whereas *L. cosentinii* and *L. hispanicus* were sensitive (Ding et al. 2018b). This discrepancy might be related to different growth systems (i.e. sand vs hydroponics) or

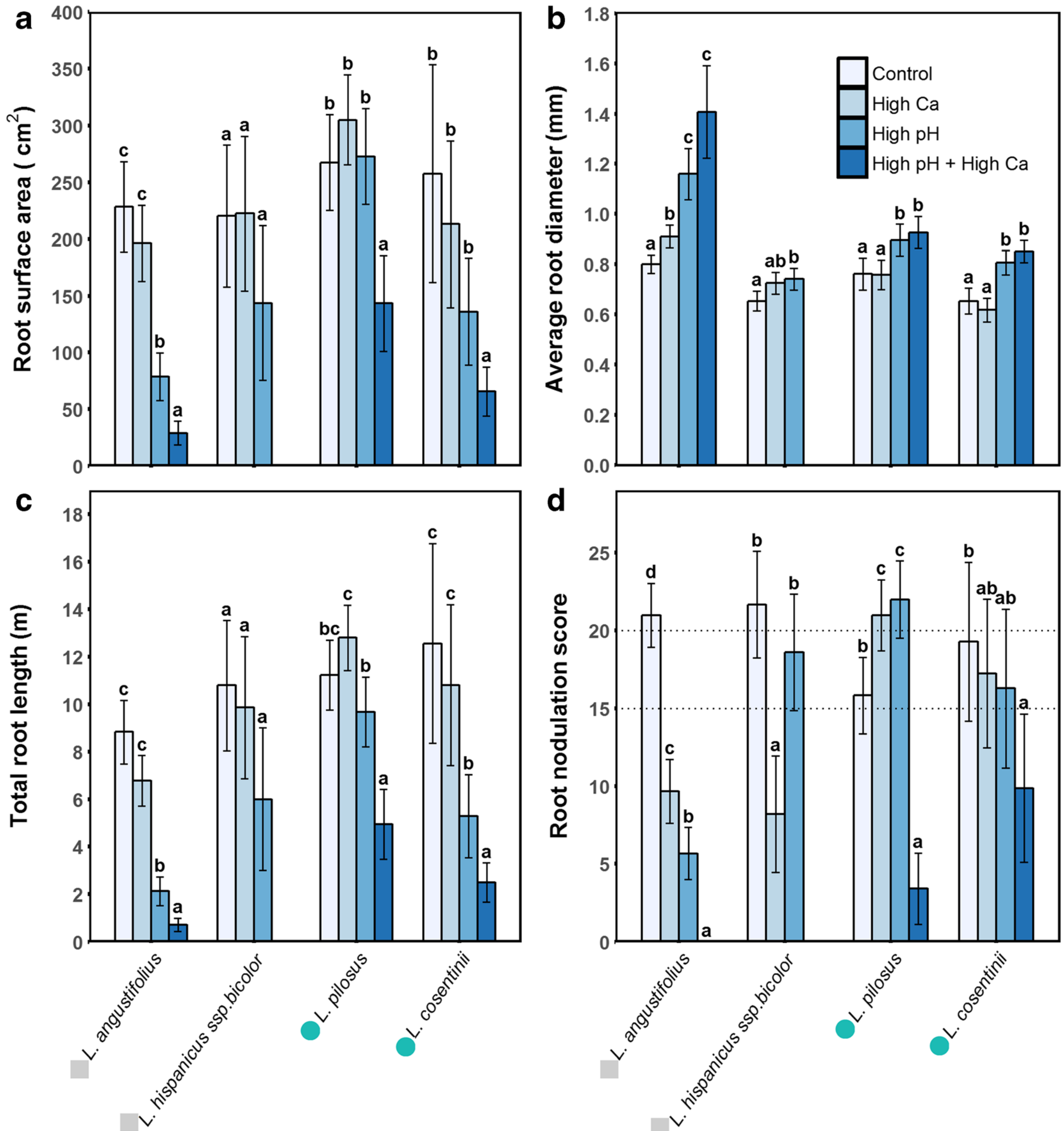

Fig. 5 Root surface area (**a**), average root diameter (**b**), total root length (**c**) and root nodulation score (**d**) of four *Lupinus* species when grown under different pH and calcium (Ca) treatments. The score above the upper grey dashed line in d represents nodulation considered as very effective, the score between upper and lower grey dashed line in d represents nodulation considered as less effective, while the score below the lower grey dashed line in d is considered as ineffective nodulation. Nodulation was assessed according to the British Columbia Ministry of Forests field guide (BCMF 1991); details are included in Materials and Methods (British Columbia Ministry of Forests 1991). No data are shown for *L. hispanicus* grown under high pH + high Ca, as they died in this treatment. Error bars represent 95% confidence intervals (Cl). Letters show significant differences of different treatments within each species (based on Tukey's post-hoc analysis, $P < 0.05$). ■ marks calcifuge species, ● marks calcicole species

different [Ca^{2+}] used in various experiments (4.8 g kg^{-1} vs 6 mM). Most likely, Ca is not the main reason why some *Lupinus* species are sensitive to calcareous soils (Tang et al. 1995a). This also explains why there was no correlation between Ca concentration in *Lupinus* species grown in Wangary calcareous soil and their leaf chlorosis or growth (Brand et al. 2000).

Leaf symptoms

The pH of the high pH and high pH + high Ca treatments was similar. Therefore, the difference in leaf chlorosis and its gradual disappearance with time among *Lupinus* species under these two treatments is likely explained by different [Ca^{2+}], [HCO_3^-] and pH buffer

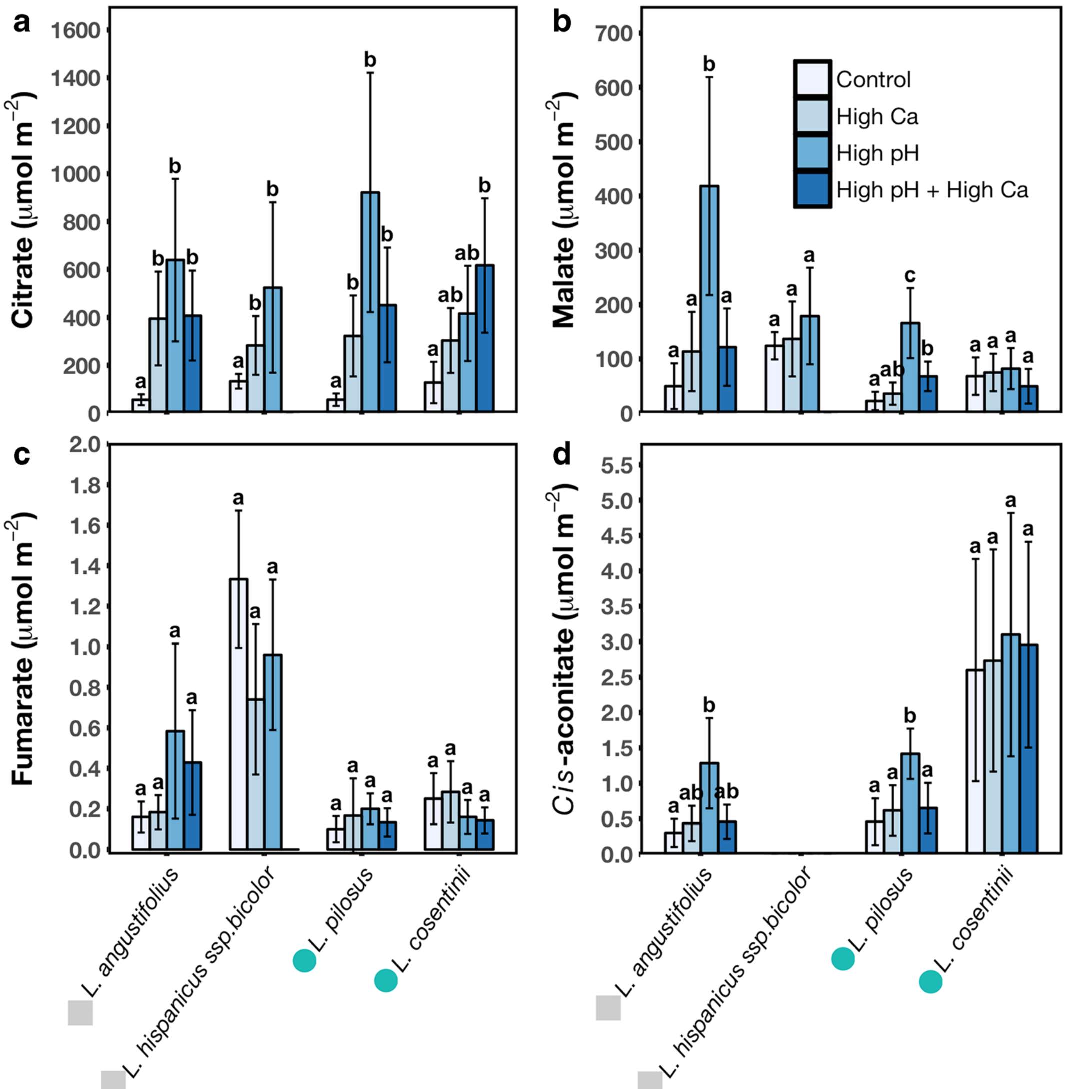

Fig. 6 Amounts of rhizosphere citrate (**a**), malate (**b**), fumarate (**c**) and *cis*-aconitate (**d**) acid of four *Lupinus* species when grown under different pH and calcium (Ca) treatments. Rhizosphere carboxylates were extracted with 0.2 mM $CaCl_2$ and amounts are expressed per unit total root surface area. No data are shown for *L. hispanicus* grown under high pH + high Ca, as they died in this treatment. Error bars represent 95% confidence intervals (Cl). Letters show significant differences of different treatments within each species (based on Tukey's post-hoc analysis, $P < 0.05$). ■ marks calcifuge species, ● marks calcicole species

capacity associated with the different [$CaCO_3$]. In addition, high [Ca^{2+}] showed no relationship with leaf chlorosis in this study. Therefore, we conclude that the gradual disappearance of the leaf chlorosis symptoms was due to a lower [HCO_3^-].

Similarly, Brand et al. (1999) also found that leaf chlorosis of *L. angustifolius* correlated with $CaCO_3$ content (which can react with CO_2 and H_2O to produce bicarbonate) in a range of calcareous soils; the pH of these calcareous soils was similar. Except for leaf chlorosis, the negative effects of high pH + high Ca treatment on the growth, as shown by the biomass, of all *Lupinus* species were generally consistent with those observed under high pH, rather than with those observed under a high Ca treatment. The effects of high pH (buffered by MES/TES or caused by high [HCO_3^-]) on the growth of *Lupinus* species were consistent in a hydroponic study as well (Ding et al., personal

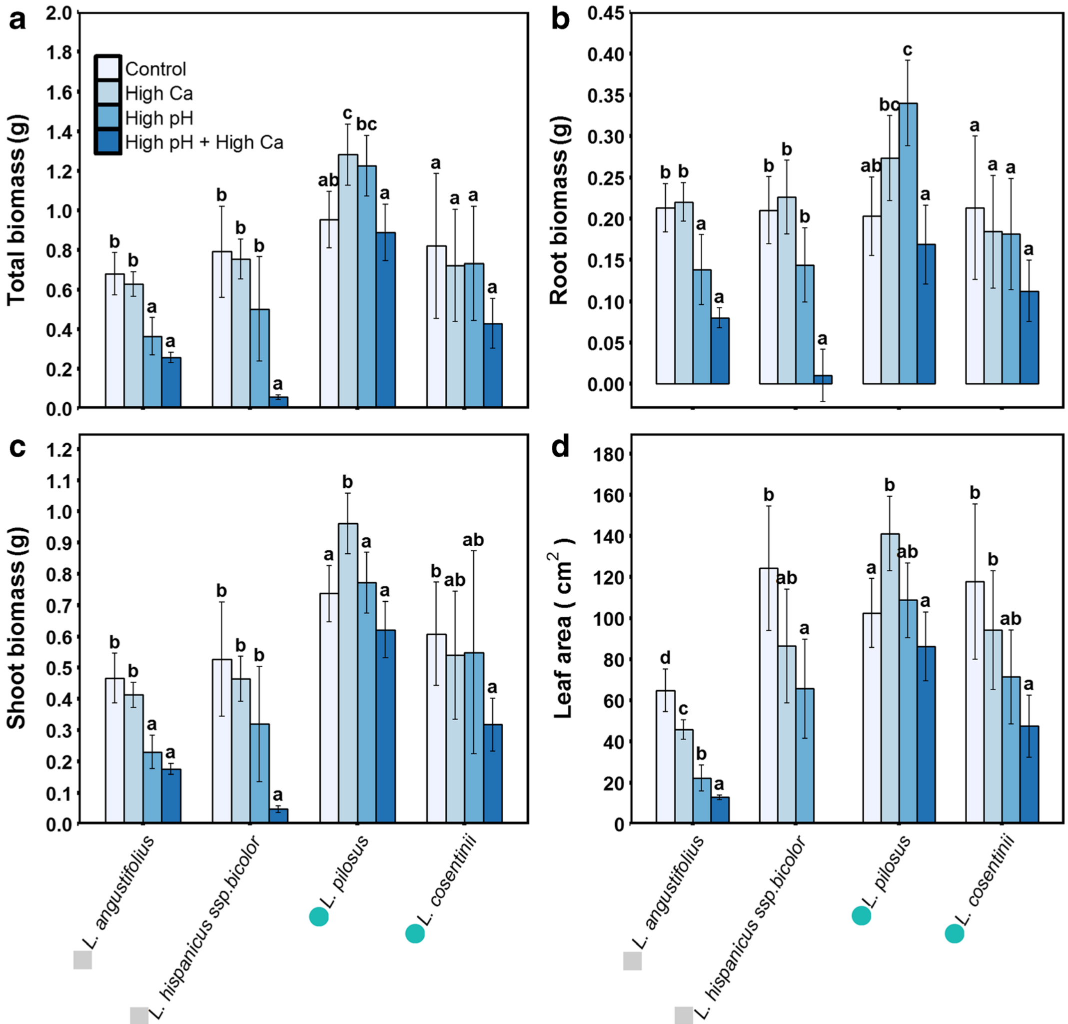

Fig. 7 Total (**a**), root (**b**), shoot (**c**) dry biomass and leaf area (**d**) of four *Lupinus* species when grown under different pH and calcium (Ca) treatments. No data are shown for the leaf area of *L. hispanicus* grown under high pH + high Ca, as they died in this treatment. Error bars represent 95% confidence intervals (CI). Letters show significant differences of different treatments within each species (based on Tukey's post-hoc analysis, $P < 0.05$). ■ marks calcifuge species, ● marks calcicole species

observations). MES/TES or bicarbonate rather than $CaCO_3$ were used in the hydroponic study to maintain a stable pH is because $CaCO_3$ is not soluble in a hydroponic system. Taken together, this indicates that high pH is the actual cause why some *Lupinus* species are sensitive to calcareous soils, while the strong buffering capacity of HCO_3^- determines if *Lupinus* species can recover from leaf chlorosis and then survive in calcareous soils or not. As the strong buffering capacity of bicarbonate could restrict rhizosphere acidification of carboxylic acids, and then limit nutrient availability. This also explains why active lime (high Ca, high HCO_3^- and high pH) tends to have greater negative effects on the growth of some calcifuge *Lupinus* species than alkalinity (high pH) (Liu and Tang 1999).

Plant growth

Among all the species, the greatest tolerance of *L. pilosus* to high pH and high pH + high Ca treatments is evidenced by the biomass under those treatments. In addition, the shoot and total biomass of *L. pilosus* under a high Ca supply was actually significantly greater than that of control plants. This confirms our finding that Ca improves the shoot growth of species that grow well under an extremely high Ca concentration (Ding et al.

2018a). The negative effects of high pH and/or high pH + high Ca on total root length of *Lupinus* species agree with the decreased lateral root growth of some *Lupinus* species grown in either a high pH (buffered by MES/TES or caused by high [HCO_3^-]) nutrient solution (Ding et al., personal observations) or limed soil (Kerley and Huyghe 2001). This is also consistent with the decreased root length of *L. angustifolius* grown in nutrient solution with high pH (>6) (Tang et al. 1992, 1993b). Thus, we can conclude that high pH is the direct reason why root growth was inhibited.

Root nodulation of all *Lupinus* species was not effective under high pH + high Ca treatment, as evidenced by nodulation scores. However, nodulation of *Lupinu*s species responded differently to high pH treatment, with that in *L. angustifolius* being ineffective under high pH, while that of calcicole species not being negatively affected by high pH. The nodule numbers of *L. angustifolius*, *L. albus* and *L. pilosus* were inhibited when grown in nutrient solution with high pH (buffered by MES/TES or caused by high [HCO_3^-]) (Tang and Robson 1993; Tang and Thomson 1996). This is inconsistent with what we found; however, as pointed out above, high pH + high Ca treatment contains larger [HCO_3^-] and [Ca^{2+}] than the high pH treatment. Therefore, the buffering capacity of HCO_3^-, that is the ability to maintain a high pH, was closely related with root nodulation of the calcicole species, while for the calcifuge species, either high [Ca] or high [HCO_3^-] inhibited root nodulation.

Carboxylates

The exudation of citrate increased in the high pH + high Ca treatment, and the amount of citrate was much greater than that of the other carboxylates under any treatment. The relatively large amounts of malate and *cis*-aconitate in the rhizosphere of *L. angustifolius* and *L. pilosus* under high-pH treatment compared with those under high pH + high Ca treatment partly explain why leaf chlorosis symptoms under a high-pH treatment decreased over time more than they did under a high pH + high Ca treatment. This is because increased carboxylate release would decrease rhizosphere pH, subsequently increasing the availability of P and micronutrients such as Fe, Mn and Zn (Dinkelaker et al. 1989; Lambers et al. 2013; Liang and Li 2003). However, the extent of rhizosphere acidification would be limited by the strong buffering capacity of HCO_3^- under a high pH + high Ca treatment. The extent of rhizosphere acidification is also shown by the rhizosphere pH (Table S1).

Leaf and root nutrient concentrations

As discussed above, root surface area, root length, and fine root growth of *L. cosentinii*, *L. angustifolius* and *L. hispanicus* were significantly reduced at high pH and/or a high pH + high Ca treatment, which would have resulted in less nutrient uptake, including Fe. In contrast, a high pH did not inhibit Fe uptake of *L. pilosus*, as the root surface area and total root length of *L. pilosus* under high pH were not affected. However, translocation of Fe and Mn from root to shoot in all *Lupinus* species was inhibited by high pH and high pH + high Ca treatments. Probably because Fe^{3+}- and/or Mn^{4+}-reductase activity was restricted by a high pH in the root apoplast which resulted in reduced Fe and Mn availability and translocation to leaves (Kosegarten and Koyro 2001; Mengel 1994; Millaleo et al. 2010; Rengel 2000; Zribi and Gharsalli 2002). Leaf Fe concentrations in these two treatments were even below the concentration in shoot dry matter considered adequate for crop growth (Kirkby 2012). This is consistent with the observed leaf chlorosis in this study, and also agrees with previous findings (Bertoni et al. 1992; White and Robson 1989).

Based on the comparison of leaf and root P concentrations of different treatments, we found the P translocation of calcifuge species from roots to shoots was inhibited by either high soil [Ca] or high pH, and this was similar for *L. cosentinii*, while P translocation in *L. pilosus* was only negatively affected by high pH + high Ca treatment. This shows only under a high pH + high Ca treatment, the strong buffering capacity of HCO_3^- could limit the extent of rhizosphere carboxylate acidification in *L. pilosus* and then result in less P availability and translocation from roots to shoots. This also explains why, compared with other species in this study, *L. pilosus* was more tolerant to calcareous soils.

Nitrogen concentration and content were clearly decreased in *L. angustifolius*, *L. albus* and *L. pilosus* grown in solution with high pH (buffered with MES and TES), especially *L. angustifolius* (Tang and Robson 1993). However, in the present study, leaf N concentrations of *L. angustifolius* were not affected by high pH and high pH + high Ca treatments. The reason for this disagreement is not yet clear; further research is needed to explain this. In addition, leaf N concentration was not consistent with root nodulation. These results suggest that the role of nodulation (rather than N_2 fixation) related with *Bradyrhizobium* sp. (*Lupinus*) WU425 in the growth of *Lupinus* species in calcareous soils is not as important as that reported before (Tang and Robson 1995).

Gas exchange

The decreased A_{max} of *L. hispanicus* under high pH and *L. pilosus* under high pH + high Ca treatment was due to reduced stomatal conductance, as it was associated with a corresponding decrease of g_s and C_i. The A_{max} and F_v/F_m of *L. angustifolius* under the high pH + high Ca treatment and of *L. cosentinii* under the high pH and high pH + high Ca treatments were significantly lower than those of plants under other treatments. In addition, the change of A_{max} and F_v/F_m of *L. angustifolius* under high pH and *L. cosentinii* under high pH + high Ca treatments was correlated with leaf Fe concentration. This indicates that the decreased PSII photochemical capacity of *L. angustifolius* under high pH + high Ca treatment and that of *L. cosentinii* under high pH and high pH + high Ca treatments was likely due to Fe deficiency caused by high [HCO_3^-] and/or high pH (Maxwell and Johnson 2000).

Conclusions

A high pH (resulting from either KOH or HCO_3^-) inhibited root growth of *Lupinus* species which resulted in less nutrient uptake (except N) and reduced shoot growth. However, the strong buffering capacity of bicarbonate determines if *Lupinus* species can survive in calcareous soils or not. Among all studied *Lupinus* species, *L. pilosus* was the most tolerant, followed by *L. cosentinii* and *L. angustifolius*, while *L. hispanicus* was the most sensitive.

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