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

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17

18 **Abstract**

19 Aims: Previous studies have shown that pH, rather than calcium (Ca), is the main reason why
20 some *Lupinus* species are sensitive to nutrient solutions mimicking calcareous soils; however,
21 a hydroponic system is quite different from soil systems, and plants may respond differently
22 to these two growing conditions. Thus, studies with *Lupinus* species grown in calcareous
23 soils are needed.

24 Methods: Two calcicole and two calcifuge species were grown in river sand with different Ca
25 forms and amounts, pH levels, and [bicarbonate (HCO_3^-)] (which is produced by calcium
26 carbonate (CaCO_3)). Leaf symptoms, leaf area, gas exchange, biomass, and root morphology
27 were recorded; whole leaf and root nutrient concentrations were analysed.

28 Results: We observed leaf chlorosis of the youngest leaves under high pH (adjusted by KOH)
29 and high pH + high Ca (representing high $[\text{HCO}_3^-]$, high pH and high Ca) treatments for all
30 *Lupinus* species. However, after two weeks, leaf chlorosis of all *Lupinus* species under high
31 pH started to disappear, with calcicole species fully, and calcifuge species only partly
32 recovering. Leaf chlorosis symptoms of calcicole species under high pH + high Ca partly
33 disappeared as well, while those of calcifuge species did not disappear at all.

34 Conclusions: High pH (resulting from either KOH or HCO_3^-) inhibited root growth, and
35 subsequently uptake of some nutrients and shoot growth of *Lupinus* species. However, the
36 strong buffering capacity of HCO_3^- is the key factor determining if *Lupinus* species can
37 survive in calcareous soils. Among all studied *Lupinus* species, *L. pilosus* was the most
38 tolerant to high $[\text{HCO}_3^-]$ and/or high pH, followed by *L. cosentinii* and *L. angustifolius*, while
39 *L. hispanicus* was the most sensitive.

40

41 **Keywords:** Calcicole, calcifuge, high pH, high bicarbonate concentration, *Lupinus*

42

43 Introduction

44 Calcareous soils are alkaline and contain free CaCO_3 , and high concentrations of, calcium
45 (Ca) and bicarbonate (HCO_3^-). The availability of phosphorus (P) and some micronutrients,
46 including iron (Fe), zinc (Zn), manganese (Mn) and copper (Cu) in calcareous soils is very
47 low (Tyler 2003). Most *Lupinus* species grow poorly on calcareous soils, and this has been
48 attributed to various factors, including Ca toxicity, sensitivity to high $[\text{HCO}_3^-]$ and high pH
49 and Fe deficiency, and poor nodulation (Abd-Alla 1999; Ding et al. 2018b; Jessop et al. 1990;
50 Kerley 2000; Tang et al. 1995b; Tang and Thomson 1996; White 1990).

51

52 Calcium toxicity has been considered one of the major causes for why some *Lupinus* species
53 are sensitive to calcareous soils (De Silva and Mansfield 1994; Jessop et al. 1990; Kerley et
54 al. 2001). For example, a high Ca supply may disturb the lamellar structure of chloroplasts,
55 and consequently negatively affect net photosynthetic rates (Chevalier and Paris 1980;
56 Chevalier and Paris-Pireyre 1984; De Silva et al. 1994). Excessive Ca may precipitate with P
57 as $\text{Ca}_3(\text{PO}_4)_2$ in plant tissues which makes both Ca and P unavailable (Ding et al. 2018b;
58 McLaughlin and Wimmer 1999; Zohlen and Tyler 2004). We found that Ca-tolerant *Lupinus*
59 species tended to have tight control over Ca uptake and/or translocation from roots to leaves,
60 or better Ca compartmentation at the cellular level (Ding et al. 2018a). These abilities likely
61 play an important role in the tolerance of some *Lupinus* species to calcareous soils, as
62 suggested for other calcicole species (He et al. 2014; Jefferies and Willis 1964; Raza et al.
63 2000; Valentinuzzi et al. 2015; Webb 1999; Wu et al. 2011).

64

65 In addition, high $[\text{HCO}_3^-]$ and high pH in calcareous soils are considered the main factors
66 affecting the sensitivity of some *Lupinus* species to calcareous soils (Coulombe et al. 1984;
67 Mengel et al. 1984; Romera et al. 1992; Tang and Thomson 1996; Waters and Troupe 2012).
68 For example, a high rhizosphere pH caused by a high $[\text{HCO}_3^-]$ in calcareous soils reduces
69 nutrient availability, especially that of P, Fe, Mn, Zn, Cu and boron (B) for root uptake, and
70 thus limits plant growth (George et al. 2012; Neumann and Römheld 2012; Parker et al.
71 1999; Tyler and Ström 1995; Yue Ao et al. 1987). In addition, a high $[\text{HCO}_3^-]$ and high pH
72 can also inhibit root growth of some *Lupinus* species (Peiter et al. 2001). Tang and Thomson
73 (1996) found that root elongation of *L. angustifolius* is inhibited by high $[\text{HCO}_3^-]$ and high
74 pH in nutrient

75 al. 1992). Our comparative study showed that there is a correlation between the decreased
76 lateral root growth and nutrient availability of some *Lupinus* species grown in nutrient
77 solution with high $[\text{HCO}_3^-]$ and/or high pH (Ding et al., personal observations).

78

79 In a series of hydroponic experiments, we found that a high Ca supply caused P deficiency in
80 some *Lupinus* species, but its effects on the growth, especially the leaf symptoms of *Lupinus*
81 species were inconsistent with what has been reported for those grown in calcareous soils
82 (Ding et al. 2018b). In contrast, the effects of high pH (buffered by MES/TES or caused by
83 high $[\text{HCO}_3^-]$) on different *Lupinus* species are generally consistent with those of plants
84 grown in calcareous soils, for example, leaf chlorosis were developed in the calcifuge
85 *Lupinus* species under high pH (Ding et al., personal observations). Considering the results of
86 additional studies (Kerley and Huyghe 2002; White and Robson 1990), we concluded that pH
87 is the main reason why some *Lupinus* species are sensitive to nutrient solutions mimicking
88 calcareous soils. However, a hydroponic system is quite different from soil systems, and
89 plants may respond differently to these two growing conditions. Therefore, in this study, we
90 aimed to test if pH is the main factor determining why some *Lupinus* species are sensitive to
91 calcareous soils, by growing different *Lupinus* species in soils with different forms of Ca, pH
92 levels, and $[\text{HCO}_3^-]$. We compared leaf symptoms, root nodulation, root and shoot biomass,
93 and root and shoot nutrient status. We hypothesised that pH, rather than Ca, is the main
94 reason why *Lupinus* species respond differently to calcareous soils. In addition, we
95 hypothesised that root surface area, root length, and fine root growth of calcifuge species will
96 be inhibited significantly in calcareous soils, while those of calcicole species will be slightly
97 inhibited or not inhibited at all.

98

99 **Materials and Methods**

100 **Plant growth**

101 Four *Lupinus* species were selected (two calcifuge species: *L. angustifolius* L. cv Mandelup
102 and *L. hispanicus* ssp. *bicolor* Boiss. and Reut. P22999; two calcicole species: *L. pilosus*
103 Murr. P27440 and *L. cosentinii* Guss. P27225) (Table 1). All seeds were obtained from the
104 Australian Lupin Collection (Department of Primary Industries and Regional Development,
105 WA, Australia). Except for *L. angustifolius*, seeds were scarified. Seeds were then sterilised

106 in 5% (v/v) sodium hypochlorite for 20 mins and rinsed thoroughly with deionised (DI)
 107 water. All seeds were pre-germinated in sterilised river sand for four days, and then three or
 108 four seeds were inoculated with Group G[®] (*Bradyrhizobium* sp. (*Lupinus*) WU425), which is
 109 effective for the inoculation of *Lupinus* species (Tang and Robson 1993), sown in prepared
 110 pots, and then later thinned to one plant. All sands were washed, sterilised and then dried
 111 first. After drying, CaCO₃ or CaSO₄ was mixed evenly with sand, as shown in Table 2. Each
 112 pot was then filled with 1.2 kg dry river sand.

113 All essential nutrients other than Ca were provided as: 189.5 mg kg⁻¹ Ca(NO₃)₂·4H₂O, 28.6
 114 mg kg⁻¹ NH₄Cl, 65.9 mg kg⁻¹ KH₂PO₄, 101.2 mg kg⁻¹ MgSO₄·7H₂O, 12.3 mg kg⁻¹
 115 MnSO₄·H₂O, 8.8 mg kg⁻¹ ZnSO₄·7H₂O, 0.7 mg kg⁻¹ H₃BO₃, 1.0 mg kg⁻¹ Na₂MoO₄, 2.0 mg kg⁻¹
 116 ¹ CuSO₄·5H₂O and 32.9 mg kg⁻¹ FeNaEDTA. This nutrient composition has been used in our
 117 lab and proved to be suitable for the growth of legumes (Pang et al. 2010; Pang et al. 2011).
 118 The N was 80 mg kg⁻¹, and this was to supply some N for young plants; when they grow
 119 bigger, they will need to rely on biological N₂ fixation. Besides, and the amount of Ca(NO₃)₂
 120 added to all the treatments was very small compared with the amount of CaSO₄ or CaCO₃
 121 added, so it hardly affected the experiment design. All these nutrients were added as a
 122 nutrient solution with DI water, and KOH was used to adjust solution pH to 6 and 8
 123 according to different treatments shown in Table 2. K₂SO₄ was used to balance [K], and the
 124 final [K] was 80 mg kg⁻¹ for all the treatments, the final [S] was 133.4 mg kg⁻¹ for control and
 125 pH 6, the final [S] was 40 mg kg⁻¹ for high Ca and high pH + high Ca treatment. After adding
 126 basic nutrition solution for one week, all pots were ready to use, and sands from three plain
 127 pots of each treatment were air-dried and passed through a 2-mm sieve to measure the actual
 128 pH (10 g dry sand in 50 ml CaCl₂ as shown in Table 2) and exchangeable Ca²⁺ of each
 129 treatment. Exchangeable Ca²⁺ was then extracted by 1 M ammonium acetate at pH 7
 130 (Rayment and Lyons 2011) and analysed by inductively coupled plasma optical emission
 131 spectrometry (ICP-OES; School of Agriculture and Environment, University of Western
 132 Australia, Perth, Australia).

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

135 **Gas exchange and chlorophyll fluorescence measurements**

136 The day before harvest, the youngest fully-expanded leaves were chosen to measure net
 137 photosynthesis rate ($A_{\max}$), stomatal conductance (g_s) and intercellular carbon dioxide

138 concentration (C_i) with a LI-6400 portable gas exchange system (Li-Cor, Lincoln, NE, USA)
139 at 1500 $\mu\text{mol quanta m}^{-2} \text{s}^{-1}$. The same leaf used to measure photosynthesis was dark-adjusted
140 in a dark room for one hour, and then the maximum photochemical quantum yield of PSII
141 (F_v/F_m) was measured using a LI-6400 portable gas exchange system as described above.

142 **Plant harvest and carboxylate extraction**

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

153 Nodulation was assessed according to the British Columbia Ministry of Forests field guide
154 (British Columbia Ministry of Forests 1991), where a ranking of 0–5 is given to each of five
155 categories, including plant growth and vigour (1 for very chlorotic plants, 2 for slightly
156 chlorotic plants, 3 for green and relatively small plants and 5 for green and vigorous plants);
157 nodule number (0 for no nodules, 2 for few nodules (<5) with slight pigmentation, 3 for few
158 nodules (<5) with pink pigmentation, 3 also for many nodules (>50) mainly white or smaller,
159 5 for many nodules (>50) with pink pigmentation, 5 for 5–50 nodules as well); nodule
160 position (1 for nodules on lateral roots only, 3 for nodules on both crown and lateral roots, 5
161 for nodules predominantly on the crown); nodule colour (0 for white or green colour, 2 for
162 some pink or whitish with green areas, 5 for predominantly pink); and nodule appearance (0
163 for ineffective, 3 for intermediate, 5 for effective). A total score was summed to evaluate the
164 effectiveness of nodulation, and a score between 20 and 25 is considered very effective, a
165 score between 15 and 20 less effective, while a score between 0 and 14 is considered
166 unsatisfactory nodulation.

167 The whole roots were then thoroughly washed to remove the remaining sand. After that,
168 plants were separated into mature leaves, immature leaves, stems (including petioles), and
169 roots. Leaf area was measured and fresh biomass was recorded. Leaf symptoms were

170 recorded by taking photos prior to harvest. Roots of all plants were scanned by an
171 Epson Perfection V700 Photo Scanner and analysed by WinRHIZO 2009a, b (Regents
172 Instruments Canada Inc, Quebec City, Quebec, QC, Canada). After that, all the plant parts
173 were dried in an oven an oven at 70°C for seven days, and dry biomass was recorded.

174

175 **Carboxylate analysis**

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

182

183 **Determination of whole leaf and root nutrient concentrations**

184 To determine whole leaf and root nutrient concentrations, all the dry mature leaves and roots
185 were ground to a fine powder with a GenoGrinder (SPEX SamplePrep LLC, Metuchen, New
186 Jersey, USA), and 0.1 g of ground material was digested with concentrated HNO₃ and HClO₄
187 (3:1) and analysed by inductively coupled plasma optical emission spectrometry (ICP-OES).
188 Nitrogen was determined by a CNS analyser (Elementar Vario Macro Elemental Analyzer,
189 Elementar Analysensystem GmbH, Hanau, Germany)

190

191 **Statistics**

192 Data and statistical analyses were performed with the R software platform (R Core Team
193 2017). As data of *L. hispanicus* grown under high pH + high Ca treatment were not included,
194 data structure of the whole experiment was unbalanced. General linear mixed-effect models
195 were used to test the differences in whole leaf and root nutrient concentrations, root surface
196 area, average root diameter, total root length, root nodulation, carboxylates, leaf area, shoot
197 biomass, root biomass and total biomass among different treatments within each species. We
198 tested the differences of gas exchange parameters and chlorophyll fluorescence with general

199 linear mixed-effect models among different treatments within each species; individual plant
200 was considered as the random effect.

201 The residuals of each model were inspected for heteroscedasticity, and in the presence of
202 heteroscedasticity, models with different variance structures were compared, and a suitable
203 variance structure significantly improved the model were specified based on Akaike
204 Information Criterion (AIC) (Burnham and Anderson 2003; Zuur et al. 2009). The effects
205 package was used to determine the mean values and 95% confidence intervals (CI) (Fox
206 2003). Significant differences were defined based on Tukey's post-hoc analysis ($P < 0.05$).

207

208 **Results**

209 **Leaf symptoms**

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

216

217 **Leaf nutrients**

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

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

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

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

238 Leaf Mn and Fe concentrations of all *Lupinus* species were significantly lower under high pH
239 and/or high pH + high Ca treatments than those of control plants and plants of high Ca
240 treatment (Figs 2E and 2F).

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

242 **Root nutrients**

243 Compared with control plants, root N concentrations of *L. angustifolius*, *L. hispanicus* and *L.*
244 *pilosus* at high Ca supply were significantly lower. Root N concentrations of *L. hispanicus*,
245 *L. pilosus* and *L. cosentinii* under high pH and high pH + high Ca treatments were
246 significantly lower than those of control plants (Fig. 3A).

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

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

256 Compared with control plants, root Mg concentrations of all *Lupinus* species at high pH
257 and/or high pH + high Ca treatments were significantly higher. The root Mg concentration of
258 *L. angustifolius* under high Ca supply was also significantly higher than that of control plants.

259 However, the root Mg concentration of *L. cosentinii* under high Ca supply was significantly
260 lower than that of the control. There were no significant differences in root Mg
261 concentrations of *L. hispanicus* and *L. pilosus* between control and the high-Ca treatments
262 (Fig. 3D).

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

269 Root Fe concentrations of *L. angustifolius* and *L. cosentinii* under high pH and high pH +
270 high Ca treatments were significantly lower than those of the control and plants of the high-
271 Ca treatments. Root Fe concentration of *L. hispanicus* under high pH was significantly lower
272 than that of the control. Root Fe concentration of *L. pilosus* under high Ca was the highest,
273 while that under high pH + high Ca was the lowest among all treatments (Fig. 3F).

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

275

276 **Gas exchange parameters**

277 Net photosynthetic rate ($A_{\max}$), stomatal conductance (g_s), and the maximum photochemical
278 quantum yield of PSII (F_v/F_m) of *L. angustifolius* under high pH + high Ca were the smallest
279 among all treatments, while the intercellular carbon dioxide concentration (C_i) was the largest
280 among all treatments. There were no significant differences in these values among the other
281 three treatments (Fig. 4).

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

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

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

291

292 **Root morphology**

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

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

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

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

314

315 **Carboxylates**

316 The amounts of rhizosphere citrate for all *Lupinus* species under high Ca, high pH, and high
 317 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 more specific shoot growth of different *Lupinus* species under different treatments, including mature leaf, immature leaf and stem biomass 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

349 this study, tolerance was mainly based on the biomass data. This is consistent with results on
 350 these species when grown in nutrient solution with high pH (buffered by MES/TES or caused
 351 by high $[\text{HCO}_3^-]$) (Ding et al., personal observations), some field and glasshouse studies, and
 352 their natural occurrence on acid or alkaline soils (Brand et al. 2002; Clements and Cowling
 353 1990; Tang et al. 1993a; Tang et al. 1995b; White 1990). However, this result is inconsistent
 354 with the Ca sensitivity of different *Lupinus* species grown in a hydroponic system, where *L.*
 355 *pilosus* and *L. angustifolius* were tolerant of high Ca, whereas *L. cosentinii* and *L. hispanicus*
 356 were sensitive (Ding et al. 2018b). This discrepancy might be related to different growth
 357 systems (*i.e.* sand vs hydroponics) or different $[\text{Ca}^{2+}]$ used in various experiments (4.8 g kg^{-1}
 358 vs 6 mM). Most likely, Ca is not the main reason why some *Lupinus* species are sensitive to
 359 calcareous soils (Tang et al. 1995a). This also explains why there was no correlation between
 360 Ca concentration in *Lupinus* species grown in Wangary calcareous soil and their leaf
 361 chlorosis or growth (Brand et al. 2000).

362

363 Leaf symptoms

364 The pH of the high pH and high pH + high Ca treatments was similar. Therefore, the
 365 difference in leaf chlorosis and its gradual disappearance with time among *Lupinus* species
 366 under these two treatments is likely explained by different $[\text{Ca}^{2+}]$, $[\text{HCO}_3^-]$ and pH buffer
 367 capacity associated with the different $[\text{CaCO}_3]$. In addition, high $[\text{Ca}^{2+}]$ showed no
 368 relationship with leaf chlorosis in this study. Therefore, we conclude that the gradual
 369 disappearance of the leaf chlorosis symptoms was due to a lower $[\text{HCO}_3^-]$.

370 Similarly, Brand et al. (1999) also found that leaf chlorosis of *L. angustifolius* correlated with
 371 CaCO_3 content (which can react with CO_2 and H_2O to produce bicarbonate) in a range of
 372 calcareous soils; the pH of these calcareous soils was similar. Except for leaf chlorosis, the
 373 negative effects of high pH + high Ca treatment on the growth, as shown by the biomass, of
 374 all *Lupinus* species were generally consistent with those observed under high pH, rather than
 375 with those observed under a high Ca treatment. The effects of high pH (buffered by
 376 MES/TES or caused by high $[\text{HCO}_3^-]$) on the growth of *Lupinus* species were consistent in a
 377 hydroponic study as well (Ding et al., personal observations). MES/TES or bicarbonate rather
 378 than CaCO_3 were used in the hydroponic study to maintain a stable pH is because CaCO_3 is
 379 not soluble in a hydroponic system. Taken together, this indicates that high pH is the actual
 380 cause why some *Lupinus* species are sensitive to calcareous soils, while the strong buffering

381 capacity of HCO_3^- determines if *Lupinus* species can recover from leaf chlorosis and then
382 survive in calcareous soils or not. As the strong buffering capacity of bicarbonate could
383 restrict rhizosphere acidification of carboxylic acids, and then limit nutrient availability. This
384 also explains why active lime (high Ca, high HCO_3^- and high pH) tends to have greater
385 negative effects on the growth of some calcifuge *Lupinus* species than alkalinity (high pH)
386 (Liu and Tang 1999).

387

388 **Plant growth**

389 Among all the species, the greatest tolerance of *L. pilosus* to high pH and high pH + high Ca
390 treatments is evidenced by the biomass under those treatments. In addition, the shoot and
391 total biomass of *L. pilosus* under a high Ca supply was actually significantly greater than that
392 of control plants. This confirms our finding that Ca improves the shoot growth of species that
393 grow well under an extremely high Ca concentration (Ding et al. 2018a). The negative effects
394 of high pH and/or high pH + high Ca on total root length of *Lupinus* species agree with the
395 decreased lateral root growth of some *Lupinus* species grown in either a high pH (buffered by
396 MES/TES or caused by high $[\text{HCO}_3^-]$) nutrient solution (Ding et al., personal observations) or
397 limed soil (Kerley and Huyghe 2001). This is also consistent with the decreased root length
398 of *L. angustifolius* grown in nutrient solution with high pH (>6) (Tang et al. 1993b; Tang et
399 al. 1992). Thus, we can conclude that high pH is the direct reason why root growth was
400 inhibited.

401

402 Root nodulation of all *Lupinus* species was not effective under high pH + high Ca treatment,
403 as evidenced by nodulation scores. However, nodulation of *Lupinus* species responded
404 differently to high pH treatment, with that in *L. angustifolius* being ineffective under high pH,
405 while that of calcicole species not being negatively affected by high pH. The nodule numbers
406 of *L. angustifolius*, *L. albus* and *L. pilosus* were inhibited when grown in nutrient solution
407 with high pH (buffered by MES/TES or caused by high $[\text{HCO}_3^-]$) (Tang and Robson 1993;
408 Tang and Thomson 1996). This is inconsistent with what we found; however, as pointed out
409 above, high pH + high Ca treatment contains larger $[\text{HCO}_3^-]$ and $[\text{Ca}^{2+}]$ than the high pH
410 treatment. Therefore, the buffering capacity of HCO_3^- , that is the ability to maintain a high
411 pH, was closely related with root nodulation of the calcicole species, while for the calcifuge
412 species, either high [Ca] or high $[\text{HCO}_3^-]$ inhibited root nodulation.

413

414 **Carboxylates**

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

426

427 **Leaf and root nutrient concentrations**

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

441 Based on the comparison of leaf and root P concentrations of different treatments, we found
442 the P translocation of calcifuge species from roots to shoots was inhibited by either high soil
443 [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 $[\text{HCO}_3^-]$ 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

475 calcareous soils or not. Among all studied *Lupinus* species, *L. pilosus* was the most tolerant,
476 followed by *L. cosentinii* and *L. angustifolius*, while *L. hispanicus* was the most sensitive.

477

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635

636

637 Table 1. *Lupinus* species and genotypes used in this study, and the collection site soil pH.
638 Calcicole or calcifuge was classified according to the literature (Gladstones 1970; Tang et al.
639 1995b; White 1990). “naturalised” means seeds of this species were collected from the nature
640 where they originally grow.

641

Species	Genotype	Breeding status	Collection site soil pH	Country of origin	Calcicole/calcifuge
<i>L. angustifolius</i>	Mandelup	cultivar	unknown	Australia	calcifuge
<i>L. hispanicus</i> ssp. <i>bicolor</i>	P22999	naturalised	5.7	Portugal	calcifuge
<i>L. pilosus</i>	P27440	naturalised	9.0	Syria	calcicole
<i>L. cosentinii</i>	P27225	naturalised	9.0	Morocco	calcicole

642

643

644

645 Table. 2 Calcium (Ca), exchangeable Ca^{2+} , and pH of the different treatments used in the
 646 experiment. The relative high or low concentration of bicarbonate was decided based on the
 647 amount of CaCO_3 added.

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

948

949

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

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

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

966 **Fig. 4** Net photosynthetic rate ($A_{\max}$) (A), stomatal conductance (g_s) (B), intercellular carbon
 967 dioxide concentration (C_i) (C) and the maximum photochemical quantum yield of PSII
 968 (F_v/F_m) (D) of four *Lupinus* species when grown under different pH and calcium (Ca)
 969 treatments. No data are shown for *L. hispanicus* grown under high pH + high Ca, as they died
 970 in this treatment. Error bars represent 95% confidence intervals (CI). Letters show significant
 971 differences of different treatments within each species (based on Tukey's post-hoc analysis, P
 972 < 0.05). ■ marks calcifuge species, ● marks calcicole species.

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

985 **Fig. 6** Amounts of rhizosphere citrate (A), malate (B), fumarate (C) and *cis*-aconitate (D) acid
 986 of four *Lupinus* species when grown under different pH and calcium (Ca) treatments.
 987 Rhizosphere carboxylates were extracted with 0.2 mM CaCl_2 and amounts are expressed per
 988 unit total root surface area. No data are shown for *L. hispanicus* grown under high pH + high
 989 Ca, as they died in this treatment. Error bars represent 95% confidence intervals (CI). Letters

990 show significant differences of different treatments within each species (based on Tukey's
991 post-hoc analysis, $P < 0.05$). ■ marks calcifuge species, ● marks calcicole species.

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

998

colour figure

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