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Title

Response-based selection of barley cultivars and legume species for complementarity: Root morphology and exudation in relation to nutrient source

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Abstract

Phosphorus (P) and nitrogen (N) use efficiency may be improved through increased biodiversity in agroecosystems. Phenotypic variation in plants' response to nutrient deficiency may influence positive complementarity in intercropping systems. A multicomponent screening approach was used to assess the influence of P supply and N source on the phenotypic plasticity of nutrient foraging traits in barley (*H. vulgare* L.) and legume species. Root morphology and exudation were determined in six plant nutrient treatments. A clear divergence in the response of barley and legumes to the nutrient treatments was observed. Root morphology varied most among legumes, whereas exudate citrate and phytase activity were most variable in barley. Changes in root morphology were minimized in plants provided with ammonium in comparison to nitrate but increased under P deficiency. Exudate phytase activity and pH varied with legume species, whereas citrate efflux, specific root length, and root diameter lengths were more variable among barley cultivars. Three legume species and four barley cultivars were identified as the most responsive to P deficiency and the most contrasting of the cultivars and species tested. Phenotypic response to nutrient availability may be a promising approach for the selection of plant combinations for minimal input cropping systems.

Highlights

- Phenotypic response to nutrient source in barley cultivars and legume species
- Divergent responses based on root morphology and exudation
- Potential plant combinations for improved nutrient acquisition identified

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

Abbreviations A, ammonium-N; A_{root}, Aitchison distance of root diameter length distribution; B, balanced nitrate-ammonium; CV, coefficient of variation; ΔpH, change in pH.; H^+, proton; HCO_3^- bicarbonate; K^+, potassium ion; N, nitrogen; NH_4^+, ammonium; NO_3^-, nitrate; P, phosphorus; P0, no P; P1, 0.5 mM P; P2, 1.0 mM P; SRL, specific root length.

1. Introduction

There is a mounting concern for the long-term viability of conventional cropping practices, which rely on non-renewable mineral phosphate supplies to maintain yields and meet the dietary requirements of a growing global population [1, 2]. Agricultural biotechnologies and practices which maximize the utilization of added and endogenous soil P supplies are therefore needed to reduce the dependence of agricultural production on external fertilizer inputs and minimize the loss of nutrients to surface waters [3]. Intercropping of cereals and legumes has been proposed as an approach to improve crop yields and nutrient use efficiency in agricultural systems through increased biodiversity, resource sharing, resilience to pests, and inter-species facilitation [4]. Understanding the response of barley cultivars and legume species to P supply and N source could therefore improve the selection of plants for biodiverse and nutrient efficient agroecosystems.

Complementarity between two or more plants in poly-culture is characterized by improved resource acquisition and productivity relative to a monoculture [5]. Facilitation and reduced competition for soil resources by plants in poly-culture occur due to reduced competition for spatial (e.g., top-soil nutrient foraging) and non-spatial soil resources (e.g., chemically distinct nutrient pools), as well as enhanced productivity through N-fixation by legumes and other environmental modifications (e.g., soil moisture retention, disease suppression) [4, 6]. The success of intercropping strategies is predicted to depend on architectural and anatomical properties of roots as well as the exudation of carboxylates and phosphatase enzymes, which optimize the extraction of soil nutrients and exploration of niche space in soil by the individual plant species [7]. If however the nutrient

acquisition strategies of two or more plants are too similar, for example targeting the same niche space or nutrient pool in soil, competitive effects may limit the success of intercropping strategies. Therefore, the plasticity of root morphology and exudation under conditions of limited or heterogeneous nutrient sources is expected to minimize competition between plants and enhance the acquisition of nutrients by individual plants and intercrops [8].

The recovery of P from organic forms is achieved by the production of phosphatases by plants or microorganisms in the soil environment. The purple acid phosphatase and histidine acid phosphatase classes of phytase have been characterized in plants [9, 10] and are reported to be expressed within the cell and exuded under conditions of P limitation [11]. Several species of grasses (e.g., *Brachiaria, Dactylis*) and legumes (e.g., *Stylosanthes, Medicago, Trifolium*) respond to P deficiency through the increased exudation of phytase from roots [11, 12]. For example, wheat plants (*Triticum* L.) with greater root-associated phosphatase activity could assimilate more P from organic forms than plants with less or no activity [13]. When constitutively expressed in transgenic plants (e.g., *Nicotiana tabacum, Trifolium* L.), various fungal phytases (e.g., *Aspergillus* sp., *Peniophora* sp.) are shown to improve the assimilation of P from sparingly available P sources *in vitro* [14, 15]. Whilst the modification of plants with single traits such as fungal phytase exudation has had a limited effect on P acquisition by plants grown in unfertilized soils [16], studies with model tobacco [17] and cereal/legume systems [8, 18] suggest that the combination of phytase/phosphatase exudation and citrate efflux could improve the ability of plants to acquire P due to the combined action of these exudates on the solubilization and mineralization of soil P [19].

Organic anions/carboxylates represent a major component of root exudates, which directly affect the diffusivity and availability of P in soils [20]. A secondary effect of carboxylate exudation is the co-transport of counter ion species (e.g., H^+, K^+, HCO_3^-) to maintain cytosolic charge balance during exudation [21]. This exudation leads to the modification of rhizosphere pH with potential consequences on the solubility of nutrients, enzyme function, and cascading effects within the

microbial community [22]. The genetic and environmental controls on carboxylate exudation (e.g., citrate, malate) have been studied extensively in cereals (e.g., *Tritucum* L., *Hordeum* L., *Zea mayes*) [23-25] and are known to depend on various nutrient deficiencies (e.g., P) [20], metal toxicities (e.g., Al^{3+}, Mn^{2+}) [26], or as a mechanism for below ground C partitioning and the facilitation of microbial community symbiosis [27]. Phosphorus deficiency leads to increased citrate efflux in several legume species (e.g., *Medicago sativa, Lupinus* spp.) [28, 29] and may be further enhanced when ammonium is supplied as the primary source of N due to rhizosphere acidification during ammonium uptake (e.g., *Lupinus albus*) [30, 31]. In contrast, nitrate acts as a signal to induce the production of organic anions in tobacco (*Nicotiana tabacum*), which act as receptors of nitrate or counter ions for the maintenance of cytosolic pH [32]. Citrate efflux in barley (*H. vulgare* L.) is primarily studied with regard to its genetic variation across cultivars or role in Al^{3+} toxicity tolerance in acid soils and is therefore typically assessed under either P sufficient or deficient conditions [33-35]. To our knowledge, there are no reports of citrate efflux among barley cultivars being affected by both P supply and N source (NH_4^+, NO_3^-).

Root plasticity in response to selective pressure (e.g., nutrient supply/source) allows plants to explore heterogeneous soil environments and forage for nutrients [7]. Common physiological responses of cereals and legumes to P deficiency include the partitioning of biomass to roots, increased production of fine roots, and the generation of 'low metabolic cost' roots, characterized by increased proportion of aerenchyma cells and greater root length relative to root biomass (i.e., specific root length, SRL; [36-42]. The initiation or inhibition of root branching and elongation is also affected by N source (NO_3^-, NH_4^+). For example in barley and wheat, the localized application of nitrate initiates the growth and extension of seminal and lateral roots [43-45]. Plants provided with ammonium can suppress root branching and elongation in the absence of P, with these effects reversed and associated with improved seedling growth at higher rates of P application [46, 47]. If yields in cereal and legume systems are significantly impacted by root architectural [5] and morphological traits, which affect the

acquisition of soil mineral nutrients (e.g., lateral root angle, rhizosheath, SRL), the selection of plants with traits appropriate to a particular growth environment will be needed [48].

The effective combination of traits for the efficient recovery of P in complementary plant systems must also consider the genotypic variation of physiological and biochemical responses of plants to nutrient availability [49]. Therefore, the objective of this study was to take a systematic approach to the selection of barley cultivars and legumes species based on the morphological and biochemical response of genotypes to P supply and N source. We assessed root exudation (citrate efflux, phytase activity, pH change) and root morphological traits (root length, specific root length, root diameter size distribution) and identified plants with the greatest potential to access sparingly available or poorly soluble P in soil.

2. Materials and methods

2.1 Plant materials

Barley seeds (*Hordeum vulgare* L.) from a genome-wide association mapping collection (144 elite European germplasm) and previously assessed for P use efficiency and rhizosheath [50, 51] were used for the initial screening in hydroponics, with a sub-set of these selected for further characterization following growth in sterile sand (Table A.1). Seeds from six pasture legumes representing a range of root morphological [36] and exudation characteristics [52] were obtained from the New South Wales Department of Primary Industries, Wagga Wagga Agricultural Institute, NSW, Australia. These legume species, originally sourced from the southern Mediterranean and studied extensively in Australian pasture systems [53], were: Subterranean clover (*Trifolium subterraneum* cv. Leura), Purple clover (*Trifolium purpureum* cv. Electra), Biserrula (*Biserrula pelecinus* cv. Casbah), Yellow serradella (*Ornithopus compressus* cv. Santorini), French serradella (*Ornithopus sativus* cv. Margurita), and Barrel medic (*Medicago truncatula* cv. Sultan; Table A.1).

2.2 Chemical and Enzyme Sources

Standard nutrient salts were sourced from Sigma-Aldrich or BDH for all plant growth experiments. *Myo*-inositol hexakisphosphate dodecasodium heptahydrate salt (InsP6; Sigma-Aldrich P8810; Gillingham, UK) was used for the determination of phytase activity in plant exudate solutions. Ammonium sulphate suspensions of lactic dehydrogenase (LDH; Sigma-Aldrich L2500), malic dehydrogenase (MDH; Sigma-Aldrich M1567), β-Nicotinamide adenine dinucleotide (NADH; Sigma-Aldrich N4505), citrate lyase from *Klebsiella pneumoniae* (CL; Roche Ltd., West Sussex, UK), and a stock citrate standard from Fluka Analytical (Seelze, Germany) were used for the analysis of citrate in plant exudate solutions.

2.3 Exudate Collection Following Growth in Hydroponics

One-hundred and forty-three of the 144 barley cultivars were screened in hydroponics for root growth and pH response to P deficiency in order to select a sub-set (n=12) for determination of citrate efflux and phytase activity in exudates. Seeds were pre-germinated on distilled water agar (1% agarose w/v). After three days, when radicles were approximately 1 cm long, 5 replicate seedlings were planted in hydroponic solutions and grown for 3 weeks in batches of 90 plants per 60 L. The standard nutrient 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-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 Na_2MoO_4, 1.0 μM $CoCl_2$) and was either supplemented with 1 mM KH_2PO_4 or left unamended. Nutrient solutions were changed on a weekly basis beginning with a quarter strength solution, followed by half strength, and then full strength nutrients for the final week of the experiment. The pH in nutrient solutions was adjusted to 5.5 using sodium hydroxide as necessary. Due to the size of the experiment, four screening cycles of 36 cultivars (5 replicates each, including one plant control, cv Optic) were carried out for each P condition. Plants were grown for three weeks under controlled conditions (22°C day 16h/14°C night, 200 W m^{-2}) and then transferred to 50 mL of P-free nutrient

solution for exudate collection over 24h. Shoot and root materials were collected for biomass weight determination after drying for 1 week (70^{o}C).

2.4 Exudate Collection Following Growth in Sterile Sand

A representative subset of 12 barley cultivars (cvs Domen, Chieftan, Dialog, Waggon, Spire, Thuringia, Kym, Prague, Aramir, Krystal, Rainbow, Kenia) and the six legumes (Table A.1) were selected for exudate screening following six weeks of growth in sterile sand. Course river sand was washed through a 500 micron sieve and potted (250 – 300 g air-dried sand) prior to sterilization by autoclaving (180^{o}C). Seeds were vapour sterilized as described previously by enclosing seeds in an airtight container for 1 h with a solution containing 100 mL hypochlorite solution (4% w/v) and 3 mL concentrated hydrochloric acid [14]. Seeds were germinated on sterile distilled water agar (0.1% m/v) for 2 d prior to planting, after which time plants were monitored for incomplete emergence and replaced with germinated seeds to achieve one plant per pot. Plants were supplied with 20 mL of full-strength nutrients each day during the 21 d growth period in a glasshouse (22^{o}C/14^{o}C day/night) with 16 h light and additional lighting provided at incident radiation less than 200 W m^{-2}. Five replicate pots were prepared for all cultivars and nutrient conditions including plant-free controls, which received nutrients for the duration of the growth period.

Plant nutrient solutions were adjusted to pH 5.5 with 10 M sodium hydroxide and filter sterilized (0.3 µm pore size) before use. The N-balanced treatment (B) included equal molarities (6 mM) of NO_3^--N and NH_4-N and other macronutrients as described for the hydroponics experiment above. The ammonium treatment (A) contained 9 mM NH_4Cl and 1 mM each of $Ca(NO_3)_2$ and KNO_3. Phosphorus was added to each N treatment as KH_2PO_4 at three concentrations (mM): 0.0 (P0), 0.5 (P1), 1.0 (P2). The resulting solutions are annotated based on the combination of nutrient conditions as follows: low P (P0XA, P0XB), intermediate P (P1XA, P1XB), and high P (P2XA, P2XB).

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^{o}C) and light. Filtered exudate
192 solutions (0.2 μm, PES) were stored immediately for analysis of pH (4^{o}C), phytase activity (4^{o}C),
193 dissolved organic C and N, and organic anion composition (-20^{o}C). Sand remaining in pots after the
194 plant harvest was stored at 4^{o}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^{o}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

solution (8 mg NAD and 7 mg $NaHCO_3$ in 1 mL water) and 2 µL of 1:1 solution of LDH and MDH were added. Samples were allowed to equilibrate for 1h at room temperature in order for natural NADH depletion to stabilize. Two µL citrate lyase (CL; 100 mg mL^{-1}) was added to half of the well replicates (n=4) and incubated for an additional hour. The concentration of NADH was measured at 340 nm. The depletion of NADH in wells treated with CL was proportional to citrate concentration in standards (0, 5, 10, 15, 20, 40, 60, 80 nmol citrate). All standard solutions were prepared in blank P0XA or P0XB solutions containing 8.33 times nutrient salts.

2.6 Shoot and Root Analysis

Plants were separated into above- (shoots) and below- (roots) ground biomass. Shoots were oven dried for 48 h (70°C) and weighed for the determination of dry weight. Roots from exudate screening experiments in sterile sand were stored at 4°C in 50% ethanol (v/v) prior to root scanning (EPSON, Hertfordshire, UK) and image analysis. Root images (300 dpi, grey scale) using the Lagarde transformation for pixel identification and analysed for total root length (cm), average diameter (mm), and root lengths in each diameter size class (in 0.1mm increments to >1.9mm) using the root architectural algorithm in WinRHIZO (Regent Instruments, Inc., Quebec, Canada). The percentage of root length in each diameter size class was calculated relative to the total root length determined for individual plant replicates.

2.7 Statistical Analysis

Means and standard errors are presented for five replicate plants and three technical replicates for citrate and phytase-activity measurements. For exudate screening in sterile sand, Tukey Least Square Difference (LSD <.05) was used to compare plant growth and exudate characteristics of cultivars within a single nutrient condition and across nutrient conditions for a single cultivar. Principal component analysis (PCA) was used to visualize and quantify the variation in plant response to the six

nutrient treatments based on physical root parameters and exudation traits. All variables were checked for normality and those not normally distributed were log-transformed prior to correlation and significance testing (Pearson pair-wise, $p<.05$). Aitchison distance was calculated to identify system wide changes in the distribution of root diameter size classes in response to nutrient treatment and defined as A_{root}. The length distributions of root diameter size classes (0 to >1.9mm, 0.1mm increments) were transformed using the isometric log ratio (*ilr*) procedure (Equation A.1) and a sequential binary partition matrix (Table A.2)[57]. Aitchison distances (Equation A.2) [58, 59] were computed for each nutrient treatment (P1XB, P2XB, P0XA, P1XA, P2XA) relative to the reference nutrient condition (P0XB) for each barley cultivar and legume species based on the averaged sum of *ilr* values (n=5). The variance of A_{root} was determined using the propagation of error procedure for the difference of means with equal variance (n=5). The 95% confidence interval (n-1=4 degrees of freedom) was determined for comparison of mean A_{root} values across plant and nutrient treatments.

3. Results

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

3.1.1 Root Morphology and Exudate pH Change

In order to evaluate the response of barley cultivars to P deficiency, root morphological characteristics and pH change of exudate solutions was assessed following three weeks growth in hydroponics with (P1) and without added P (P0). Phosphorus deficiency led to significant changes in the morphological characteristics of roots among the 143 barley cultivars (5 replicates each) tested. Averaged across all cultivars, root dry weight, root surface area, and total root length were significantly larger in P-deficient plants compared to plants grown with P ($p<.001$; Table 1). The proportion of roots smaller than 0.5 mm and larger than 3 mm in diameter increased due to P-deficiency, whereas intermediate diameter roots (0.5 – 3 mm) either decreased or stayed the same (Table 1). For roots greater than 3

mm in diameter, the length of subsequent size classes increased progressively from 23.5% to 111%. The lengthening of thicker roots due to P deficiency is also reflected by increases in total root length, dry weight, and surface area. These trends represent the average response of the entire population to P-deficiency and a large variability of root morphological traits among individual cultivars.

Differences in the P0 and P1 values of root diameter proportions and total surface area have been used here to indicate the response of individual cultivars to P-deficiency, whereby positive differences indicate root elongation or increased surface area, and negative differences indicate shortening or loss of surface area. There were significant positive relationships between the change in total root length with dry weight ($r=0.61$, $p<.0001$) and surface area ($r=0.56$, $p<.0001$) due to P-deficiency for the entire population (Table 1). Difference values for the proportion of roots in specific diameter size classes displayed significant positive relationships with total root surface area ($r>0.51$, $p<.0001$; Table 1) with the exception of roots <0.5mm in diameter. On average, roots less than 0.5 mm in diameter were ~6% more abundant in P0 relative to the P1 condition (Table 1); however, the greater length of <0.5mm roots was related to the net loss of root surface area ($r=-0.57$, $p<.0001$). On average, specific root length (m g^{-1}) was approximately 12% larger in barley cultivars provided with P ($p<.0001$; Table 1) with 58% of cultivars increasing SRL in response to P deficiency. Therefore, the lengthening of thicker roots and an increased proportion of fine roots dominated the physiological response of barley cultivars with a large variation among cultivars identified based on the SRL (Fig. 1).

The growth of barley cultivars in P0 and P1 hydroponics solutions resulted in significant differences in the ability of P0 and P1 plants to affect the pH of exudate collection solutions. Although the average pH in exudate solutions from P-deficient plants (6.28±0.29) was not significantly different from P-sufficient plants (6.12±0.29), exudate solutions from plants supplied with P contained a wider range of pH (4.12-7.19) in comparison to P-deficient plants (5.07-6.85) and were generally more 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),

representing various combinations of P supply (P0=0, P1=0.5, P2=1.0 mM) and N source (A=Ammonium; B='Balanced' nitrate-ammonium N).

3.2.1 Shoot and root Growth of Barley Cultivars in Sterile Sand

Phosphorus supply ($p<.0001$) and N source ($p=0.0003$; Table 2) had a significant effect on shoot biomass and R:S ratios in barley. Barley cultivars were responsive to P supply by increasing the length of roots with no added P (P0) and greater shoot biomass accumulation with the greatest P addition (P2). Across nutrient treatments, shoot biomass in barley ranged from 0.05 to 0.69 g dry weight and increased with greater P supply ($p<.0001$; Table 2, Table 3). Under P deficient conditions, there was no significant difference in shoot dry weight among barley cultivars supplied with ammonium or balanced N (CV=0.20; Table 2), with the exception of the large biomass of cv Waggon (0.23 g) and small biomass of cv Prague in P0XB (0.05 g; Fig. 2). Root to shoot ratios were, on average, 3.4-fold larger in the P deficient treatments (P0XA: 0.36; P0XB: 0.51) compared to P sufficient treatments (P2XA: 0.13; P2XB: 0.13; Fig. 2), indicating the partitioning of resources to root biomass in response to P deficiency.

There was significant variation in shoot biomass ($p=0.0002$) and R:S ratio ($p<.0001$) among individual barley cultivars (Table 2). With respect to R:S, the interaction identified between cultivar and nutrient treatment ($p<.0001$; Table 2) was related to the greater variability of root and shoot biomass measurements among cultivars supplied with balanced N in comparison to ammonium-fed plants. Shoot biomass of barley was significantly greater when plants were supplied with a balanced 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. 2). The coefficients of variation for shoot dry weights in the P1 and P2 treatments were also larger among cultivars supplied with balanced N (P1XB: CV=0.28; P2XB: CV=0.22) in comparison to the ammonium-fed plants (P1XA: CV=0.25; P2XA: CV=0.13).

Specific root length ranged from 54 (cv Prague, P1XB) to 519 m g^{-1} (cv Rainbow, P2XB) and varied significantly across nutrient treatments ($p<.0001$; Table 2, Fig. 2). On average, SRL was consistently greater when plants were provided with ammonium and minimal P (in m g^{-1} P0XA: 280; P0XB: 220; P1XA: 270; P1XB: 123; P2XA: 217; P2XB: 258; Table 2). This was in part due to root dry weights in ammonium treatments, which were 1.3 to 2.3-fold less than plants supplied with balanced N across P treatments. Nitrogen source had a greater effect on SRL ($p=0.0001$) than P supply ($p=0.0166$), but interacted with P supply ($p=0.0003$) to significantly affect SRL in the population of barley cultivars tested (Table 2).

Aitchison distance (A_{root}) was derived from the length of roots in the various root diameter size classes of the barley cultivars. A_{root} was used to compare the root morphology of cultivars in P0XB (reference condition) to plants grown in the other nutrient treatments (Fig. 4). Nutrient treatments with increasing P and N provided as ammonium significantly affected the distribution of root lengths in the various diameter size classes for the majority of barley cultivars tested, including Aramir, Chieftan, Kenia, Krystal, Kym, Prague, Rainbow, Spire, and Waggon ($p<.05$; Fig. 4). In contrast, there was no significant change in A_{root} among Dialog, Domen, and Thuringia cultivars, relative to plants grown in P0XB ($p<.05$; Fig. 4). A_{root} increased with increasing levels of P for Aramir, Kenia, Krystal, Spire, and Waggon cultivars, however this affect was more pronounced when ammonium was provided as the primary N source. The increasing trend in A_{root} with greater P is reflected in the raw proportions of root lengths in the smallest diameter classes, for example in the 0-0.1 and 0.1-0.2 mm (Fig. A.2). The response of barley cultivars to the nutrient treatments was therefore associated with a global changes to root morphology, including a shift in the proportion of roots from larger to smaller diameter size classes.

3.2.2 Shoot and Root Growth of Legumes in Sterile Sand

In terms of shoot biomass, legumes responded to P supply ($p<.0001$) and N source ($p=0.0124$), with significant differences identified between cultivars and across all nutrient treatments ($p<.0001$; Table 2). Legume species increased shoot biomass with increasing P supply from a minimum of 0.08 in the P0XB treatment (*T. purpureum*) to 0.25 g dry wt. in P2XB (*T. subterraneum*; Fig. 3). Under P-deficient conditions, legumes provided with ammonium produced larger shoot biomasses (0.16±0.00 g dry wt.) compared to balanced N (0.10±0.01 g dry wt.), whereas at larger P treatments, legumes provided with balanced N were larger (e.g., in g dry wt. P2XA: 0.14, P2XB: 0.20; Fig. 3). Legumes responded to P deficiency by partitioning more biomass to roots, as indicated by larger R:S ratios in the lowest P treatments (Fig. 3) and significant interactions of R:S with P supply and N source ($p<.0001$; Table 2). Across P treatments, average R:S ratios of legumes provided with ammonium as the primary N source were 1.5 to 2-fold greater than plants provided with balanced N (Fig. 3). However, unlike barley, legume species did not have a significant effect on R:S ratios ($p=0.1812$; Table 2).

Specific root length of individual legume species ranged from 0.3 to 38.2 m g^{-1} dry wt. and was significantly affected by P supply, N source, and cultivar type ($p<.0001$), with no interactions identified between nutrient treatment and cultivar ($p=0.2538$; Fig. 3, Table 2). Averaged across legume species, SRL was greatest for plants supplied with ammonium as the primary N source and 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). The effect of the N source was more pronounced for some species, such as *M. truncatula*, *O. sativus*, and *T. subterraneum*, which in terms of SRL, responded to increasing P more dramatically when supplied with ammonium as the primary source of N (Fig. 3).

Aitchison distances of root lengths in various diameter size classes were larger and more variable (A_{root}: -5.7 to 15.4) than the barley cultivars tested (A_{root}: -6.0 to 7.1; Fig. 4). All legume species responded to the nutrient treatments through a change in the distribution of root diameter length distributions at the greatest P levels (P2XA, P2XB). *Medicago truncatula* cv Sultan was the only

legume to show a significant shift in A_{root} under all nutrient conditions relative to the reference. There was an increasing trend of A_{root} with P supply in the ammonium treatments of *O. sativus* and *T. subterranem*, with a significant difference found between P0XA and P2XA treatments only (Fig. 4). The A_{root} of *O. sativus* and *T. subterranem* corresponded to SRL, which increased with P supply in the ammonium treatments (Fig. 3). This was in contrast to *Medicago*, which displayed the greatest increase in SRL with P supply despite having similar A_{root} values at P0XA and P2XA (Fig. 4).

3.2.3 Exudation Response of Barley cultivars to P and N Treatments

Relative to uncultivated controls, the average pH change of the sterile sand media (ΔpH) by barley cultivars ranged from -0.23 to +0.38 pH units depending on nutrient treatment (Fig. 5). P supply had a more significant effect on ΔpH ($p<.0001$) than N supply alone ($p=0.4105$; Table 3). In general, the average pH change caused by barley cultivars was positive and most pronounced in P deficient treatments (P0XA: +0.23±0.12; P0XB: +0.13±0.08), whereas plants supplied with P did not significantly affect the pH of the sand media (P2XA: -0.07±0.06; P2XB: 0.02±0.05; Fig. 5). There was a significant interaction between P supply and N source on ΔpH by barley ($p<.0001$; Table 2). For example, ΔpH of plants provided with ammonium as the primary source of N was greater than in the N-balanced plant treatment under P deficiency, whereas small differences between N treatments were observed as P addition increased (Fig. 5). Consistent with results of the barley screening in hydroponics (Fig. 1), there was significant variation in the ability of individual cultivars to affect pH of the growth media under different nutrient treatments ($p<.0001$; Table 2).

As for ΔpH, citrate efflux was significantly affected by P supply ($p=0.0408$), and not N source ($p=0.1974$), with a significant interaction between P supply and N source identified in barley ($p=0.0317$; Table 2). On average, citrate efflux by barley cultivated under P deficiency did not differ significantly between N treatments, but was 2.4 fold greater in plants provided with balanced N at the largest P additions (Fig. 5). The interaction of P supply and N source is evident when considering

citrate efflux by plants provided with ammonium as the primary source of N, which was greatest under P deficiency and declined with increasing P (in μmol g^{-1} dry wt. h^{-1} P0XA: 44.0±20.8; P2XA: 14.9±7.9). In contrast, plants cultivated under balanced N displayed the opposite trend, with the greatest citrate efflux being measured in the largest P treatment (in μmol g^{-1} dry wt. h^{-1} P0XB: 26.1±10.5; P2XB: 36.7±13.8; Fig. 5). A significant variation in the ability of individual barley cultivars to exude citrate was identified (p=0.0005) and was found to depend on the nutrient treatment provided (p=0.0005; Table 2); for example, in the extreme cases of cvs Krystal (8.9 μmol g^{-1} dry wt. 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 Aramir (63.1 μmol g^{-1} dry wt. h^{-1}) in the P2XB nutrient treatment (Fig. 5).

Phytase activity ranged from 0.02 to 0.23 nKat g^{-1} root dry wt. h^{-1} and was not detected in all nutrient treatments for the barley cultivars tested (Fig. 5). P supply did not have a significant effect (p=0.4787), whereas N source (p=0.0028) and its interaction with P supply (p=0.0062) were significant factors affecting exudate phytase activity in barley (Table 2). On average, phytase activity was greatest for plants grown under P deficient conditions with ammonium as the primary N source (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 wt. h^{-1}; Fig. 5). In contrast, plants provided with balanced N displayed less exudate phytase activity under P deficiency (0.09±0.05 nKat g^{-1} root dry wt. h^{-1}; Fig. 5) and did not vary significantly with P treatment. There was no significant effect of cultivar on the phytase activity of barley exudates (p=0.4503), however individual cultivars did respond differently to the various nutrient treatments (p=0.0119; Table 2); for example, cv Prague, which varied considerably with N source (P0XA: 0.05; P0XB: 0.15 nKat g^{-1} root dry wt. h^{-1}), or cv Waggon, which did not differ in exudate phytase activity across nutrient treatments (Fig. 5).

3.2.4 Exudation Response of Legumes to P and N Treatments

All legume species and nutrient treatments led to a decline in pH of the sterile sand growth media (Fig. 6). P supply affected ΔpH in the exudate solutions of legumes ($p<.0001$), whereas N source did not ($p=0.4105$); however, a significant interaction between P supply and N source was observed ($p<.0001$; Table 2). On average, there was no difference in ΔpH for legumes cultivated with ammonium and balanced N under P deficiency (-0.40 to -0.46 pH units) or the intermediate P addition (-0.14 to -0.15 pH units); however, plants in the P2 treatment showed a significant acidification of the sand media when provided with ammonium (-0.66±0.01) in comparison to balanced N (-0.17±0.02; Fig. 5). Significant differences between legume species were observed ($p=0.0037$) with the response of individual legumes depending on the nutrient treatment ($p<.0001$; Table 2). For example, acidification by *O. sativus* relative to other legumes in the balanced N treatments was greater under P deficient (-0.19 to -0.31) than under P sufficient (-0.04 to -0.22) conditions (Fig. 6).

Citrate efflux ranged from 2.4 to 74.0 μmol g^{-1} dry wt. h^{-1} and was significantly affected by P supply, N source, and the interaction of nutrient factors ($p<.0001$; Fig. 6, Table 2). As for ΔpH, there was no difference between citrate efflux between the N treatments in the P deficient condition (10.3 to 10.7 μmol g^{-1} dry wt. h^{-1} on average). However, as P supply increased, the average difference between citrate efflux in the two N treatments increased by 2-fold at P1 and 4-fold at P2 (Fig. 5). Legume species did not significantly affect citrate efflux ($p=0.1412$) unless nutrient treatment was also considered ($p<.0001$), as illustrated by the increasing variation in citrate efflux P supply by legumes provided with balanced N and the greatest amount of P (e.g., in μmol g^{-1} dry wt. h^{-1}: *B. pelecinus*: 2.4 vs *O. sativus*: 74.0; Fig. 6, Table 2).

Phytase activity occurred in a similar range for legumes as for barley (0.01 to 0.25 nkat g^{-1} root dry wt. h^{-1}; Fig. 6); however in contrast to barley, legume phytase activity was effected by P supply ($p=0.0429$) rather than N source ($p=0.1238$) and no interaction was found between the two nutrient conditions ($p=0.1315$; Table 2). On average, legume phytase activity was greatest in the P deficient

condition and did not differ significantly between N treatments (in nKat g^{-1} root dry wt. h^{-1} P0XA: 0.13±0.03; P0XB: 0.10±0.06). The variation between individual legume species was weakly significant (p=0.0482) and individual legume species responded differently to the various nutrient treatments in terms of phytase activity (p=0.0004; Table 2). For example, *T. subterraneum* plants provided with ammonium as the primary N source had greater phytase activity in exudates compared to balanced N plants across P treatments (e.g., in nKat g^{-1} root dry wt. h^{-1} P0XA: 0.17; P0XB: 0.08). In contrast, phytase activity was not detected in the exudates of *O. sativus* at P0 but increased to a maximum among legumes at P2, particularly when provided with balanced N (0.23 nKat g^{-1} root dry wt. h^{-1}; Fig. 6).

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

Principal component analysis was used to assess the contribution of plant-induced pH change, citrate efflux, exudate phytase activity and SRL to the variation in response of barley cultivars and legume species to P supply and N source. Principal component 1 (PC1) accounted for 48.6% of the variation between treatments and was primarily explained by SRL (0.854), citrate efflux (0.781), and ΔpH 0.749), whereas PC2 (27.0%) was primarily influenced by differences in phytase activity (0.944; Fig. 7). The shift in values along the PC1 axis illustrates the contrasting responses of barley cultivars and legume species to N source regarding citrate efflux, which was most pronounced under P deficient conditions but greatest in legumes with balanced N (Fig. 7). The response of barley to ammonium is observed in a shift to more positive loading values along the PC1 and PC2 axes, corresponding to increased citrate efflux and exudate phytase activities, particularly under P deficiency (Fig. 7). In contrast, the distribution of legume loadings shows a large variation in ΔpH and exudate phytase activity and a more restricted response of plants in terms of SRL and citrate efflux (Fig. 7).

4. Discussion

We investigated root morphological and biochemical responses of several barley cultivars and legume species to P limitation and N source in order to identify plant combinations for complementarity and facilitation. Root morphology (R:S, SRL, A_{root}) and exudation (citrate efflux, phytase activity) varied with P supply and N source, as well as plant cultivar and species. We identified significant effects and interactions of these factors on the measured root traits, with contrasting responses to six nutrient treatments among barley cultivars and legume species, specifically with regards to citrate efflux, pH change, and root diameter size distribution (A_{root}). Whilst the response of barley and legume varieties to the nutrient treatments were generally consistent with the literature (e.g., root elongation response to P deficiency, stimulation/inhibition of root growth with ammonium), our results provide additional information on the conservation and plasticity of biochemical (e.g., citrate, phytase) and morphological (e.g., SRL) root traits, as well as a compositional metric for describing the entire distribution of root lengths in various diameter size classes (A_{root}). Based on this analysis, we identify promising barley cultivars and legume species for testing some of the questions and ecological principles pertaining to complementarity and growth facilitation between multiple plant species and further discuss the potential importance of selecting companion plants with contrasting responses to nutrient source.

4.1 Conservation of Specific Root Length in Legumes Across Nutrient Treatments

Yang et al. [36] reported SRL in legume varieties following six weeks growth in defined soil mixtures with rhizobial inoculation and superphosphate amendment, which were one to two orders of magnitude larger than those measured in the current study and which followed the order (in m g^{-1}): *T. subterraneum* 159; *T. purpureum* 177; *M. sativa* 209; *B. pelecinus* 299; *O. compressus* 307; *O. sativus* 320. Our results indicate that the relative ranking of legumes based on SRL was consistent across nutrient treatments and followed the order (in m g^{-1}): *M. truncatula* 19; *T. subterraneum* 10; *T. purpureum* 9; *O. sativus* 8; *B. pelecinus* 2; *O. compressus* 1 (Fig. 3). This is consistent with the prediction that, although the response of these legumes to nutrient availability may vary, the relative

ranking of intrinsic root traits such as SRL should be conserved [36]. We can also confirm that the relationship between the length of fine roots (<0.1mm diam.) and SRL is conserved across nutrient treatments for these legume varieties ($r=0.84$, $p<.0001$; Table 3). However, the rankings of *Medicago* and *Biserulla* relative to other legume genera differed in this study relative to the report of Yang et al. [36]. An important difference between these studies was the use of rhizobial inoculants. In the current study, legumes were cultivated in sterile sand and provided with N in order to optimize the recovery of root carboxylates, which, as a labile source of C, are readily degraded by soil microorganisms. Rhizobia play an important role in nodulation as well as root proliferation, branching and pathogen resistance in legumes [60]. In *Vigna* spp. for example, root length, number, branch points, and weight were 67 to 100% reduced in uninoculated plant treatments [61] with similar effects on root biomass accumulation reported in soybean (*Glycine max*)[62]. This indicates a significant effect of rhizobia on the physical development and absolute magnitude of SRL, which may be exacerbated in plants cultivated in sterile sand. This warrants further investigation into the dependence of individual legumes on rhizobia for stimulating root growth as well as SRL values and ranking among other legume varieties.

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

The proportion of root lengths of particular diameter size classes represents a compositional dataset with a sum equivalent to one. As for other compositional datasets, changes in the length of one diameter class will affect the relative proportion of the others [57, 58]. This was observed in the initial analysis of barley cultivars in hydroponics as simultaneous changes in the smallest and thickest root diameters (on the basis of both % and absolute length) in response to P deficiency (Table 1). Aitchison distance, a univariate compositional metric, has been used as a statistical approach for treating compositional data including the distribution of soil P species and fractions [58], soil aggregate size distribution [63], and microbial community compositions [64].

Here, we used Aitchison distance (A_{root}) to assess global changes in the distribution of root lengths of various diameters in response to changes in P supply and N source for each of the barley cultivars and legume species tested. A_{root} is independent of unit (length or %), provides a single representation of all root diameter size classes, and can therefore be used to statistically verify global changes to the distribution of thick and fine roots simultaneously. Furthermore, the metric is defined relative to a reference condition, in this case, the P0XB nutrient treatment. A_{root} values that are significantly different to the P0XB condition represent a change in the distribution of root lengths in the various diameter size classes. Large positive or negative A_{root} values may therefore be interpreted as belonging to plants with highly plastic root systems. In the current study, legume species displayed the largest magnitude and range of A_{root} values despite having smaller roots and SRL relative to the barley cultivars (Fig. 4). This illustrates the scale-independence of the A_{root} measure as an indicator of root morphological plasticity. Limited phenotypic plasticity among barley cultivars has been reported and is linked to a narrow range of selective pressures during the domestication of wild and land-race varieties [65]. In contrast, the large plasticity of legumes based on A_{root} values were consistent with changes observed in root size classes less than 0.1 mm in diameter and SRL, particularly in response to P availability (Fig. 3, Table 3). However, in contrast to the SRL ranking described above, the patterns of A_{root} response to nutrient treatment were not conserved among legumes. Though not investigated in the current study, measures of root diameter size length distributions using A_{root} could provide additional insight into fine-scale differences in root morphology and root biomass partitioning, which cannot be captured by gross measures such as SRL.

4.3 Mechanisms of Plant Response to Nutrient Availability

Barley cultivars responded to P deficiency by an increased partitioning of biomass to roots, alkalization of the growth media, and increasing citrate efflux and phytase activity in exudates. This is consistent with previous accounts of root biomass accumulation in response to nutrient deficiencies 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

inorganic anions ($H_2PO_4^-$/HPO_4^{3-} and NO_3^-) and exchange with alkaline counter ions (HCO_3^-, OH^-), proton sequestration by organic anions (e.g., citrate, maleate, oxalate), and ammonification processes. Conversely, acidification results from the uptake of inorganic cations (NH_4^+) and export of protons, atmospheric N_2 fixation by microbial symbionts, and denitrification processes [70]. Rhizosphere alkalization by cereals and grasses is typically explained by the uptake of nitrate and release of hydroxyl/bicarbonate ions [71], however considering the large concentration and affinity of phosphate transporters in barley, alkalization is likely to be associated with phosphate transport as well [66]. In the current study, plants provided with balanced N consistently increased rhizosphere pH with increasing P supply and did not vary significantly in terms of citrate efflux (Fig. 5). The limited effect of P deficiency on citrate efflux by barley (*H. vulgare* cv Marie) provided with a balanced source of N was recently reported for a single cultivar [72]. In contrast, plants provided with ammonium as the primary source of N appear to have reduced the pH of the growth media at the largest P supply, possibly through the release of acidic counter ions during the uptake of ammonium in larger plants. Citrate efflux was positively correlated with ΔpH among barley cultivars in the ammonium treatment ($r=0.2303$, $p=0.0193$). The relationship of pH and citrate efflux in the ammonium treatment supports a secondary mechanism of alkalization, whereby citrate sequesters or is coupled with the efflux of protons during ammonium uptake by barley [21].

Extracellular release of barley histidine acid phosphatase (HAP) has been linked to the ability of cultivars to grow on phytate due to constitutive levels of exudation regardless of P supply or source [10]. Low levels of phytase activity were measured in barley exudates with contrasting levels of activity, which were found to depend on P supply and N source. Consistent with the results of Ciereszko et al. (2011), no difference in phytase activity was found across P supply when a balanced source of N was provided (Fig. 5). In contrast, plants provided with ammonium responded to P deficiency by increasing exudate phytase activity, which was positively correlated with citrate efflux ($r=0.75$; $p<.0001$) and ΔpH ($r=0.32$; $p=0.0113$; Fig. 5). Similar interactions between P supply and N

source have been reported based on root and soil acid phosphatase (APase) activity in ryegrass (*Lolium perenne*) and tall fescue (*Festuca arundinaceae*)[73]. To our knowledge, this is the first report that the induction of phytase exudation by P deficiency in barley may depend on N source.

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

When combined in intercropping systems, species with contrasting responses to nutrient source and availability are expected to contain a greater range of adaptations for improved P acquisition [4, 8]. Our results indicate that barley and legumes both respond to increasing P supply through physiological (increased SRL) and biochemical traits (increased phytase activity; $r=0.27$, $p<.0001$), particularly with ammonium as the primary source of N (Fig. 5, Fig. 6). Contrasting responses of barley cultivars and legume species include greater acidification by legumes and the interaction of P supply and N source in controlling citrate efflux by these varieties (Fig. 6). Larger rates of acid production by legumes in comparison to barley are expected based on the relatively greater physiological demand for N by legumes, higher rates of N uptake, and, under ammonium treatment, increased proton export [74, 75].

Contrasting responses to P deficiency among plant species based on citrate efflux have been reported to occur in barley (*H. vulgare* L. cv Heder), canola (*Brassica napus* cv Marie), and potato (*Solanum tuberosum* cv Pimpernel), whereby canola was the only species with the greatest citrate efflux in the absence of P [72]. We found citrate efflux by legumes to vary as a function of P supply only when provided with balanced N (Fig. 6) and found no relationship between citrate and pH in either N treatment. In contrast, citrate efflux by barley varied with P supply only with ammonium as the primary source (Fig. 5) and was likely linked to an acid tolerance mechanism induced in response to ammonium nutrition. These results indicate that intercropping of barley and legume species with contrasting responses to N source could improve the adaptation of plants to P deficiency, sub-optimal

soil pH, and the heterogeneous distribution of nutrients in soil while promoting the expression of citrate and phytase exudation in one or both plant species.

Barley and legume varieties with the ability to respond to local nutrient conditions represent promising candidates for improving nutrient efficiency in multi-crop and biodiverse agroecosystems [7]. However, response-based approaches for the selection of complementary plant varieties should consider the morphological and biochemical bounds of response, which will likely vary across species. As would be expected for comparisons made at the species versus cultivar level, differences in SRL, phytase activity, citrate efflux, and pH were more significant among legume species in comparison to the variation identified within the *H. vulgare* L. cultivars (Fig. 5, Table 2).

Li et al. [46] reported greater plasticity of leguminous root systems (e.g., faba bean, chickpea) in comparison to graminoids (e.g., maize, wheat) in response to nutritional variation. Consistent with the analysis of Li et al. [46], we found limited variation in the distribution of root diameter sizes among cultivars of the single *Hordeum* species tested (Fig. 2), and considerably more variability in A_{root} values among legumes in all of the nutrient treatments (Fig. 3, Table 2). In contrast, the variation among barley cultivars was considerably greater than legumes in the P-deficient condition with regards to plant-induced pH change, citrate efflux, and phytase activity (Fig. 5, Table 2).

The contrasting responses of barley and legumes to P deficiency indicate differences in the morphological and biochemical adaptations of these species to acquire soil nutrients [76]. In the case of domesticated barley, the limited morphological plasticity of roots implies that plants must respond to changes in nutrient availability through exudation and modifications to the chemical environment [65]. In contrast, the greater root morphological plasticity of legumes may allow for the physical exploration of soils, but at the cost of biochemical plasticity. Through the identification of contrasting nutrient acquisition strategies such as these, complementary plant combinations may be selected to minimize competition between plants for soil resources (i.e., niche space, nutrients) and maximize

productivity within sustainable cropping systems [5, 6]. The selection of complementary plant combinations may therefore be improved through an understanding of plant genetic variation and phenotypic response to nutrient source and limitation.

5. Conclusion

This study investigated the variation of root exudation and morphological traits among barley cultivars and legume species in order to identify plants with contrasting responses to P supply and N source. The selected traits were based on those previously linked to the capacity of plants to acquire P from poorly soluble and organic forms of P in soils (citrate efflux, exudate phytase activity, pH, root diameter size distribution, specific root length). Three legume species (*M. truncatula*, *T. subterraneum*, *O. sativus*) and four barley cultivars (cvs Prague, Waggon, Spire, Krystal; Fig. 7) displayed the greatest variation in root responses to nutrient supply and represent promising candidates for future facilitation and complementarity studies. It is likely that the selection of complementary cereal and legume varieties will not only depend on intrinsic or constitutive expression of root traits, but condition-specific trade-offs in the expression of these traits between individual plants in the combination. The optimized selection of plant species and cultivars for nutrient-efficient and biodiverse cropping systems will be critical for improving the productivity and export of nutritional resources (e.g., carbohydrates, protein, micronutrients) amidst declining global soil fertility and loss of arable land area.

6. Appendices

Fig. A.1 Characteristics of shoot, root, and exudate solutions of barley cultivars (n=143) grown in hydroponics under P-deficient (P0) and sufficient (P1) conditions.

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

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

Table A.2 Sequential binary partition used for the calculation of isometric log ratios (*ilr*) associated with root diameter size classes of barley cultivars and legume species cultivated in sterile sand.

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

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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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

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).

	Phosphorus treatment			
Root morphological or exudation property	**P0**	**P1**	**% change**	**P-value**
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.

903

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 ($p<.05$).

906

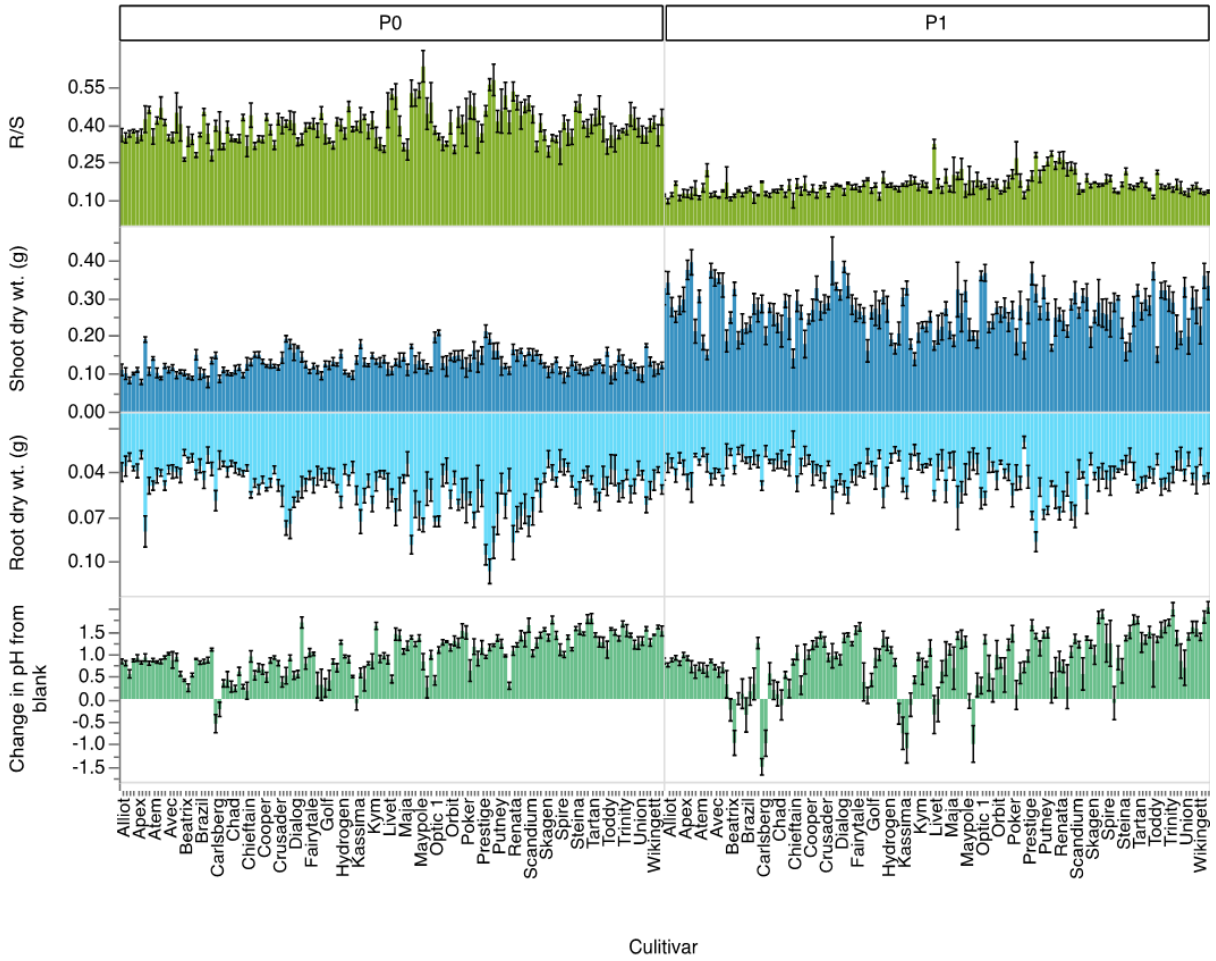
	Factors and interactions	Shoot dry wt.	R:S	SRL	ΔpH	Citrate Efflux	Phytase Activity
All plants	P supply	<.0001*	0.0083*	0.1427	<.0001	0.3341	0.0322*
	N source	0.0413*	0.0428*	0.0013*	0.0245*	0.9276	0.0035*
	P supply × N source	0.3842	0.7128	0.0101*	<.0001	0.1392	0.0236*
	Nutrient Treatment	<.0001*	<.0001*	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

P supply × N source	<.0001*	0.0171*	0.0161*	0.0017*	<.0001*	0.1315
Nutrient Treatment	<.0001*	<.0001*	<.0001*	<.0001	0.2861	0.4216
Cultivar	<.0001*	0.1812	<.0001*	0.0037*	0.1412	0.0482*
Nutrient treatment × Cultivar	<.0001*	<.0001*	0.2538	<.0001	<.0001*	0.0004*

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	- -	+ +							Significantly correlated (p<.05) among barley cultivars and legume species			
ΔpH	- +	+	+ +						Significantly correlated (p<.05) among barley cultivars only			
Citrate efflux nmol g^{-1} root dry wt. h^{-1}	-	- +							Significantly correlated (p<.05) among legume species only			
Phytase activity nKat g^{-1} root dry wt. h^{-1}	-	- -	-		+ -			+ -	Positive or negative correlation; left and right symbols correspond to barley and legume if different			
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	+ +	+	-	+		- -	+ +	+ +	+	+	+	+ +

927



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929 **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
930 sufficient (P1) conditions.

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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

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

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

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

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

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

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

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

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 length distributions of the reference nutrient conditions (P0XB) relative to the other nutrient treatments (P1XB, P2XB, P0XA, P1XA, P2XA) within a barley cultivar or legume species. The computation of A_{root} is made as follows (Egozcue and Pawlowsky-Glahn, 2006):

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

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 the transposed matrix.

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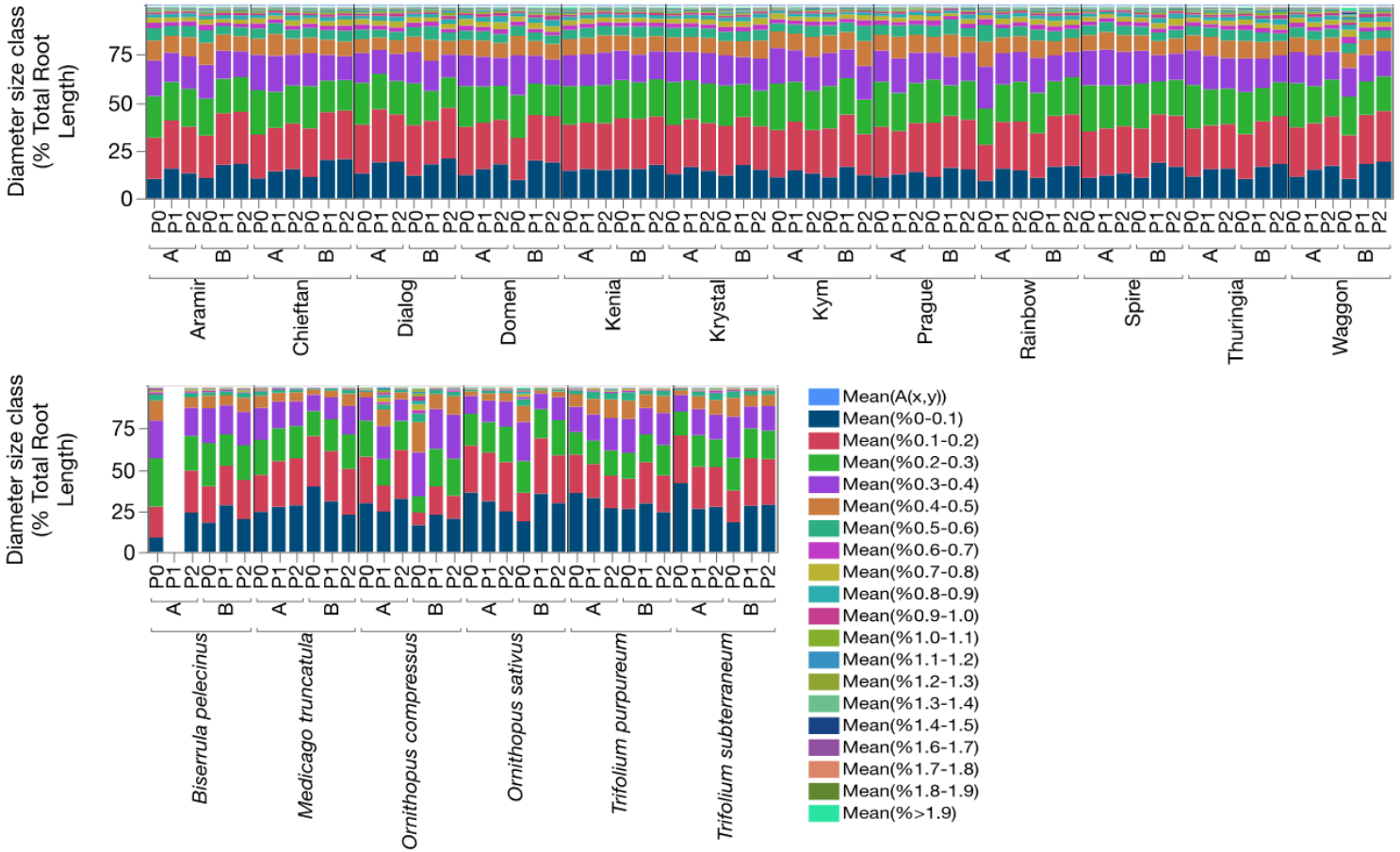

Figure A.2 Root diameter size class, length distribution of barley cultivars (*H. vulgare* L.; top) and legume species (bottom) grown under three phosphorus treatments (P0=0, P1=0.5, P2=1.0mM) with ammonium as the primary source of nitrogen (A) or with balanced nitrate-ammonium (B).

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