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1   *Title*

2   Response-based selection of barley cultivars and legume species for complementarity: Root

3   morphology and exudation in relation to nutrient source

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24 *Abstract*

25 Phosphorus (P) and nitrogen (N) use efficiency may be improved through increased biodiversity in

26 agroecosystems. Phenotypic variation in plants' response to nutrient deficiency may influence

27 positive complementarity in intercropping systems. A multicomponent screening approach was used

28 to assess the influence of P supply and N source on the phenotypic plasticity of nutrient foraging traits

29 in barley (*H. vulgare* L.) and legume species. Root morphology and exudation were determined in six

30 plant nutrient treatments. A clear divergence in the response of barley and legumes to the nutrient

31 treatments was observed. Root morphology varied most among legumes, whereas exudate citrate and

32 phytase activity were most variable in barley. Changes in root morphology were minimized in plants

33 provided with ammonium in comparison to nitrate but increased under P deficiency. Exudate phytase

34 activity and pH varied with legume species, whereas citrate efflux, specific root length, and root

35 diameter lengths were more variable among barley cultivars. Three legume species and four barley

36 cultivars were identified as the most responsive to P deficiency and the most contrasting of the

37 cultivars and species tested. Phenotypic response to nutrient availability may be a promising approach

38 for the selection of plant combinations for minimal input cropping systems.

39 *Highlights*

40 • Phenotypic response to nutrient source in barley cultivars and legume species

41 • Divergent responses based on root morphology and exudation

42 • Potential plant combinations for improved nutrient acquisition identified

43 *Keywords* barley, legumes, plant nutrition, root morphology, exudation

44   *Abbreviations* A, ammonium-N; A_{root}, Aitchison distance of root diameter length distribution; B,

45   balanced nitrate-ammonium; CV, coefficient of variation; ΔpH, change in pH.; H^+, proton; HCO_3^-

46   bicarbonate; K^+, potassium ion; N, nitrogen; NH_4^+, ammonium; NO_3^-, nitrate; P, phosphorus; P0,

47   no P; P1, 0.5 mM P; P2, 1.0 mM P; SRL, specific root length.

48   *1. Introduction*

49   There is a mounting concern for the long-term viability of conventional cropping practices, which rely

50   on non-renewable mineral phosphate supplies to maintain yields and meet the dietary requirements of

51   a growing global population [1, 2]. Agricultural biotechnologies and practices which maximize the

52   utilization of added and endogenous soil P supplies are therefore needed to reduce the dependence of

53   agricultural production on external fertilizer inputs and minimize the loss of nutrients to surface

54   waters [3]. Intercropping of cereals and legumes has been proposed as an approach to improve crop

55   yields and nutrient use efficiency in agricultural systems through increased biodiversity, resource

56   sharing, resilience to pests, and inter-species facilitation [4]. Understanding the response of barley

57   cultivars and legume species to P supply and N source could therefore improve the selection of plants

58   for biodiverse and nutrient efficient agroecosystems.

59   Complementarity between two or more plants in poly-culture is characterized by improved resource

60   acquisition and productivity relative to a monoculture [5]. Facilitation and reduced competition for

61   soil resources by plants in poly-culture occur due to reduced competition for spatial (e.g., top-soil

62   nutrient foraging) and non-spatial soil resources (e.g., chemically distinct nutrient pools), as well as

63   enhanced productivity through N-fixation by legumes and other environmental modifications (e.g.,

64   soil moisture retention, disease suppression) [4, 6]. The success of intercropping strategies is

65   predicted to depend on architectural and anatomical properties of roots as well as the exudation of

66   carboxylates and phosphatase enzymes, which optimize the extraction of soil nutrients and

67   exploration of niche space in soil by the individual plant species [7]. If however the nutrient

68 acquisition strategies of two or more plants are too similar, for example targeting the same niche

69 space or nutrient pool in soil, competitive effects may limit the success of intercropping strategies.

70 Therefore, the plasticity of root morphology and exudation under conditions of limited or

71 heterogeneous nutrient sources is expected to minimize competition between plants and enhance the

72 acquisition of nutrients by individual plants and intercrops [8].

73 The recovery of P from organic forms is achieved by the production of phosphatases by plants or

74 microorganisms in the soil environment. The purple acid phosphatase and histidine acid phosphatase

75 classes of phytase have been characterized in plants [9, 10] and are reported to be expressed within the

76 cell and exuded under conditions of P limitation [11]. Several species of grasses (e.g., *Brachiaria,*

77 *Dactylis*) and legumes (e.g., *Stylosanthes, Medicago, Trifolium*) respond to P deficiency through the

78 increased exudation of phytase from roots [11, 12]. For example, wheat plants (*Triticum* L.) with

79 greater root-associated phosphatase activity could assimilate more P from organic forms than plants

80 with less or no activity [13]. When constitutively expressed in transgenic plants (e.g., *Nicotiana*

81 *tabacum*, *Trifolium* L.), various fungal phytases (e.g., *Aspergillus* sp., *Peniophora* sp.) are shown to

82 improve the assimilation of P from sparingly available P sources *in vitro* [14, 15]. Whilst the

83 modification of plants with single traits such as fungal phytase exudation has had a limited effect on P

84 acquisition by plants grown in unfertilized soils [16], studies with model tobacco [17] and

85 cereal/legume systems [8, 18] suggest that the combination of phytase/phosphatase exudation and

86 citrate efflux could improve the ability of plants to acquire P due to the combined action of these

87 exudates on the solubilization and mineralization of soil P [19].

88 Organic anions/carboxylates represent a major component of root exudates, which directly affect the

89 diffusivity and availability of P in soils [20]. A secondary effect of carboxylate exudation is the co-

90 transport of counter ion species (e.g., H^+, K^+, HCO_3^-) to maintain cytosolic charge balance during

91 exudation [21]. This exudation leads to the modification of rhizosphere pH with potential

92 consequences on the solubility of nutrients, enzyme function, and cascading effects within the

93   microbial community [22]. The genetic and environmental controls on carboxylate exudation (e.g.,

94   citrate, malate) have been studied extensively in cereals (e.g., *Tritucum* L., *Hordeum* L., *Zea mayes*)

95   [23-25] and are known to depend on various nutrient deficiencies (e.g., P) [20], metal toxicities (e.g.,

96   Al^{3+}, Mn^{2+}) [26], or as a mechanism for below ground C partitioning and the facilitation of microbial

97   community symbiosis [27]. Phosphorus deficiency leads to increased citrate efflux in several legume

98   species (e.g., *Medicago sativa, Lupinus* spp.) [28, 29] and may be further enhanced when ammonium

99   is supplied as the primary source of N due to rhizosphere acidification during ammonium uptake (e.g.,

100 *Lupinus albus*) [30, 31]. In contrast, nitrate acts as a signal to induce the production of organic anions

101 in tobacco (*Nicotiana tabacum*), which act as receptors of nitrate or counter ions for the maintenance

102 of cytosolic pH [32]. Citrate efflux in barley (*H. vulgare* L.) is primarily studied with regard to its

103 genetic variation across cultivars or role in Al^{3+} toxicity tolerance in acid soils and is therefore

104 typically assessed under either P sufficient or deficient conditions [33-35]. To our knowledge, there

105 are no reports of citrate efflux among barley cultivars being affected by both P supply and N source

106 (NH_4^+, NO_3^-).

107 Root plasticity in response to selective pressure (e.g., nutrient supply/source) allows plants to explore

108 heterogeneous soil environments and forage for nutrients [7]. Common physiological responses of

109 cereals and legumes to P deficiency include the partitioning of biomass to roots, increased production

110 of fine roots, and the generation of 'low metabolic cost' roots, characterized by increased proportion

111 of aerenchyma cells and greater root length relative to root biomass (i.e., specific root length, SRL;

112 [36-42]. The initiation or inhibition of root branching and elongation is also affected by N source

113 (NO_3^-, NH_4^+). For example in barley and wheat, the localized application of nitrate initiates the growth

114 and extension of seminal and lateral roots [43-45]. Plants provided with ammonium can suppress root

115 branching and elongation in the absence of P, with these effects reversed and associated with

116 improved seedling growth at higher rates of P application [46, 47]. If yields in cereal and legume

117 systems are significantly impacted by root architectural [5] and morphological traits, which affect the

5

118   acquisition of soil mineral nutrients (e.g., lateral root angle, rhizosheath, SRL), the selection of plants

119   with traits appropriate to a particular growth environment will be needed [48].

120   The effective combination of traits for the efficient recovery of P in complementary plant systems

121   must also consider the genotypic variation of physiological and biochemical responses of plants to

122   nutrient availability [49]. Therefore, the objective of this study was to take a systematic approach to

123   the selection of barley cultivars and legumes species based on the morphological and biochemical

124   response of genotypes to P supply and N source. We assessed root exudation (citrate efflux, phytase

125   activity, pH change) and root morphological traits (root length, specific root length, root diameter size

126   distribution) and identified plants with the greatest potential to access sparingly available or poorly

127   soluble P in soil.

128   *2. Materials and methods*

129   2.1 Plant materials

130   Barley seeds (*Hordeum vulgare* L.) from a genome-wide association mapping collection (144 elite

131   European germplasm) and previously assessed for P use efficiency and rhizosheath [50, 51] were used

132   for the initial screening in hydroponics, with a sub-set of these selected for further characterization

133   following growth in sterile sand (Table A.1). Seeds from six pasture legumes representing a range of

134   root morphological [36] and exudation characteristics [52] were obtained from the New South Wales

135   Department of Primary Industries, Wagga Wagga Agricultural Institute, NSW, Australia. These

136   legume species, originally sourced from the southern Mediterranean and studied extensively in

137   Australian pasture systems [53], were: Subterranean clover (*Trifolium subterraneum* cv. Leura),

138   Purple clover (*Trifolium purpureum* cv. Electra), Biserrula (*Biserrula pelecinus* cv. Casbah), Yellow

139   serradella (*Ornithopus compressus* cv. Santorini), French serradella (*Ornithopus sativus* cv.

140   Margurita), and Barrel medic (*Medicago truncatula* cv. Sultan; Table A.1).

6

141   2.2 Chemical and Enzyme Sources

142   Standard nutrient salts were sourced from Sigma-Aldrich or BDH for all plant growth experiments.

143   *Myo*-inositol hexakisphosphate dodecasodium heptahydrate salt (InsP6; Sigma-Aldrich P8810;

144   Gillingham, UK) was used for the determination of phytase activity in plant exudate solutions.

145   Ammonium sulphate suspensions of lactic dehydrogenase (LDH; Sigma-Aldrich L2500), malic

146   dehydrogenase (MDH; Sigma-Aldrich M1567), β-Nicotinamide adenine dinucleotide (NADH;

147   Sigma-Aldrich N4505), citrate lyase from *Klebsiella pneumoniae* (CL; Roche Ltd., West Sussex,

148   UK), and a stock citrate standard from Fluka Analytical (Seelze, Germany) were used for the analysis

149   of citrate in plant exudate solutions.

150   2.3 Exudate Collection Following Growth in Hydroponics

151   One-hundred and forty-three of the 144 barley cultivars were screened in hydroponics for root growth

152   and pH response to P deficiency in order to select a sub-set (n=12) for determination of citrate efflux

153   and phytase activity in exudates. Seeds were pre-germinated on distilled water agar (1% agarose w/v).

154   After three days, when radicles were approximately 1 cm long, 5 replicate seedlings were planted in

155   hydroponic solutions and grown for 3 weeks in batches of 90 plants per 60 L. The standard nutrient

156   solution (pH 5.5) contained 3 mM NH_4Cl, 4 mM $Ca(NO_3)_2$, 4 mM KNO_3, 3 mM $MgSO_4$, 0.1 mM Fe-

157   EDTA with micronutrients (6 μM $MnCl_2$, 23 μM H_3BO_3, 0.6 μM $ZnCl_2$, 1.6 μM $CuSO_4$, 1.0 μM

158   Na_2MoO_4, 1.0 μM $CoCl_2$) and was either supplemented with 1 mM KH_2PO_4 or left unamended.

159   Nutrient solutions were changed on a weekly basis beginning with a quarter strength solution,

160   followed by half strength, and then full strength nutrients for the final week of the experiment. The pH

161   in nutrient solutions was adjusted to 5.5 using sodium hydroxide as necessary. Due to the size of the

162   experiment, four screening cycles of 36 cultivars (5 replicates each, including one plant control, cv

163   Optic) were carried out for each P condition. Plants were grown for three weeks under controlled

164   conditions (22°C day 16h/14°C night, 200 W m^{-2}) and then transferred to 50 mL of P-free nutrient

7

165 solution for exudate collection over 24h. Shoot and root materials were collected for biomass weight

166 determination after drying for 1 week ($70^{o}C$).

167 2.4 Exudate Collection Following Growth in Sterile Sand

168 A representative subset of 12 barley cultivars (cvs Domen, Chieftan, Dialog, Waggon, Spire,

169 Thuringia, Kym, Prague, Aramir, Krystal, Rainbow, Kenia) and the six legumes (Table A.1) were

170 selected for exudate screening following six weeks of growth in sterile sand. Course river sand was

171 washed through a 500 micron sieve and potted (250 – 300 g air-dried sand) prior to sterilization by

172 autoclaving ($180^{o}C$). Seeds were vapour sterilized as described previously by enclosing seeds in an

173 airtight container for 1 h with a solution containing 100 mL hypochlorite solution (4% w/v) and 3 mL

174 concentrated hydrochloric acid [14]. Seeds were germinated on sterile distilled water agar (0.1% m/v)

175 for 2 d prior to planting, after which time plants were monitored for incomplete emergence and

176 replaced with germinated seeds to achieve one plant per pot. Plants were supplied with 20 mL of full-

177 strength nutrients each day during the 21 d growth period in a glasshouse ($22^{o}C/14^{o}C$ day/night) with

178 16 h light and additional lighting provided at incident radiation less than 200 W m^{-2}. Five replicate

179 pots were prepared for all cultivars and nutrient conditions including plant-free controls, which

180 received nutrients for the duration of the growth period.

181 Plant nutrient solutions were adjusted to pH 5.5 with 10 M sodium hydroxide and filter sterilized (0.3

182 μm pore size) before use. The N-balanced treatment (B) included equal molarities (6 mM) of NO_3^--N

183 and NH_4-N and other macronutrients as described for the hydroponics experiment above. The

184 ammonium treatment (A) contained 9 mM NH_4Cl and 1 mM each of $Ca(NO_3)_2$ and KNO_3.

185 Phosphorus was added to each N treatment as KH_2PO_4 at three concentrations (mM): 0.0 (P0), 0.5

186 (P1), 1.0 (P2). The resulting solutions are annotated based on the combination of nutrient conditions

187 as follows: low P (P0XA, P0XB), intermediate P (P1XA, P1XB), and high P (P2XA, P2XB).

8

188   At the end of the growth period, plants were carefully removed from the sand pots and rinsed

189   thoroughly with tap water for removal of sand. Plants were transferred to 30 mL of the P-free nutrient

190   solution corresponding to the appropriate N treatment (P0XA or P0XB). Plant exudates were

191   collected for 2 h in the laboratory at ambient temperature (approx. $20^{\circ}C$) and light. Filtered exudate

192   solutions (0.2 µm, PES) were stored immediately for analysis of pH ($4^{\circ}C$), phytase activity ($4^{\circ}C$),

193   dissolved organic C and N, and organic anion composition ($-20^{\circ}C$). Sand remaining in pots after the

194   plant harvest was stored at $4^{\circ}C$ for pH determination in 0.01 M $CaCl_2$ (1:2 w/v).

195   2.5 Exudate Analysis

196   The pH of exudate solutions was measured within one week of collection using a combination

197   electrode (Mettler Toledo, Ltd., Leicester UK) and compared to blank P-free collection solutions to

198   determine the relative ability of plants to alkalize or acidify the starting solution from pH 5.5.

199   Exudates collected from plants grown in sterile sand were assayed for phytase activity and citrate.

200   Phytase activity was measured as described by Hayes et al. [54] and modified by Giles et al. [14].

201   Briefly, 240 µL of exudates were combined with 30 µL150 mM MES (pH 5.5) and 30 µL of 20 mM

202    $Na_{12}IHP$ and incubated at $37^{\circ}C$ for one hour. The reaction was stopped immediately (t=0) or after one

203   hour (t = 60 min) by adding equal parts of incubation solution to chilled 10 % trichloroacetic acid.

204   Phosphate in stopped reaction solutions was measured by malachite green colorimetry [55]. The

205   difference in phosphate concentration for a given sample was proportional to phytase activity as

206   expressed in nKat and normalized to root dry weight and the exudate collection period (nKat g^{-1} root

207   dry wt. h^{-1}).

208   Citrate was assayed enzymatically according to Dagley [56] with the following modifications. Freeze-

209   dried exudate solutions were reconstituted at 8.33 times the original concentration by adding 1 mL

210   MilliQ water and 125 µL Tris-HCl (1 mM, pH 8). To 250 µL of exudate solutions, 4 µL NAD

9

211   solution (8 mg NAD and 7 mg NaHCO$_3$ in 1 mL water) and 2 μL of 1:1 solution of LDH and MDH

212   were added. Samples were allowed to equilibrate for 1h at room temperature in order for natural

213   NADH depletion to stabilize. Two μL citrate lyase (CL; 100 mg mL^{-1}) was added to half of the well

214   replicates (n=4) and incubated for an additional hour. The concentration of NADH was measured at

215   340 nm. The depletion of NADH in wells treated with CL was proportional to citrate concentration in

216   standards (0, 5, 10, 15, 20, 40, 60, 80 nmol citrate). All standard solutions were prepared in blank

217   P0XA or P0XB solutions containing 8.33 times nutrient salts.

218   2.6 Shoot and Root Analysis

219   Plants were separated into above- (shoots) and below- (roots) ground biomass. Shoots were oven

220   dried for 48 h (70°C) and weighed for the determination of dry weight. Roots from exudate screening

221   experiments in sterile sand were stored at 4°C in 50% ethanol (v/v) prior to root scanning (EPSON,

222   Hertfordshire, UK) and image analysis. Root images (300 dpi, grey scale) using the Lagarde

223   transformation for pixel identification and analysed for total root length (cm), average diameter (mm),

224   and root lengths in each diameter size class (in 0.1mm increments to >1.9mm) using the root

225   architectural algorithm in WinRHIZO (Regent Instruments, Inc., Quebec, Canada). The percentage of

226   root length in each diameter size class was calculated relative to the total root length determined for

227   individual plant replicates.

228   2.7 Statistical Analysis

229   Means and standard errors are presented for five replicate plants and three technical replicates for

230   citrate and phytase-activity measurements. For exudate screening in sterile sand, Tukey Least Square

231   Difference (LSD <.05) was used to compare plant growth and exudate characteristics of cultivars

232   within a single nutrient condition and across nutrient conditions for a single cultivar. Principal

233   component analysis (PCA) was used to visualize and quantify the variation in plant response to the six

234   nutrient treatments based on physical root parameters and exudation traits. All variables were checked

235   for normality and those not normally distributed were log-transformed prior to correlation and

236   significance testing (Pearson pair-wise, p<.05). Aitchison distance was calculated to identify system

237   wide changes in the distribution of root diameter size classes in response to nutrient treatment and

238   defined as A_{root}. The length distributions of root diameter size classes (0 to >1.9mm, 0.1mm

239   increments) were transformed using the isometric log ratio (*ilr*) procedure (Equation A.1) and a

240   sequential binary partition matrix (Table A.2)[57]. Aitchison distances (Equation A.2) [58, 59] were

241   computed for each nutrient treatment (P1XB, P2XB, P0XA, P1XA, P2XA) relative to the reference

242   nutrient condition (P0XB) for each barley cultivar and legume species based on the averaged sum of

243   *ilr* values (n=5). The variance of A_{root} was determined using the propagation of error procedure for the

244   difference of means with equal variance (n=5). The 95% confidence interval (n-1=4 degrees of

245   freedom) was determined for comparison of mean A_{root} values across plant and nutrient treatments.

246   ***3. Results***

247   3.1 Hydroponics Screening of Barley under P-Deficient and P-Sufficient Conditions

248   3.1.1 Root Morphology and Exudate pH Change

249   In order to evaluate the response of barley cultivars to P deficiency, root morphological characteristics

250   and pH change of exudate solutions was assessed following three weeks growth in hydroponics with

251   (P1) and without added P (P0). Phosphorus deficiency led to significant changes in the morphological

252   characteristics of roots among the 143 barley cultivars (5 replicates each) tested. Averaged across all

253   cultivars, root dry weight, root surface area, and total root length were significantly larger in P-

254   deficient plants compared to plants grown with P (p<.001; Table 1). The proportion of roots smaller

255   than 0.5 mm and larger than 3 mm in diameter increased due to P-deficiency, whereas intermediate

256   diameter roots (0.5 – 3 mm) either decreased or stayed the same (Table 1). For roots greater than 3

257 mm in diameter, the length of subsequent size classes increased progressively from 23.5% to 111%.

258 The lengthening of thicker roots due to P deficiency is also reflected by increases in total root length,

259 dry weight, and surface area. These trends represent the average response of the entire population to

260 P-deficiency and a large variability of root morphological traits among individual cultivars.

261 Differences in the P0 and P1 values of root diameter proportions and total surface area have been used

262 here to indicate the response of individual cultivars to P-deficiency, whereby positive differences

263 indicate root elongation or increased surface area, and negative differences indicate shortening or loss

264 of surface area. There were significant positive relationships between the change in total root length

265 with dry weight (r=0.61, p<.0001) and surface area (r=0.56, p<.0001) due to P-deficiency for the

266 entire population (Table 1). Difference values for the proportion of roots in specific diameter size

267 classes displayed significant positive relationships with total root surface area (r>0.51, p<.0001; Table

268 1) with the exception of roots <0.5mm in diameter. On average, roots less than 0.5 mm in diameter

269 were ~6% more abundant in P0 relative to the P1 condition (Table 1); however, the greater length of

270 <0.5mm roots was related to the net loss of root surface area (r=-0.57, p<.0001). On average, specific

271 root length (m g^{-1}) was approximately 12% larger in barley cultivars provided with P (p<.0001; Table

272 1) with 58% of cultivars increasing SRL in response to P deficiency. Therefore, the lengthening of

273 thicker roots and an increased proportion of fine roots dominated the physiological response of barley

274 cultivars with a large variation among cultivars identified based on the SRL (Fig. 1).

275 The growth of barley cultivars in P0 and P1 hydroponics solutions resulted in significant differences

276 in the ability of P0 and P1 plants to affect the pH of exudate collection solutions. Although the

277 average pH in exudate solutions from P-deficient plants (6.28±0.29) was not significantly different

278 from P-sufficient plants (6.12±0.29), exudate solutions from plants supplied with P contained a wider

279 range of pH (4.12-7.19) in comparison to P-deficient plants (5.07-6.85) and were generally more

280 acidic (Table 1, Fig. 1). Individual cultivars varied in their ability to change the pH from the starting

281 value of 5.5 in the P0 (ΔpH range: -0.57 - +1.81 pH units) and P1 treatments (ΔpH range: -1.48 -

282 +2.00 pH units; Fig. 1, Fig. A.1).

283 3.1.2 Selection of Barley Cultivars for Further Study

284 A subset of 12 barley cultivars were selected for the screening of citrate efflux and exudate phytase

285 activity based on changes in specific root length and exudate pH in response to P-deficiency (Table 1,

286 Fig. 1). Fig. 1 shows the wide range of responses among individual cultivars based on these two

287 variables. More than 50% of cultivars alkalized the pH of exudate collection solutions in response to

288 P-deficiency, whereas less than 25% responded by acidifying the media. Phosphorus deficiency led to

289 increased SRL in less than half of the population with changes ranging from +100 to -250 m g^{-1} root

290 dry wt. (Fig. 1). The cultivars selected for further screening included those representing extremes in

291 pH change (cv Domen, -0.67; cv Kenia, +1.8) and SRL (cv Aramir, -251.5 m g^{-1}; cv Chieftain, +65.9

292 m g^{-1}) as well as cultivars with a minimal response to P deficiency based on one or both of these

293 metrics (e.g., cvs Waggon, Spire, Kym; Fig. 1). Five cultivars responded to P deficiency with gains in

294 SRL, which were associated with acidification (cvs Chieftan, Dialog, Spire) or alkalization of exudate

295 solutions (cvs Prague, Rainbow). Of the seven cultivars that expressed reduced SRL due to P

296 deficiency, one acidified (cv Domen), three had no effect on pH (cvs Waggon, Kym, Thuringia), and

297 three alkalized the media (cvs Aramir, Krystal, Kenia; Fig. 1).

298 3.2 Screening of Barley Cultivars and Legume Species for Root Morphological Characteristics and

299 Exudation of Citrate and Phytase

300 Twelve barley cultivars and six legume species were grown in sterile washed river sand for 3 weeks in

301 order to evaluate shoot and root growth, root morphological characteristics, and the exudation of

302 citrate and phytase in response to 6 nutrient conditions (P0XA, P1XA, P2XA, P0XB, P1XB, P2XB),

303 representing various combinations of P supply (P0=0, P1=0.5, P2=1.0 mM) and N source

304 (A=Ammonium; B='Balanced' nitrate-ammonium N).

305 3.2.1 Shoot and root Growth of Barley Cultivars in Sterile Sand

306 Phosphorus supply (p<.0001) and N source (p=0.0003; Table 2) had a significant effect on shoot

307 biomass and R:S ratios in barley. Barley cultivars were responsive to P supply by increasing the

308 length of roots with no added P (P0) and greater shoot biomass accumulation with the greatest P

309 addition (P2). Across nutrient treatments, shoot biomass in barley ranged from 0.05 to 0.69 g dry

310 weight and increased with greater P supply (p<.0001; Table 2, Table 3). Under P deficient conditions,

311 there was no significant difference in shoot dry weight among barley cultivars supplied with

312 ammonium or balanced N (CV=0.20; Table 2), with the exception of the large biomass of cv Waggon

313 (0.23 g) and small biomass of cv Prague in P0XB (0.05 g; Fig. 2). Root to shoot ratios were, on

314 average, 3.4-fold larger in the P deficient treatments (P0XA: 0.36; P0XB: 0.51) compared to P

315 sufficient treatments (P2XA: 0.13; P2XB: 0.13; Fig. 2), indicating the partitioning of resources to root

316 biomass in response to P deficiency.

317 There was significant variation in shoot biomass (p=0.0002) and R:S ratio (p<.0001) among

318 individual barley cultivars (Table 2). With respect to R:S, the interaction identified between cultivar

319 and nutrient treatment (p<.0001; Table 2) was related to the greater variability of root and shoot

320 biomass measurements among cultivars supplied with balanced N in comparison to ammonium-fed

321 plants. Shoot biomass of barley was significantly greater when plants were supplied with a balanced

322 N source (in g dry wt. P1XA: 0.41±0.10; P1XB: 0.49±0.13; P2XA: 0.48±0.06; P2XB: 0.53±0.11; Fig.

323 2). The coefficients of variation for shoot dry weights in the P1 and P2 treatments were also larger

324 among cultivars supplied with balanced N (P1XB: CV=0.28; P2XB: CV=0.22) in comparison to the

325 ammonium-fed plants (P1XA: CV=0.25; P2XA: CV=0.13).

14

326   Specific root length ranged from 54 (cv Prague, P1XB) to 519 m g^{-1} (cv Rainbow, P2XB) and varied

327   significantly across nutrient treatments (p<.0001; Table 2, Fig. 2). On average, SRL was consistently

328   greater when plants were provided with ammonium and minimal P (in m g^{-1} P0XA: 280; P0XB: 220;

329   P1XA: 270; P1XB: 123; P2XA: 217; P2XB: 258; Table 2). This was in part due to root dry weights in

330   ammonium treatments, which were 1.3 to 2.3-fold less than plants supplied with balanced N across P

331   treatments. Nitrogen source had a greater effect on SRL (p=0.0001) than P supply (p=0.0166), but

332   interacted with P supply (p=0.0003) to significantly affect SRL in the population of barley cultivars

333   tested (Table 2).

334   Aitchison distance (A_{root}) was derived from the length of roots in the various root diameter size classes

335   of the barley cultivars. A_{root} was used to compare the root morphology of cultivars in P0XB (reference

336   condition) to plants grown in the other nutrient treatments (Fig. 4). Nutrient treatments with

337   increasing P and N provided as ammonium significantly affected the distribution of root lengths in the

338   various diameter size classes for the majority of barley cultivars tested, including Aramir, Chieftan,

339   Kenia, Krystal, Kym, Prague, Rainbow, Spire, and Waggon (p<.05; Fig. 4). In contrast, there was no

340   significant change in A_{root} among Dialog, Domen, and Thuringia cultivars, relative to plants grown in

341   P0XB (p<.05; Fig. 4). A_{root} increased with increasing levels of P for Aramir, Kenia, Krystal, Spire,

342   and Waggon cultivars, however this affect was more pronounced when ammonium was provided as

343   the primary N source. The increasing trend in A_{root} with greater P is reflected in the raw proportions of

344   root lengths in the smallest diameter classes, for example in the 0-0.1 and 0.1-0.2 mm (Fig. A.2). The

345   response of barley cultivars to the nutrient treatments was therefore associated with a global changes

346   to root morphology, including a shift in the proportion of roots from larger to smaller diameter size

347   classes.

348   3.2.2 Shoot and Root Growth of Legumes in Sterile Sand

349   In terms of shoot biomass, legumes responded to P supply ($p<.0001$) and N source ($p=0.0124$), with

350   significant differences identified between cultivars and across all nutrient treatments ($p<.0001$; Table

351   2). Legume species increased shoot biomass with increasing P supply from a minimum of 0.08 in the

352   P0XB treatment (*T. purpureum*) to 0.25 g dry wt. in P2XB (*T. subterraneum*; Fig. 3). Under P-

353   deficient conditions, legumes provided with ammonium produced larger shoot biomasses (0.16 ± 0.00

354   g dry wt.) compared to balanced N (0.10 ± 0.01 g dry wt.), whereas at larger P treatments, legumes

355   provided with balanced N were larger (e.g., in g dry wt. P2XA: 0.14, P2XB: 0.20; Fig. 3). Legumes

356   responded to P deficiency by partitioning more biomass to roots, as indicated by larger R:S ratios in

357   the lowest P treatments (Fig. 3) and significant interactions of R:S with P supply and N source

358   ($p<.0001$; Table 2). Across P treatments, average R:S ratios of legumes provided with ammonium as

359   the primary N source were 1.5 to 2-fold greater than plants provided with balanced N (Fig. 3).

360   However, unlike barley, legume species did not have a significant effect on R:S ratios ($p=0.1812$;

361   Table 2).

362   Specific root length of individual legume species ranged from 0.3 to 38.2 m g^{-1} dry wt. and was

363   significantly affected by P supply, N source, and cultivar type ($p<.0001$), with no interactions

364   identified between nutrient treatment and cultivar ($p=0.2538$; Fig. 3, Table 2). Averaged across

365   legume species, SRL was greatest for plants supplied with ammonium as the primary N source and

366   increased with added P (e.g., in m g^{-1} root dry wt. P0XA: 7.8, P0XB: 4.5; P2XA: 20.8, P2XB: 8.3).

367   The effect of the N source was more pronounced for some species, such as *M. truncatula*, *O. sativus*,

368   and *T. subterraneum*, which in terms of SRL, responded to increasing P more dramatically when

369   supplied with ammonium as the primary source of N (Fig. 3).

370   Aitchison distances of root lengths in various diameter size classes were larger and more variable

371   (A_{root}: -5.7 to 15.4) than the barley cultivars tested (A_{root}: -6.0 to 7.1; Fig. 4). All legume species

372   responded to the nutrient treatments through a change in the distribution of root diameter length

373   distributions at the greatest P levels (P2XA, P2XB). *Medicago truncatula* cv Sultan was the only

374 legume to show a significant shift in A_{root} under all nutrient conditions relative to the reference. There

375 was an increasing trend of A_{root} with P supply in the ammonium treatments of *O. sativus* and *T.*

376 *subterranem*, with a significant difference found between P0XA and P2XA treatments only (Fig. 4).

377 The A_{root} of *O. sativus* and *T. subterranem* corresponded to SRL, which increased with P supply in the

378 ammonium treatments (Fig. 3). This was in contrast to *Medicago*, which displayed the greatest

379 increase in SRL with P supply despite having similar A_{root} values at P0XA and P2XA (Fig. 4).

380 3.2.3 Exudation Response of Barley cultivars to P and N Treatments

381 Relative to uncultivated controls, the average pH change of the sterile sand media (ΔpH) by barley

382 cultivars ranged from -0.23 to +0.38 pH units depending on nutrient treatment (Fig. 5). P supply had a

383 more significant effect on ΔpH (p<.0001) than N supply alone (p=0.4105; Table 3). In general, the

384 average pH change caused by barley cultivars was positive and most pronounced in P deficient

385 treatments (P0XA: +0.23±0.12; P0XB: +0.13±0.08), whereas plants supplied with P did not

386 significantly affect the pH of the sand media (P2XA: -0.07±0.06; P2XB: 0.02±0.05; Fig. 5). There

387 was a significant interaction between P supply and N source on ΔpH by barley (p<.0001; Table 2).

388 For example, ΔpH of plants provided with ammonium as the primary source of N was greater than in

389 the N-balanced plant treatment under P deficiency, whereas small differences between N treatments

390 were observed as P addition increased (Fig. 5). Consistent with results of the barley screening in

391 hydroponics (Fig. 1), there was significant variation in the ability of individual cultivars to affect pH

392 of the growth media under different nutrient treatments (p<.0001; Table 2).

393 As for ΔpH, citrate efflux was significantly affected by P supply (p=0.0408), and not N source

394 (p=0.1974), with a significant interaction between P supply and N source identified in barley

395 (p=0.0317; Table 2). On average, citrate efflux by barley cultivated under P deficiency did not differ

396 significantly between N treatments, but was 2.4 fold greater in plants provided with balanced N at the

397 largest P additions (Fig. 5). The interaction of P supply and N source is evident when considering

398   citrate efflux by plants provided with ammonium as the primary source of N, which was greatest

399   under P deficiency and declined with increasing P (in μmol g^{-1} dry wt. h^{-1} P0XA: 44.0±20.8; P2XA:

400   14.9±7.9). In contrast, plants cultivated under balanced N displayed the opposite trend, with the

401   greatest citrate efflux being measured in the largest P treatment (in μmol g^{-1} dry wt. h^{-1} P0XB:

402   26.1±10.5; P2XB: 36.7±13.8; Fig. 5). A significant variation in the ability of individual barley

403   cultivars to exude citrate was identified (p=0.0005) and was found to depend on the nutrient treatment

404   provided (p=0.0005; Table 2); for example, in the extreme cases of cvs Krystal (8.9 μmol g^{-1} dry wt.

405   h^{-1}) and Waggon (81.6 μmol g^{-1} dry wt. h^{-1}) in P0XA or cvs Spire (12.5 μmol g^{-1} dry wt. h^{-1}) and

406   Aramir (63.1 μmol g^{-1} dry wt. h^{-1}) in the P2XB nutrient treatment (Fig. 5).

407   Phytase activity ranged from 0.02 to 0.23 nKat g^{-1} root dry wt. h^{-1} and was not detected in all nutrient

408   treatments for the barley cultivars tested (Fig. 5). P supply did not have a significant effect

409   (p=0.4787), whereas N source (p=0.0028) and its interaction with P supply (p=0.0062) were

410   significant factors affecting exudate phytase activity in barley (Table 2). On average, phytase activity

411   was greatest for plants grown under P deficient conditions with ammonium as the primary N source

412   (0.16±0.06 nKat g^{-1} root dry wt. h^{-1}) and declined as P increased (P2XA: 0.08±0.04 nKat g^{-1} root dry

413   wt. h^{-1}; Fig. 5). In contrast, plants provided with balanced N displayed less exudate phytase activity

414   under P deficiency (0.09±0.05 nKat g^{-1} root dry wt. h^{-1}; Fig. 5) and did not vary significantly with P

415   treatment. There was no significant effect of cultivar on the phytase activity of barley exudates

416   (p=0.4503), however individual cultivars did respond differently to the various nutrient treatments

417   (p=0.0119; Table 2); for example, cv Prague, which varied considerably with N source (P0XA: 0.05;

418   P0XB: 0.15 nKat g^{-1} root dry wt. h^{-1}), or cv Waggon, which did not differ in exudate phytase activity

419   across nutrient treatments (Fig. 5).

420   3.2.4 Exudation Response of Legumes to P and N Treatments

421   All legume species and nutrient treatments led to a decline in pH of the sterile sand growth media

422   (Fig. 6). P supply affected ΔpH in the exudate solutions of legumes (p<.0001), whereas N source did

423   not (p=0.4105); however, a significant interaction between P supply and N source was observed

424   (p<.0001; Table 2). On average, there was no difference in ΔpH for legumes cultivated with

425   ammonium and balanced N under P deficiency (-0.40 to -0.46 pH units) or the intermediate P addition

426   (-0.14 to -0.15 pH units); however, plants in the P2 treatment showed a significant acidification of the

427   sand media when provided with ammonium (-0.66±0.01) in comparison to balanced N (-0.17±0.02;

428   Fig. 5). Significant differences between legume species were observed (p=0.0037) with the response

429   of individual legumes depending on the nutrient treatment (p<.0001; Table 2). For example,

430   acidification by *O. sativus* relative to other legumes in the balanced N treatments was greater under P

431   deficient (-0.19 to -0.31) than under P sufficient (-0.04 to -0.22) conditions (Fig. 6).

432   Citrate efflux ranged from 2.4 to 74.0 µmol g^{-1} dry wt. h^{-1} and was significantly affected by P supply,

433   N source, and the interaction of nutrient factors (p<.0001; Fig. 6, Table 2). As for ΔpH, there was no

434   difference between citrate efflux between the N treatments in the P deficient condition (10.3 to 10.7

435   µmol g^{-1} dry wt. h^{-1} on average). However, as P supply increased, the average difference between

436   citrate efflux in the two N treatments increased by 2-fold at P1 and 4-fold at P2 (Fig. 5). Legume

437   species did not significantly affect citrate efflux (p=0.1412) unless nutrient treatment was also

438   considered (p<.0001), as illustrated by the increasing variation in citrate efflux P supply by legumes

439   provided with balanced N and the greatest amount of P (e.g., in µmol g^{-1} dry wt. h^{-1}: *B. pelecinus*: 2.4

440   vs *O. sativus*: 74.0; Fig. 6, Table 2).

441   Phytase activity occurred in a similar range for legumes as for barley (0.01 to 0.25 nkat g^{-1} root dry

442   wt. h^{-1}; Fig. 6); however in contrast to barley, legume phytase activity was effected by P supply

443   (p=0.0429) rather than N source (p=0.1238) and no interaction was found between the two nutrient

444   conditions (p=0.1315; Table 2). On average, legume phytase activity was greatest in the P deficient

445   condition and did not differ significantly between N treatments (in nKat g^{-1} root dry wt. h^{-1} P0XA:

446   0.13±0.03; P0XB: 0.10±0.06). The variation between individual legume species was weakly

447   significant (p=0.0482) and individual legume species responded differently to the various nutrient

448   treatments in terms of phytase activity (p=0.0004; Table 2). For example, *T. subterraneum* plants

449   provided with ammonium as the primary N source had greater phytase activity in exudates compared

450   to balanced N plants across P treatments (e.g., in nKat g^{-1} root dry wt. h^{-1} P0XA: 0.17; P0XB: 0.08).

451   In contrast, phytase activity was not detected in the exudates of *O. sativus* at P0 but increased to a

452   maximum among legumes at P2, particularly when provided with balanced N (0.23 nKat g^{-1} root dry

453   wt. h^{-1}; Fig. 6).

454   3.3 Multivariate Analysis of Root Morphological and Exudation Traits in Barley and Legumes

455   Principal component analysis was used to assess the contribution of plant-induced pH change, citrate

456   efflux, exudate phytase activity and SRL to the variation in response of barley cultivars and legume

457   species to P supply and N source. Principal component 1 (PC1) accounted for 48.6% of the variation

458   between treatments and was primarily explained by SRL (0.854), citrate efflux (0.781), and ΔpH

459   0.749), whereas PC2 (27.0%) was primarily influenced by differences in phytase activity (0.944; Fig.

460   7). The shift in values along the PC1 axis illustrates the contrasting responses of barley cultivars and

461   legume species to N source regarding citrate efflux, which was most pronounced under P deficient

462   conditions but greatest in legumes with balanced N (Fig. 7). The response of barley to ammonium is

463   observed in a shift to more positive loading values along the PC1 and PC2 axes, corresponding to

464   increased citrate efflux and exudate phytase activities, particularly under P deficiency (Fig. 7). In

465   contrast, the distribution of legume loadings shows a large variation in ΔpH and exudate phytase

466   activity and a more restricted response of plants in terms of SRL and citrate efflux (Fig. 7).

467   **4. Discussion**

20

468   We investigated root morphological and biochemical responses of several barley cultivars and legume

469   species to P limitation and N source in order to identify plant combinations for complementarity and

470   facilitation. Root morphology (R:S, SRL, A_{root}) and exudation (citrate efflux, phytase activity) varied

471   with P supply and N source, as well as plant cultivar and species. We identified significant effects and

472   interactions of these factors on the measured root traits, with contrasting responses to six nutrient

473   treatments among barley cultivars and legume species, specifically with regards to citrate efflux, pH

474   change, and root diameter size distribution (A_{root}). Whilst the response of barley and legume varieties

475   to the nutrient treatments were generally consistent with the literature (e.g., root elongation response

476   to P deficiency, stimulation/inhibition of root growth with ammonium), our results provide additional

477   information on the conservation and plasticity of biochemical (e.g., citrate, phytase) and

478   morphological (e.g., SRL) root traits, as well as a compositional metric for describing the entire

479   distribution of root lengths in various diameter size classes (A_{root}). Based on this analysis, we identify

480   promising barley cultivars and legume species for testing some of the questions and ecological

481   principles pertaining to complementarity and growth facilitation between multiple plant species and

482   further discuss the potential importance of selecting companion plants with contrasting responses to

483   nutrient source.

484   4.1 Conservation of Specific Root Length in Legumes Across Nutrient Treatments

485   Yang et al. [36] reported SRL in legume varieties following six weeks growth in defined soil mixtures

486   with rhizobial inoculation and superphosphate amendment, which were one to two orders of

487   magnitude larger than those measured in the current study and which followed the order (in m g^{-1}): *T.*

488   *subterraneum* 159; *T. purpureum* 177; *M. sativa* 209; *B. pelecinus* 299; *O. compressus* 307; *O. sativus*

489   320. Our results indicate that the relative ranking of legumes based on SRL was consistent across

490   nutrient treatments and followed the order (in m g^{-1}): *M. truncatula* 19; *T. subterraneum* 10; *T.*

491   *purpureum* 9; *O. sativus* 8; *B. pelecinus* 2; *O. compressus* 1 (Fig. 3). This is consistent with the

492   prediction that, although the response of these legumes to nutrient availability may vary, the relative

21

493   ranking of intrinsic root traits such as SRL should be conserved [36]. We can also confirm that the

494   relationship between the length of fine roots (<0.1mm diam.) and SRL is conserved across nutrient

495   treatments for these legume varieties (r=0.84, p<.0001; Table 3). However, the rankings of *Medicago*

496   and *Biserulla* relative to other legume genera differed in this study relative to the report of Yang et al.

497   [36]. An important difference between these studies was the use of rhizobial inoculants. In the current

498   study, legumes were cultivated in sterile sand and provided with N in order to optimize the recovery

499   of root carboxylates, which, as a labile source of C, are readily degraded by soil microorganisms.

500   Rhizobia play an important role in nodulation as well as root proliferation, branching and pathogen

501   resistance in legumes [60]. In *Vigna* spp. for example, root length, number, branch points, and weight

502   were 67 to 100% reduced in uninoculated plant treatments [61] with similar effects on root biomass

503   accumulation reported in soybean (*Glycine max*)[62]. This indicates a significant effect of rhizobia on

504   the physical development and absolute magnitude of SRL, which may be exacerbated in plants

505   cultivated in sterile sand. This warrants further investigation into the dependence of individual

506   legumes on rhizobia for stimulating root growth as well as SRL values and ranking among other

507   legume varieties.

508   4.2 Plasticity of Root Diameter Size Distribution in Response to Nutrient Availability

509   The proportion of root lengths of particular diameter size classes represents a compositional dataset

510   with a sum equivalent to one. As for other compositional datasets, changes in the length of one

511   diameter class will affect the relative proportion of the others [57, 58]. This was observed in the initial

512   analysis of barley cultivars in hydroponics as simultaneous changes in the smallest and thickest root

513   diameters (on the basis of both % and absolute length) in response to P deficiency (Table 1).

514   Aitchison distance, a univariate compositional metric, has been used as a statistical approach for

515   treating compositional data including the distribution of soil P species and fractions [58], soil

516   aggregate size distribution [63], and microbial community compositions [64].

517   Here, we used Aitchison distance (A_{root}) to assess global changes in the distribution of root lengths of

518   various diameters in response to changes in P supply and N source for each of the barley cultivars and

519   legume species tested. A_{root} is independent of unit (length or %), provides a single representation of all

520   root diameter size classes, and can therefore be used to statistically verify global changes to the

521   distribution of thick and fine roots simultaneously. Furthermore, the metric is defined relative to a

522   reference condition, in this case, the P0XB nutrient treatment. A_{root} values that are significantly

523   different to the P0XB condition represent a change in the distribution of root lengths in the various

524   diameter size classes. Large positive or negative A_{root} values may therefore be interpreted as belonging

525   to plants with highly plastic root systems. In the current study, legume species displayed the largest

526   magnitude and range of A_{root} values despite having smaller roots and SRL relative to the barley

527   cultivars (Fig. 4). This illustrates the scale-independence of the A_{root} measure as an indicator of root

528   morphological plasticity. Limited phenotypic plasticity among barley cultivars has been reported and

529   is linked to a narrow range of selective pressures during the domestication of wild and land-race

530   varieties [65]. In contrast, the large plasticity of legumes based on A_{root} values were consistent with

531   changes observed in root size classes less than 0.1 mm in diameter and SRL, particularly in response

532   to P availability (Fig. 3, Table 3). However, in contrast to the SRL ranking described above, the

533   patterns of A_{root} response to nutrient treatment were not conserved among legumes. Though not

534   investigated in the current study, measures of root diameter size length distributions using A_{root} could

535   provide additional insight into fine-scale differences in root morphology and root biomass

536   partitioning, which cannot be captured by gross measures such as SRL.

537   4.3 Mechanisms of Plant Response to Nutrient Availability

538   Barley cultivars responded to P deficiency by an increased partitioning of biomass to roots,

539   alkalization of the growth media, and increasing citrate efflux and phytase activity in exudates. This

540   is consistent with previous accounts of root biomass accumulation in response to nutrient deficiencies

541   by several cereal crops including barley [66], maize (*Zea mays* L.) [47], and wheat (*Triticum aestivum*

542   L.) [67]. P deficiency resulted in diminished SRL in a limited number of spring barley and wheat

543   varieties [68] and in some cultivars of this study (e.g., cvs Prague, Krystal). However, in both

544   hydroponics and sterile sand media, the response of barley to P deficiency was highly variable and

545   was not reflected as a decrease in SRL in all cases (Fig. 2). Barley cultivars provided with ammonium

546   as the primary source of N had the greatest response to P deficiency, including larger SRL, citrate

547   efflux and phytase activity and smaller average R:S in comparison to plants in the balanced N

548   treatment. Drew [43] reported the inhibition of lateral root growth in response to localized

549   applications of ammonium to barley. Similar responses have been shown in wheat, with the inhibitory

550   effects of ammonium reversed with greater applications of P [46]. The localized application of

551   ammonium and P is recommended as an approach for improving root growth, rhizosphere

552   acidification, and nutrient acquisition in calcareous soils with maize and other cereal/legume systems

553   [31, 46]. Whilst the application of ammonium may inhibit root growth in the absence of P, it is also

554   associated with improved leaf expansion and chlorophyll content as P supply increases [31]. This

555   effect was not evident in the shoot biomass measurements of the barley or legume cultivars tested, but

556   may explain the greater citrate efflux (and possibly other photosynthates) of some barley cultivars in

557   the ammonium treatment.

558   Under P deficiency, the smaller SRL of barley cultivars provided with ammonium was due to

559   diminished root biomass and a relatively constant distribution of root diameter lengths (Fig. 2). SRL

560   of the barley cultivars tested in the current study were similar in magnitude, but more variable than

561   those reported for spring barley varieties previously (186-329 m g^{-1} root dry wt.)[68]. Whereas Løes

562   and Gahoonia [68] reported minimal variation in SRL in 35 accessions from Scandanavia and

563   Norway, other studies have indicated large variations in as few as eight cultivars in glass-house [37]

564   and field conditions [69]; however, those studies were based only on fertilization with nitrate.

565   Although barley generally alkalized the growth media, this effect was dampened in the presence of

566   ammonium with the greatest P supply (Fig. 5). Rhizosphere alkalization occurs during the uptake of

24

567   inorganic anions ($H_2PO_4^-$/HPO_4^{3-} and NO_3^-) and exchange with alkaline counter ions (HCO_3^-, OH^-),

568   proton sequestration by organic anions (e.g., citrate, maleate, oxalate), and ammonification processes.

569   Conversely, acidification results from the uptake of inorganic cations (NH_4^+) and export of protons,

570   atmospheric N_2 fixation by microbial symbionts, and denitrification processes [70]. Rhizosphere

571   alkalization by cereals and grasses is typically explained by the uptake of nitrate and release of

572   hydroxyl/bicarbonate ions [71], however considering the large concentration and affinity of phosphate

573   transporters in barley, alkalization is likely to be associated with phosphate transport as well [66]. In

574   the current study, plants provided with balanced N consistently increased rhizosphere pH with

575   increasing P supply and did not vary significantly in terms of citrate efflux (Fig. 5). The limited effect

576   of P deficiency on citrate efflux by barley (*H. vulgare* cv Marie) provided with a balanced source of N

577   was recently reported for a single cultivar [72]. In contrast, plants provided with ammonium as the

578   primary source of N appear to have reduced the pH of the growth media at the largest P supply,

579   possibly through the release of acidic counter ions during the uptake of ammonium in larger plants.

580   Citrate efflux was positively correlated with ΔpH among barley cultivars in the ammonium treatment

581   (r=0.2303, p=0.0193). The relationship of pH and citrate efflux in the ammonium treatment supports a

582   secondary mechanism of alkalization, whereby citrate sequesters or is coupled with the efflux of

583   protons during ammonium uptake by barley [21].

584   Extracellular release of barley histidine acid phosphatase (HAP) has been linked to the ability of

585   cultivars to grow on phytate due to constitutive levels of exudation regardless of P supply or source

586   [10]. Low levels of phytase activity were measured in barley exudates with contrasting levels of

587   activity, which were found to depend on P supply and N source. Consistent with the results of

588   Ciereszko et al. (2011), no difference in phytase activity was found across P supply when a balanced

589   source of N was provided (Fig. 5). In contrast, plants provided with ammonium responded to P

590   deficiency by increasing exudate phytase activity, which was positively correlated with citrate efflux

591   (r=0.75; p<.0001) and ΔpH (r=0.32; p=0.0113; Fig. 5). Similar interactions between P supply and N

592 source have been reported based on root and soil acid phosphatase (APase) activity in ryegrass

593 (*Lolium perenne*) and tall fescue (*Festuca arundinaceae*)[73]. To our knowledge, this is the first

594 report that the induction of phytase exudation by P deficiency in barley may depend on N source.

595 4.4 Selection of Complementary Barley and Legume Varieties Based on Contrasting Responses to

596 Nutrient Availability

597 When combined in intercropping systems, species with contrasting responses to nutrient source and

598 availability are expected to contain a greater range of adaptations for improved P acquisition [4, 8].

599 Our results indicate that barley and legumes both respond to increasing P supply through

600 physiological (increased SRL) and biochemical traits (increased phytase activity; r=0.27, p<.0001),

601 particularly with ammonium as the primary source of N (Fig. 5, Fig. 6). Contrasting responses of

602 barley cultivars and legume species include greater acidification by legumes and the interaction of P

603 supply and N source in controlling citrate efflux by these varieties (Fig. 6). Larger rates of acid

604 production by legumes in comparison to barley are expected based on the relatively greater

605 physiological demand for N by legumes, higher rates of N uptake, and, under ammonium treatment,

606 increased proton export [74, 75].

607 Contrasting responses to P deficiency among plant species based on citrate efflux have been reported

608 to occur in barley (*H. vulgare* L. cv Heder), canola (*Brassica napus* cv Marie), and potato (*Solanum*

609 *tuberosum* cv Pimpernel), whereby canola was the only species with the greatest citrate efflux in the

610 absence of P [72]. We found citrate efflux by legumes to vary as a function of P supply only when

611 provided with balanced N (Fig. 6) and found no relationship between citrate and pH in either N

612 treatment. In contrast, citrate efflux by barley varied with P supply only with ammonium as the

613 primary source (Fig. 5) and was likely linked to an acid tolerance mechanism induced in response to

614 ammonium nutrition. These results indicate that intercropping of barley and legume species with

615 contrasting responses to N source could improve the adaptation of plants to P deficiency, sub-optimal

616 soil pH, and the heterogeneous distribution of nutrients in soil while promoting the expression of

617 citrate and phytase exudation in one or both plant species.

618 Barley and legume varieties with the ability to respond to local nutrient conditions represent

619 promising candidates for improving nutrient efficiency in multi-crop and biodiverse agroecosystems

620 [7]. However, response-based approaches for the selection of complementary plant varieties should

621 consider the morphological and biochemical bounds of response, which will likely vary across

622 species. As would be expected for comparisons made at the species versus cultivar level, differences

623 in SRL, phytase activity, citrate efflux, and pH were more significant among legume species in

624 comparison to the variation identified within the *H. vulgare* L. cultivars (Fig. 5, Table 2).

625 Li et al. [46] reported greater plasticity of leguminous root systems (e.g., faba bean, chickpea) in

626 comparison to graminoids (e.g., maize, wheat) in response to nutritional variation. Consistent with the

627 analysis of Li et al. [46], we found limited variation in the distribution of root diameter sizes among

628 cultivars of the single *Hordeum* species tested (Fig. 2), and considerably more variability in A_{root}

629 values among legumes in all of the nutrient treatments (Fig. 3, Table 2). In contrast, the variation

630 among barley cultivars was considerably greater than legumes in the P-deficient condition with

631 regards to plant-induced pH change, citrate efflux, and phytase activity (Fig. 5, Table 2).

632 The contrasting responses of barley and legumes to P deficiency indicate differences in the

633 morphological and biochemical adaptations of these species to acquire soil nutrients [76]. In the case

634 of domesticated barley, the limited morphological plasticity of roots implies that plants must respond

635 to changes in nutrient availability through exudation and modifications to the chemical environment

636 [65]. In contrast, the greater root morphological plasticity of legumes may allow for the physical

637 exploration of soils, but at the cost of biochemical plasticity. Through the identification of contrasting

638 nutrient acquisition strategies such as these, complementary plant combinations may be selected to

639 minimize competition between plants for soil resources (i.e., niche space, nutrients) and maximize

640 productivity within sustainable cropping systems [5, 6]. The selection of complementary plant

641 combinations may therefore be improved through an understanding of plant genetic variation and

642 phenotypic response to nutrient source and limitation.

5. Conclusion

644 This study investigated the variation of root exudation and morphological traits among barley

645 cultivars and legume species in order to identify plants with contrasting responses to P supply and N

646 source. The selected traits were based on those previously linked to the capacity of plants to acquire P

647 from poorly soluble and organic forms of P in soils (citrate efflux, exudate phytase activity, pH, root

648 diameter size distribution, specific root length). Three legume species (*M. truncatula*, *T.*

649 *subterraneum*, *O. sativus*) and four barley cultivars (cvs Prague, Waggon, Spire, Krystal; Fig. 7)

650 displayed the greatest variation in root responses to nutrient supply and represent promising

651 candidates for future facilitation and complementarity studies. It is likely that the selection of

652 complementary cereal and legume varieties will not only depend on intrinsic or constitutive

653 expression of root traits, but condition-specific trade-offs in the expression of these traits between

654 individual plants in the combination. The optimized selection of plant species and cultivars for

655 nutrient-efficient and biodiverse cropping systems will be critical for improving the productivity and

656 export of nutritional resources (e.g., carbohydrates, protein, micronutrients) amidst declining global

657 soil fertility and loss of arable land area.

6. Appendices

659 **Fig. A.1** Characteristics of shoot, root, and exudate solutions of barley cultivars (n=143) grown in

660 hydroponics under P-deficient (P0) and sufficient (P1) conditions.

661 **Table A.1** Barley cultivars and legume species used in the study.

662   **Equation A.1** Isometric log-ratio transformation (*ilr*)

663   **Table A.2** Sequential binary partition used for the calculation of isometric log ratios (*ilr*) associated

664   with root diameter size classes of barley cultivars and legume species cultivated in sterile sand.

665   **Equation A.2** Aitchison distance (A_{root})

666   **Figure A.2** Root diameter size class, length distribution of barley cultivars (*H. vulgare* L.; top) and

667   legume species (bottom) grown under three phosphorus treatments (P0=0, P1=0.5, P2=1.0mM) with

668   ammonium as the primary source of nitrogen (A) or with balanced nitrate-ammonium (B).

669   *7.   Acknowledgements*

673   *8.   References*

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867   *Figure captions*

868   **Fig. 1** The effect of phosphorus deficiency on specific root length (SRL, m g^{-1}) and the ability of barley cultivars (n=143) to affect the pH of 24 h exudate
869   collection solutions following growth in hydroponics (A). The ranked distribution values of SRL (B) and pH (C) responses of barley cultivars to P deficiency
870   are based on the difference between cultivars grown with (1 mM) or without added P (subset of cultivars listed). Quartiles are defined based on pH response
871   in 24 h exudate collection solutions (pH 5.5). Labelled symbols represent cultivars that were selected for further screening of exudate citrate and phytase
872   activity.

873   **Fig. 2** Shoot dry weight, root to shoot ratio, and specific root length (SRL) of the listed barley cultivars (*H. vulgare* L.) grown under three phosphorus
874   treatments (P0=0mM, P1=0.5mM, P2=1.0mM) with ammonium as the primary source of nitrogen (XA) or with balanced nitrate-ammonium (XB). Box
875   (interquartile range) and whiskers (1.5*quartile 1 or 3) accompanied by mean values across cultivars.

876   **Fig. 3** Shoot dry weight, root to shoot ratio, specific root length (SRL), and root diameter length distribution expressed in terms of Aitchison distance (A$_{roor}$)
877   of legume species cultivated under three phosphorus treatments (P0=0mM, P1=0.5mM, P2=1.0mM) with ammonium as the primary source of nitrogen (XA)
878   or with balanced nitrate-ammonium (XB). Box (interquartile range) and whiskers (1.5*quartile 1 or 3) accompanied by mean values across species.

879   **Fig. 4** Response of (A) barley cultivars (*H. vulgare* L.) and (B) legume species (*Biserulla* sp. cv Casbah, *Medicago* sp. cv Sultan, *Ornithopus compressus* cv
880   Santorini, *O. sativus* cv Margarita, *Trifolium purpereum* cv Electra, *T. subterraneum* cv Leura) to nutrient treatments based on changes in root diameter size
881   length distribution as represented by Aitchison distance (A$_{root}$). A$_{root}$ values are calculated relative to the reference treatment (P0XB) for plants grown under
882   three phosphorus treatments (P0=0, P1=0.5, P2=1.0mM) with ammonium as the primary source of nitrogen (XA) or with balanced nitrate-ammonium (XB).
883   Error bars represent the 95% confidence interval (n=5). *A$_{root}$ values significantly different from the reference treatment (α=0.05).

884   **Fig. 5** Plant-induced change in pH of the sand growth media, citrate efflux, and phytase activity of root exudates collected from barley (*H. vulgare* L.)
885   cultivated under three phosphorus treatments (P0=0mM, P1=0.5mM, P2=1.0mM) with ammonium as the primary source of nitrogen (XA) or with balanced
886   nitrate-ammonium (XB). Box (interquartile range) and whiskers (1.5*quartile 1 or 3) accompanied by mean values across cultivars.

887   **Fig. 6** Plant-induced change in pH of the sand growth media, citrate efflux, and phytase activity of root exudates collected from legume species cultivated
888   under three phosphorus treatments (P0=0mM, P1=0.5mM, P2=1.0mM) with ammonium as the primary source of nitrogen (XA) or with balanced nitrate-
889   ammonium (XB). Box (interquartile range) and whiskers (1.5*quartile 1 or 3) accompanied by mean values across species.

890   **Fig. 7** Principal components analysis of barley cultivars and legume species based on plant-induced pH change in sand, exudate phytase activity, specific root
891   length, and citrate efflux. The PCA illustrates the divergent response of barley and legume species to nutrient treatments [No P (P0), 0.5mM P (P1), 1.0mM P

34

892   (P2) with ammonium rich (XA) or balanced nitrate-ammonium (XB) supply] based on root morphological and exudation properties. * indicates location of *O.*
893   *sativus* in PCA plot.

894   **Table 1.** Root morphological and exudation properties of barley cultivars grown in hydroponics with (P1, 1 mM P) and without added P (P0).

Root morphological or exudation property	Phosphorus treatment		% change	P-value
	P0	P1		
Exudate solution pH	6.277	6.125	+2.5	0.0065
Exudate solution pH change	0.975	0.805	+21.1	0.0096
Root dry wt. g	0.051	0.041	+22.6	<.0001
Root surface area cm^2	148.9	128.2	+16.1	0.0006
Total root length cm	688.4	612.8	+12.3	<.0001
Specific root length m g^{-1} root dry wt.	140.0	159.6	-12.3	0.0001
Specific surface area cm^2 g^{-1}	3054.2	3196.5		0.2333
% root length <0.5mm diam.	62.37	59.11	+5.5	0.0082
% root length 0.5-1.0mm diam.	25.68	29.70	-13.5	<.0001
% root length 1.0-1.5mm diam.	6.203	6.253		0.8652
% root length 1.5-2.0mm diam.	2.297	2.182		0.3835

% root length 2.0-2.5mm diam.	1.276	1.146		0.1119
% root length 2.5-3.0mm diam.	0.735	0.636		0.0620
% root length 3.0-3.5mm diam.	0.421	0.341	+23.5	0.0187
% root length 3.5-4.0mm diam.	0.287	0.217	+32.0	0.0119
% root length 4.0-4.5mm diam.	0.205	0.135	+51.4	0.0017
% root length >4.5mm diam.	0.454	0.215	+111.4	0.0073

Oneway ANOVA of paired means by P treatment. % root length data were checked for normality and log-transformed prior to statistical comparisons.

% change represents percentage increase in P0 condition above that measured in P1.

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904 **Table 2** Factors and interactions affecting shoot dry wt. (g), root to shoot ratio (R:S), specific root length (SRL, m g^{-1} root d.w.), pH change in sand (ΔpH),
905 citrate efflux (nmol g^{-1} root d.w. h^{-1}), and exudate phytase activity (nKat g^{-1} root d.w. h^{-1}). * indicates significant effects and interactions (p<.05).

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	Factors and interactions	Shoot dry wt.	R:S	SRL	ΔpH	Citrate Efflux	Phytase Activity
All plants	P supply	<.0001*	0.0083*	0.1427	<.0001	0.3341	0.0322*
	N source	0.0413*	0.0428*	0.0013*	0.0245*	0.9276	0.0035*
	P supply × N source	0.3842	0.7128	0.0101*	<.0001	0.1392	0.0236*
	Nutrient Treatment	<.0001*	<.0001*	0.8496	0.0011*	0.9831	0.3285
	Genus species	<.0001*	<.0001*	<.0001*	<.0001*	<.0001*	0.3185
	Nutrient treatment × Genus species	<.0001*	<.0001*	0.858	<.0001*	0.0012*	0.0162*
Barley	P supply	<.0001*	<.0001*	0.0166*	<.0001*	0.0408*	0.4787
	N source	0.0003*	0.0131*	0.0001*	0.4105	0.1974	0.0028*
	P supply × N source	0.1526	0.1561	0.0003*	0.0017*	0.0317*	0.0062*
	Nutrient Treatment	<.0001*	<.0001*	<.0001*	<.0001*	<.0001*	0.8654
	Cultivar	0.0002*	<.0001*	0.0004*	<.0001*	0.0005*	0.4503
	Nutrient treatment × Cultivar	0.4273	<.0001*	<.0001*	<.0001*	0.0005*	0.0119*
Legume	P supply	<.0001*	<.0001*	<.0001*	<.0001	<.0001*	0.0429*
	N source	0.0124*	<.0001*	<.0001*	0.4105	<.0001*	0.1238

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P supply × N source	<.0001*	0.0171*	0.0161*	0.0017*	<.0001*	0.1393
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Nutrient Treatment	<.0001*	<.0001*	<.0001*	<.0001	0.2861	0.4216
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Cultivar	<.0001*	0.1812	<.0001*	0.0037*	0.1412	0.0482*
Nutrient treatment × Cultivar	<.0001*	<.0001*	0.2538	<.0001	<.0001*	0.0004*
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925 **Table 3** Pair-wise correlations between biomass and root exudate properties of barley cultivars and legume species cultivated in sterile sand for 21 days with
926 six nutrient treatments containing 3 P X 2 N conditions. Empty cells indicate no correlation between variables.

Barley/Legume	Shoot dry wt. g	Root dry wt. g	R:S	ΔpH	Citrate efflux	Phytase activity	Total root length cm	Root length (0-0.1 mm diam.)	Root length % (0-0.1 mm diam.)	Root length (>1.9 mm diam.)	Root length % (>1.9 mm diam.)	SRL m g^{-1}
Root dry wt. g	+ +											
R:S	- -	+ +										
ΔpH	- +	+	+ +									
Citrate efflux nmol g^{-1} root dry wt. h^{-1}	-	- +										
Phytase activity nKat g^{-1} root dry wt. h^{-1}	-	- -	-		+ -							
Total root length cm	+ +	+	-	+		- -						
Root length (0-0.1 mm diam.)	+ +	+	-	+		- -	+ +					
Root length % (0-0.1 mm diam.)	+	+					+	+ +				
Root length (>1.9 mm diam.)		+					-	+	+			
Root length % (>1.9 mm diam.)	-		+	+			-	+		+ +		
SRL m g^{-1}		- -	- -		+	+	+ +	+				
Avg. Root diam. mm	+ +	+	-	+		- -	+ +	+ +	+	+	+	+ +

Legend:

- Significantly correlated (p<.05) among barley cultivars and legume species
- Significantly correlated (p<.05) among barley cultivars only
- Significantly correlated (p<.05) among legume species only
- + - Positive or negative correlation; left and right symbols correspond to barley and legume if different

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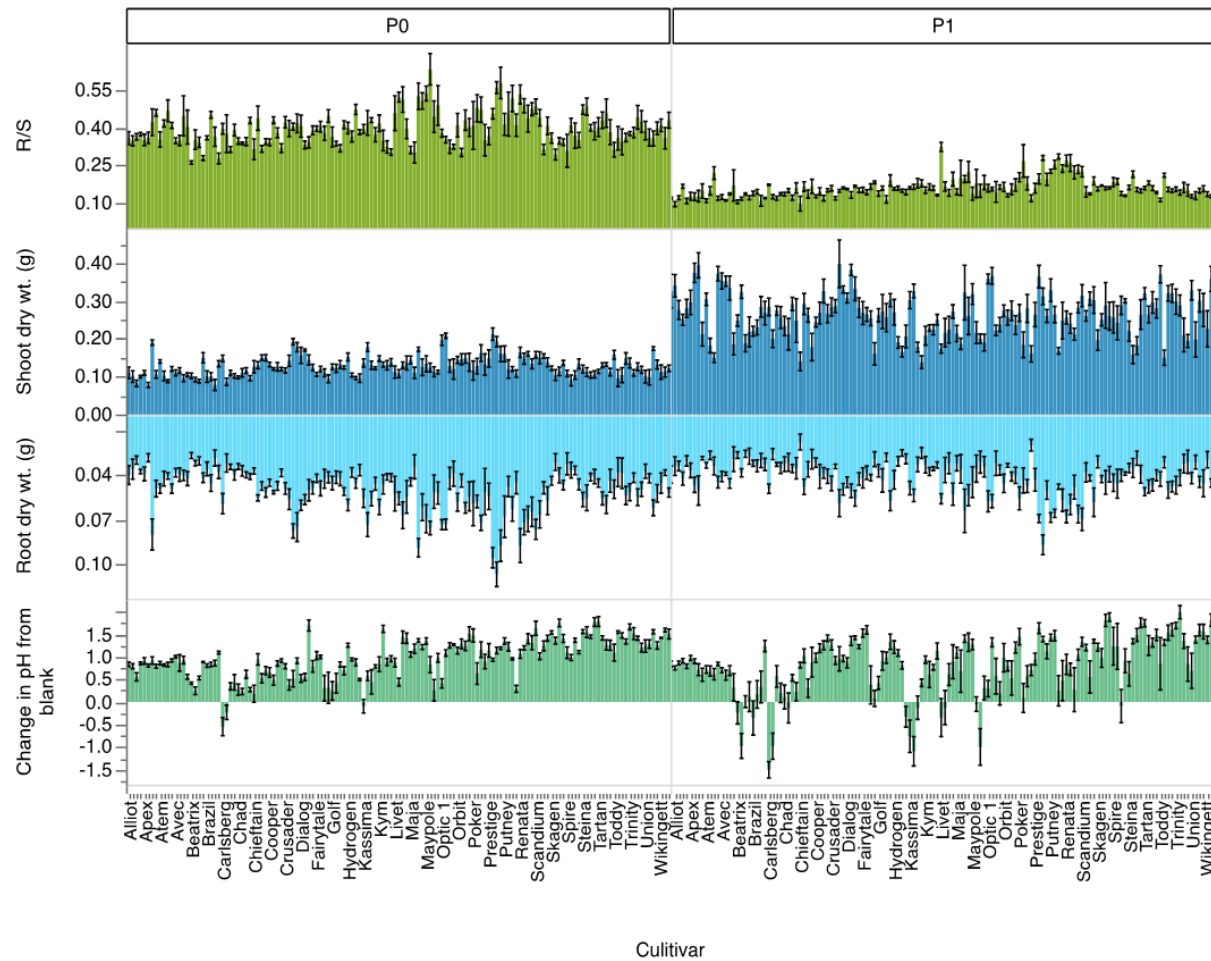
Figure A.1 Shoot and root dry weight and exudate solution pH of barley cultivars (n=143) grown in hydroponics under P-deficient (P0) and sufficient (P1) conditions.

932 **Table A.1** Barley cultivars and legume species used in the study.

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Genus species (common name)	Cultivar
Hordeum vulgare L. (spring barley)	Akka, Alabama, Alexis, Alliot, Aluminium, Anais, Annabell, Apex, Appaloosa, **Aramir**, Armelle, Atem, Athena, Athos, Atribut, Avec, Balga, Barke, Baronesse, Beatrix, Berenice, Berwick, Beryllium, Brazil, Camargue, Campala, Carafe, Carlsberg, Cellar, Centurion, Century, Chad, Chalice, Chariot, Chaser, **Chieftain**, Chime, Class, Colada, Cooper, Corniche, CPBT B76, Cristalia, **Kym**, Landlord, Latvijas Vietejie, Linga, Livet, Lysiba, Lysimax, Macaw, Maja, Maresi, Maris Mink, Marthe, Maypole, Meltan, Midas, Novello, Optic, Orbit, Perun, Pewter, Pitcher, Poker, Potter, Power, **Prague**, Prestige, Prisma, Proctor, Publican, Putney, Quench, **Rainbow**, Reggae, Renata, Riviera, Romi, Rummy, Saloon, Scandium, Scarlett, Sebastian, Simba, Skagen, Skittle, Spartan, Spey, **Spire**, Crusader, Danuta, Decanter, Derkado, **Dialog**, **Domen**, Doyen, Drum, Fairytale, Georgie, Gitane, Golden Promise, Golf, Hanka, Hellas, Heron, Hydrogen, Imidis, Isabella, Isaria, Kassima, **Kenia**, Koral, **Krystal**, Starlight, Static, Steffi, Steina, Sultan, SW SCANIA, Taphouse, Tartan, Tavern, **Thuringia**, Tocada, Toddy, Torup, Toucan, Tremois, Trinity, Triumph, Trosa, Tyne, Union, Vegas, **Waggon**, Westminster, Wikingett, Wisa, Zephyr
Trifolium subterraneum (Subterraneum clover)	Leura
Trifolium purpureum (Purple clover)	Electra
Biserrula pelecinus (Biserrula)	Casbah
Ornithopus compressus (Yellow serradella)	Santorini

Ornithopus sativus (French serradella)   Margurita

Medicago truncatula (Barrel clover)   Sultan

934   **Equation A.1** Isometric log-ratio transformation (Egozcue et al., 2003):

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$$ilr_i = \sqrt{\frac{rs}{r+s}} \ln \frac{g(x_i^+)}{g(x_i^-)}$$

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938   ilr_i is the ith balance between two sub-compositions: i [1, D-1]

939   r is the number of components in the numerator position of the subset (+)

940   s is the number of components in the denominator position of the subset (-)

941    $g(x_i^+)$ and $g(x_i^-)$ are the geometric means of the components in r and s subsets, respectively

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943   The selection of subsets for the root diameter class length compositions are defined by the sequential binary partition matrix provided in Table A.2.

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960 **Table A.2** Sequential binary partition used for the calculation of isometric log ratios (ilr) associated with root diameter size classes of barley
961 cultivars and legume species cultivated in sterile sand. The sequential binary partition is based on the length of roots (cm) in each root diameter size
962 class (mm).

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	Root diameter size class (mm)																						
	0-0.1	0.1-0.2	0.2-0.3	0.3-0.4	0.4-0.5	0.5-0.6	0.6-0.7	0.7-0.8	0.8-0.9	0.9-1.0	1.0-1.1	1.1-1.2	1.2-1.3	1.3-1.4	1.4-1.5	1.5-1.6	1.6-1.7	1.7-1.8	1.8-1.9	>1.9	r	s	coefficient
ilr1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	-1	19	1	0.975
ilr2	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	-1	0	18	1	0.973
ilr3	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	-1	0	0	17	1	0.972
ilr4	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	-1	0	0	0	16	1	0.970
ilr5	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	-1	0	0	0	0	15	1	0.968
ilr6	1	1	1	1	1	1	1	1	1	1	1	1	1	1	-1	0	0	0	0	0	14	1	0.966
ilr7	1	1	1	1	1	1	1	1	1	1	1	1	1	-1	0	0	0	0	0	0	13	1	0.964
ilr8	1	1	1	1	1	1	1	1	1	1	1	1	-1	0	0	0	0	0	0	0	12	1	0.961
ilr9	1	1	1	1	1	1	1	1	1	1	1	-1	0	0	0	0	0	0	0	0	11	1	0.957
ilr10	1	1	1	1	1	1	1	1	1	1	-1	0	0	0	0	0	0	0	0	0	10	1	0.953
ilr11	1	1	1	1	1	1	1	1	1	-1	0	0	0	0	0	0	0	0	0	0	9	1	0.949
ilr12	1	1	1	1	1	1	1	1	-1	0	0	0	0	0	0	0	0	0	0	0	8	1	0.943
ilr13	1	1	1	1	1	1	1	-1	0	0	0	0	0	0	0	0	0	0	0	0	7	1	0.935
ilr14	1	1	1	1	1	1	-1	0	0	0	0	0	0	0	0	0	0	0	0	0	6	1	0.926
ilr15	1	1	1	1	1	-1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	5	1	0.913
ilr16	1	1	1	1	-1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	4	1	0.894
ilr17	1	1	1	-1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	3	1	0.866
ilr18	1	1	-1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2	1	0.816
ilr19	1	-1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0.707

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972 Equation A.2 Aitchison distance (A_{root}) is calculated based on ilr values (Equation A.1, Table A.2) and compares the composition of root diameter
973 length distributions of the reference nutrient conditions (P0XB) relative to the other nutrient treatments (P1XB, P2XB, P0XA, P1XA, P2XA) within a
974 barley cultivar or legume species. The computation of A_{root} is made as follows (Egozcue and Pawlowsky-Glahn, 2006):

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$$A = \sqrt{\sum_{i=1}^{D-1} \left(ilr_i^x - ilr_j^y \right)^2} = \sqrt{\left(ilr_i^x - ilr_i^y \right)^T I^{-1} \left(ilr_i^x - ilr_i^y \right)}$$

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979 where ilr_i^x and ilr_i^y correspond to the ith balances of the diagnosed (x) and reference (y) compositions, respectively, I is the identity matrix, and T is
980 the transposed matrix.

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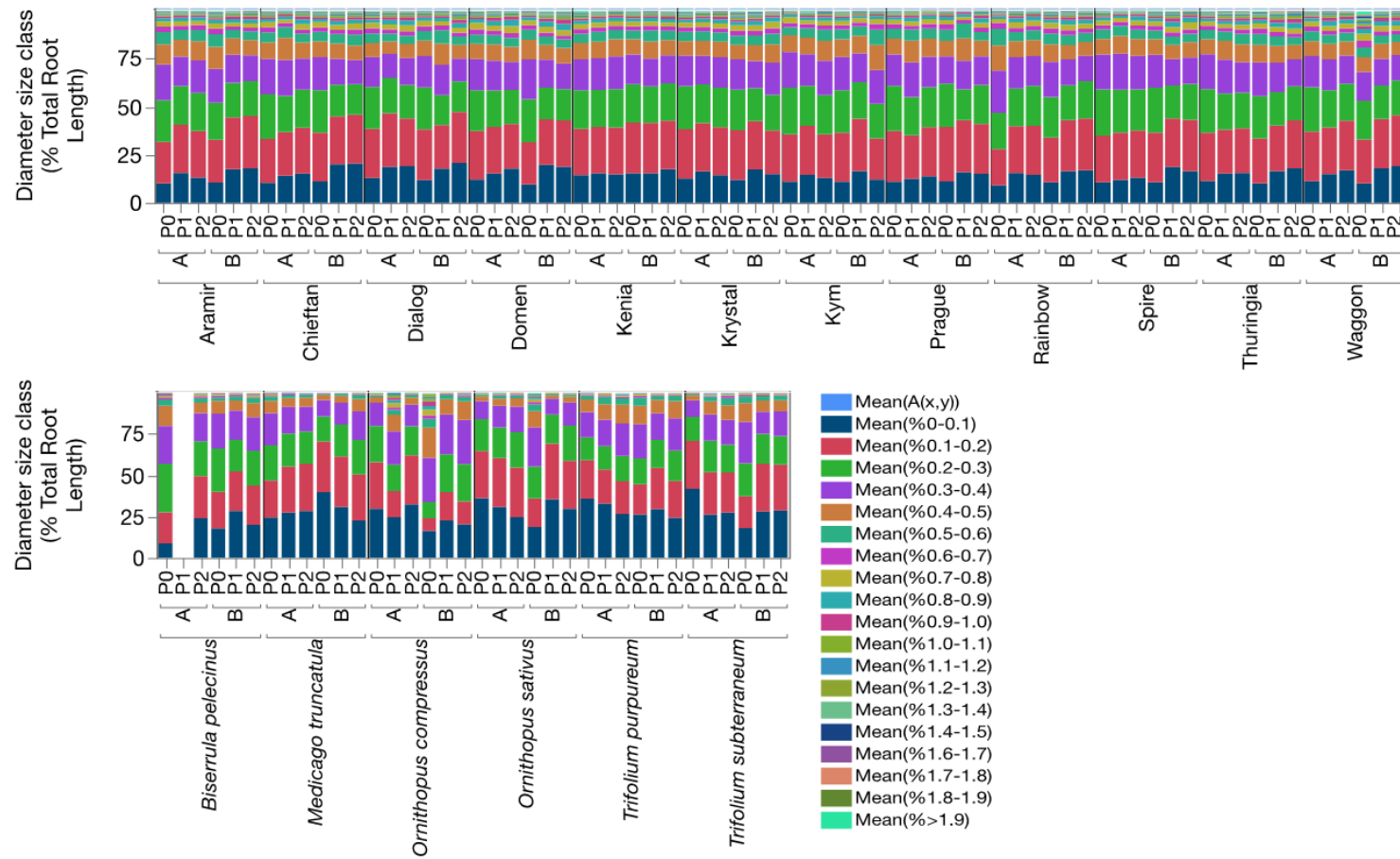
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Figure A.2 Root diameter size class, length distribution of barley cultivars (*H. vulgare* L.; top) and legume species (bottom) grown under three phosphorus treatments (P0=0, P1=0.5, P2=1.0mM) with ammonium as the primary source of nitrogen (A) or with balanced nitrate-ammonium (B).

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