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Belowground Interactions in a Barley Cultivar Mixture: Root Distribution and Arbuscular Mycorrhizal Contributions to Uptake of Heterogeneous Phosphorus

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ABSTRACT

Cultivar mixtures have the potential to mitigate abiotic stress and stabilize crop yields, but their belowground dynamics remain poorly understood. We evaluated phosphorus (P) uptake by two contrasting spring barley (*Hordeum vulgare* L.) cultivars (“Anneli” and “Feedway”), grown either in 50:50 mixture or as pure stand. The cultivars were grown in mesocosms under four P fertilization treatments: low-P, homogeneous high-P (90 mg P/kg), and localized P hotspots (100 mg P) placed either in the topsoil (5 cm) or subsoil (35 cm). To trace P uptake pathways, the hotspots were labeled with ³³P and enclosed in mesh bags allowing only mycorrhizal hyphae (25 µm) or both roots and hyphae (2 mm) to access the hotspot. After 35 days, we measured aboveground biomass, total P content, ³³P specific activity, and root biomass, length, diameter, and arbuscular mycorrhiza fungi (AMF) root colonization. In the mixture, reduced P uptake by “Feedway” led to lower overall performance compared to pure stand. Root modifications in the mixture did not enhance biomass or P acquisition, potentially due to decreased AMF colonization. Although different P placements altered P uptake patterns, they did not increase total P uptake. Roots accessed the P hotspots and acquired ³³P without notable proliferation in the enriched zones. Our findings underscore the complexity of belowground interactions involving root distribution, competition for P, and AMF, and highlight the need for future research to optimize nutrient acquisition and performance in cultivar mixtures.

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1 | Introduction

Plant roots play an essential role for the efficient use of resources in agricultural crop systems. Root architecture (the spatial and temporal structure or deployment of roots in soil) influences the exploration for and acquisition of soil nutrients along the soil profile (Lynch 1995). This spatial impact can be of particular importance for the uptake of nutrients with a low mobility in soil, such as phosphorus (P) (Richardson et al. 2011). In many agricultural systems, soil P concentrations are suboptimal for crop production and P is often heterogeneously distributed in soils due to both management and soil biochemical processes. For example, common P fertilizer management options include shallow broadcasting with or without subsequent incorporation, placement near the seed (starter-P), and concentrated placement in subsoil layers (deep bands) (Nkebiwe et al. 2016). For the latter, many dryland systems under conservation agriculture show strong surface stratification of P, limiting crop yields when P and water become spatially disjunct due to rapid topsoil drying (Angus et al. 2019). Placing P in deeper soil layers allows continued P uptake by colocating the fertilizer with subsoil water (Ma et al. 2009). Secondly, the concentrated placement locally saturates soil sorption sites, leaving relatively more P as plant available (Nkebiwe et al. 2016; Freiling et al. 2022). Under these heterogeneous conditions, the ability of plants to deploy roots in a P-rich layer or hotspot and take up P will be a major determinant of final crop productivity (van der Bom et al. 2020).

Cereal crop responses to placed P can vary significantly, depending not only on root architecture but also on factors such as soil P status, the location and size of a hotspot, and the availability of P elsewhere in the soil profile (McBeath et al. 2012; van der Bom, Williams, Borrell, et al. 2023). For example, shallowly placed P can enhance plant P uptake directly through its fertilizer content or indirectly by promoting early shoot and root growth, in turn enabling a larger root system to access deeper soil layers and intercept otherwise unavailable P. Although recent studies have explored this spatiotemporal interaction between placed P and root growth, they have primarily compared plant responses to a hotspot versus those to a control treatment (van der Bom, Williams, Borrell, et al. 2023; van der Bom et al. 2024). However, this “P difference” approach does not provide a direct measure of the contribution of each P hotspot to plant P uptake. Further, arbuscular mycorrhizal fungi (AMF) have been shown to enhance P acquisition under nutrient-limited conditions (Smith and Smith 2011), and the foraging activity of mycorrhizal hyphae may increase the probability of finding a hotspot, thus enabling plants to exploit a P hotspot earlier and more effectively (Cui and Caldwell 1996). Further, the metabolic cost of AMF hyphae may be much less than that of proliferating roots, such that providing assimilates to AMF could be economical to explore and exploit P hotspots (Hodge 2006). However, AMF can represent a significant carbon cost, potentially at the expense of plant growth in high-P environments (Jakobsen 1999; Gavito et al. 2019), especially for cereals (Grace et al. 2009). The use of isotopic tracers can provide direct quantification of these processes.

Mixed cultivar systems are proposed as a promising approach to reduce the impacts of abiotic and biotic stresses and to stabilize

yields across a range of environments (Tooker and Frank 2012; Wuest et al. 2021; Huang et al. 2024). Mixtures may change their productivity compared to pure stands through mechanisms defined by the 4C framework: (1) competition—when plants are using the same pool of abiotic resources in space and time; (2) complementarity—when plants grown together have different requirements for abiotic resources in space, time or form; (3) co-operation—when the modification of the environment by one species is beneficial to the other(s), and (4) compensation—when reduced growth of one species coincides with improved growth by the other(s) because they differ in their sensitivity to abiotic and/or biotic stress (Justes et al. 2021). A potential benefit of novel cultivar mixtures is that they may be easily implemented into existing mechanized cropping systems, where the adoption of other polycultures (e.g., intercropping systems) tends to be constrained by biophysical and socioeconomic factors, including the need for specialized equipment.

The optimization of cultivar mixtures has gained increasing attention as a strategy to improve agricultural performance (Kiær et al. 2009, 2012). For example, mixture effectiveness may be improved by selecting components that respond differently to environmental factors. To date, studies have primarily focused on effects such as resistance toward specific diseases (Borg et al. 2018) and contrasting aboveground traits such as canopy height or growth duration (Kong and Zhao 2023; Huang et al. 2024). A still largely unexplored opportunity lies in combining cultivars based on belowground traits. For example, novel mixtures could integrate cultivars with inherently different root systems. Simulations with polycultures suggest such mixtures may deliver overyielding through root spatial complementarity (Postma and Lynch 2012). However, plant root systems can flexibly adapt to local conditions (i.e., architectural plasticity) and it is unclear how mixing cultivars may affect characteristics such as root distributions and root AMF colonization.

To improve the understanding of interactions between belowground dynamics and P sharing between mixed cultivars, we conducted a mesocosm experiment with ^{33}P in different placements. We quantified root distributions, AMF contribution and P uptake from each source (soil and hotspot) by two phenotypically contrasting barley (*Hordeum vulgare* L.) cultivars, grown in mixture or in pure stand. We hypothesized that mixing two phenotypically contrasting barley cultivars on the basis of contrasting above and belowground traits would improve crop performance compared to pure stands. More specifically, contrasting genotypes will (i) increase aboveground biomass and P uptake under low P conditions, and (ii) modify root distribution to explore complementary niches, with implications for AMF colonization. Furthermore, we hypothesized that (iii) topsoil P fertilization will allow deeper rooting systems for both cultivars by virtue of providing P during early root growth.

2 | Materials and Methods

2.1 | Plant Material

The two barley (*H. vulgare*) cultivars Feedway (breeder NOS 110.352–51, Nordic Seeds) and Anneli (breeder SWA 09090, Lantmännen SW Seed SIA) were selected based on a series of

previous studies in which they demonstrated differential above and belowground traits. Firstly, both cultivars were included in the Swedish national variety trials (2014–2020), in which Feedway had a greater average yield (8.2 t ha^{-1}) and lower height (62.0 cm) than Anneli (6.7 t ha^{-1} and 84.9 cm). Subsequently, a group of nine diverse cultivars was selected from the national field trials and phenotyped in rhizoboxes, in which Feedway and Anneli displayed strongly contrasting root systems (Germon et al. 2023). Briefly, Feedway rapidly developed a steep root system, whereas Anneli developed a relatively shallow root system. Based on these combined observations, the two cultivars were expected to be suitable model cultivars for the investigation of competitive and complementary interactions.

2.2 | Soil

A low fertility soil was collected from the plough layer of the unfertilized treatment of the CRUCIAL trial (Magid et al. 2006) at the University of Copenhagen's experimental farm in Taastrup, Denmark ($55^{\circ}40' \text{ N}$, $12^{\circ}17' \text{ E}$). The soil had a sandy loam texture with 262 g kg^{-1} coarse sand ($0.2\text{--}2 \text{ mm}$), 436 g kg^{-1} fine sand ($0.02\text{--}0.2 \text{ mm}$), 143 g kg^{-1} silt ($2\text{--}20 \mu\text{m}$), and 126 g kg^{-1} clay ($< 2 \mu\text{m}$) (Lemming et al. 2019) and is classified as an Albic Luvisol (Epidystric) in the FAO system (IUSS 2006). Total C and N concentrations were 11.3 and 1.3 g kg^{-1} measured on an elemental analyzer (VARIO CUBE, Elementar Analysensysteme GmbH). The total P concentration was 0.4 g kg^{-1} (measured by ICP-OES, Agilent 5100) and the Olsen P concentration was $11.2 \pm 0.3 \text{ mg P kg}^{-1}$. The water holding capacity was 32% (gravimetric water content, measured on a saturated sand bed, modified after ISO 14238 Annex A).

The soil was air-dried, crushed with a shovel and sieved through an 8 mm mesh. Mineral nutrients, except P, were added to batches of 11.0 kg (dry weight) of soil, in liquid solution, as follows: K_2SO_4 370 mg kg^{-1} , CaCl_2 75 mg kg^{-1} , MnSO_4 0.5 mg kg^{-1} , ZnSO_4 5.4 mg kg^{-1} , CuSO_4 2.1 mg kg^{-1} , Na_2MoO_4 0.18 mg kg^{-1} , NH_4NO_3 286 mg kg^{-1} (100 mg N kg^{-1}) and MgSO_4 405 mg kg^{-1} to ensure that all nutrients except P were in adequate supply (Raymond et al. 2018). Each batch of soil was then air-dried and thoroughly mixed to ensure a homogeneous nutrient distribution.

2.3 | Mesocosm Experiment

A mesocosm experiment was conducted in an unbalanced randomized block design with three cropping systems and four P treatments, combined with two mesh sizes to differentiate between the contributions of roots and AMF. All treatments were replicated four times in blocks. The mesocosms consisted of bottom-closed tubes of 16 cm diameter and 50 cm height (Figure 1), with a $100 \mu\text{m}$ thick LDPE liner and filled with 11.0 kg batch (dry weight) of the prepared soil.

The cropping systems were:

1. Anneli in pure stand,
2. Feedway in pure stand,
3. Anneli and Feedway in 50:50 mixture.

The P treatments included:

1. Low P (without any P fertilization),
2. High P (homogeneously distributed throughout the soil volume),
3. A P hotspot in the topsoil (5 cm),
4. A P hotspot in the subsoil (35 cm).

For the high-P treatment, $395 \text{ mg kg}^{-1} \text{ KH}_2\text{PO}_4$ (corresponding to 90 mg P kg^{-1} or 990 mg P per tube) was mixed into the soil for each batch as part of the basal fertilizer procedure, and the application of K_2SO_4 was reduced to maintain the targeted K concentration. The P hotspots were prepared to contain 100 mg P (per tube). For reference, an average MAP fertilizer granule weighs approx. 44 mg (data not shown), which gives 10 mg P per granule (at 21.9% P). Consequently, 100 mg P would equate to a hotspot of 9–10 fertilizer granules stacked closely together. Further, 100 mg on a 16 cm diameter circle (cross-sectional area of the mesocosms) corresponds to 50 kg ha^{-1} . Each P hotspot treatment was conducted with two mesh size treatments:

1. $25 \mu\text{m}$ to allow only AMF hyphae to access the hotspot.
2. 2 mm to allow AMF hyphae and roots to access the hotspot.

To prepare the P hotspot, 30 g of soil (dry weight) was thoroughly mixed for 2 min with 21 g of KH_2PO_4 (100 mg P) and incubated for 5 days. Then, 0.5 mL of a solution containing carrier-free ^{33}P with a radioactivity of 4.0 MBq mL^{-1} was added to each sample and thoroughly mixed for 2 min. The resulting Olsen P concentration in the mesh bags was measured at $1.4 \text{ g P kg soil}^{-1}$. The radioactive soil was then placed in square mesh bags with 3 cm sides, closed with staples, and placed in a larger mesh bag with 5 cm sides together with 90 g of unlabelled soil. The outer mesh bag with unlabelled soil acted as a buffer to prevent unwanted uptake of ^{33}P by root hairs (in the hyphal compartment treatment) (Cruz-Paredes and Gavito 2020). The mesh bags were placed at the desired depth during filling of the mesocosms.

All mesocosms were watered to 80% water holding capacity and kept in a temperature-controlled greenhouse ($22^{\circ}\text{C}/17^{\circ}\text{C}$ day/night with 16 h of light). After watering, the mesocosms were incubated in the greenhouse for one week. For each cultivar, seeds were imbibed in petri dishes with wet filter paper in the fridge for 26 h and then left in the dark at room temperature overnight. Seeds with a small protruding radicle were selected and planted at a depth of 2 cm. In each mesocosm, four seeds were planted in a circle (two of each cultivar in the mixture) using a template to ensure uniform spacing among all mesocosms. Throughout the experiment, soil water content was maintained at 80% water holding capacity by regular weighing and topsoil watering. Weeds were removed by hand as soon as detected. Onset of radioactivity in the aboveground biomass was recorded every 2–3 days with a Geiger counter.

2.4 | Harvest and Sampling

Plants were destructively harvested after 38 days of growth, which corresponded to the late tillering stage. Tillers were

X 4 replicated blocks

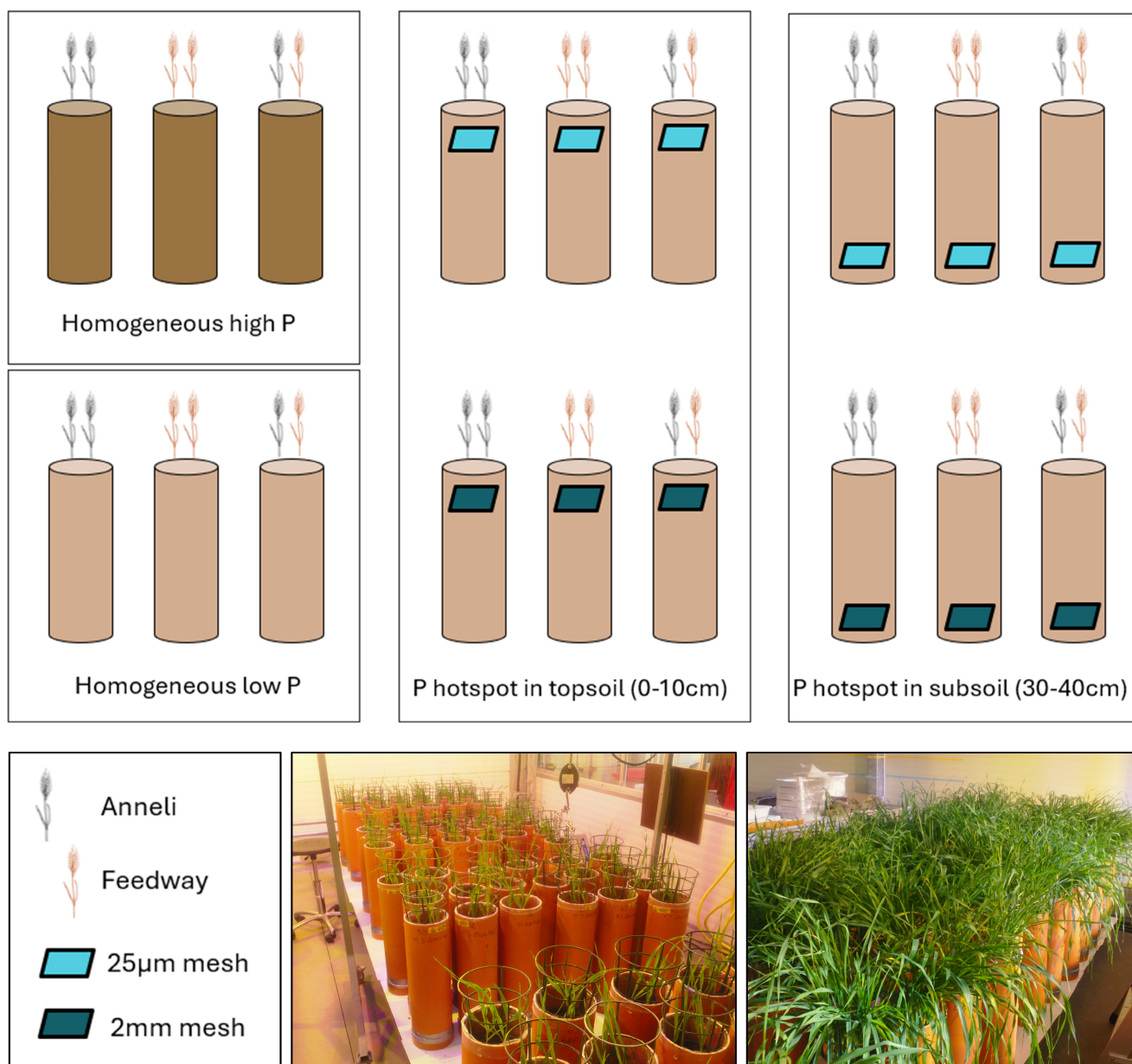


FIGURE 1 | Schematic of experimental design. Two barley cultivars were grown in pure stand or as a mixture in four replicates. Plants were grown under four P placements: Homogeneous low or high P, a hotspot placed at 5 cm depth (topsoil) and a hotspot placed at 35-cm depth (subsoil). The hotspots were placed in mesh bags of either 2 mm or 25 µm.

counted on each plant. Aboveground plant parts were cut at the base of the stem and immediately put in an oven at 75°C. The two cultivars were collected separately for the mixture treatments.

After sampling, the plastic sleeve containing the soil core was carefully removed from the mesocosm. The soil core was cut into 10-cm segments (five layers per mesocosm) and stored at 4°C until roots could be recovered. Each segment was placed on a 1 mm sieve and roots were carefully washed to remove soil and organic matter. For the top 10-cm samples, the stubble was separated from the roots, oven-dried, and added to the respective aboveground plant parts. A representative subsample of the roots was taken for AMF analyses and stored at 50% ethanol at

4°C. The rest of the roots from the topsoil segment and all the roots from the other segments were stored in 30% ethanol at 4°C until further analyses.

2.5 | Sample Analyses

Dried aboveground plant parts were weighed to obtain biomass, after which subsamples of 100 ± 10 mg were ashed in small crucibles at 550°C for 1 h for determination of the P concentrations. The ash was transferred to a 50 mL centrifuge tube, shaken on an end-over-end shaker with 50 mL of 0.5 M H_2SO_4 for 16 h, and filtered through Whatman No. 42 filter papers. The ortho-P content of the extracts was measured by a flow injection analyzer

(FIAstar 5000, FOSS). The plant digests were analyzed for ^{33}P beta-emission after mixing with a scintillation liquid (Ultima gold TM, Perkin Elmer) using a liquid scintillation counter (Tri-Carb 2910TR, Perkin Elmer).

Root samples from blocks 1, 2, and 3 were spread on a transparent tray with deionized water and scanned at 600 dpi with a flatbed scanner (Epson Expression 12000XL). The images were analyzed with RhizoVision Explorer version 2.0.3 (Seethepalli and York 2021) to determine total root length (m), average root diameter (mm) and root length (m) per diameter classes (0–0.1, 0.1–0.2, 0.2–0.3, 0.3–0.4, 0.4–0.5, 0.5–1, 1–1.5, 1.5–2, and above 2 mm). In the analysis and data presentation of root distribution, samples from the lowest layer (40–50 cm) have been excluded because roots that reached the bottom of the mesocosm and continued growing obscure the relative distributions in this layer. All root samples were dried at 75°C and then weighed to obtain root biomass.

Root colonization by AMF was quantified using the line-intercept method by Giovannetti and Mosse (1980) after clearing and staining the roots according to Kormanik and McGraw (1982), except acid fuchsin was replaced by trypan blue.

2.6 | Data Analyses

The total P concentration was multiplied by the biomass to get the P uptake on a per plant basis. The ^{33}P data were corrected for decay using ^{33}P decay tables (Cruz-Paredes and Gavito 2020). The specific activity (SA) in the shoots (kBq mg^{-1}) was calculated as:

$$\text{SA}_{\text{shoot}} = \frac{\text{shoot activity (kBq)}}{\text{shoot P uptake (mg)}}$$

The same principle was used to calculate SA in bicarbonate extractable P (Olsen P) of soil in the hotspot. The amount of P derived from the hotspot (mg) corresponds to the pool of isotopically available P taken up by plants, and was calculated as:

$$\text{P}_{\text{hotspot}} = \frac{\text{shoot activity (kBq)}}{\text{SA}_{\text{hotspot}} (\text{kBq mg}^{-1})}$$

The contribution of AMF to P uptake from the hotspots (%) was then derived from the amounts of P taken up from the hotspot with the 2 mm mesh size (roots and hyphae) (mg P plant^{-1}) and 25 μm mesh size (hyphae only) (mg P plant^{-1}).

Root length density (RLD, m cm^{-3}) was calculated as the ratio of the total length to the soil sample volume, and specific root length (SRL, m g^{-1}) was calculated as the ratio of scanned root length to root dry mass. The contribution of AMF to P uptake from the hotspots (%) was calculated as the percentage of ^{33}P uptake in mesocosms with the 25 μm mesh size (mg P plant^{-1}) divided by ^{33}P uptake in mesocosms with the 2 mm mesh.

To test the performance of the mixture compared to the pure stands, the relative yield total (RYT) was calculated as (de Wit 1960; Satorre and Snaydon 1992):

$$\text{RYT} = \frac{Y_{ij}}{Y_{ii}} + \frac{Y_{ji}}{Y_{jj}}$$

where Y_{ij} and Y_{ji} are the aboveground biomass (or the P uptake) per mesocosm of cultivar i and j , when grown as a mixture; Y_{ii} and Y_{jj} are the aboveground biomass (or the P uptake) per mesocosm of cultivar i and j , when grown in pure stand.

To test the competitive ability of cultivar i with regard to cultivar j , the aggressivity (AGR) was calculated as (McGilchrist and Trenbath 1971):

$$\text{AGR} = \left(\frac{Y_{ij}}{Y_{ii}} - \frac{Y_{ji}}{Y_{jj}} \right) \times \frac{1}{2}$$

A high AGR value corresponds to a high competitive ability of cultivar i when mixed with cultivar j .

2.7 | Statistical Analyses

All statistical analyses were performed with R software (version 4.3.1) (R Core Team 2020), using the packages *lme4* and *car*. Linear mixed models were fitted with the replicated blocks as random factors to test: (1) the effects of the cultivar, cropping system, P treatment and their interactions (fixed factors) on the aboveground biomass per plant, the uptake of P derived from the hotspot, the uptake of P derived from soil, and the percent AMF root colonization; and (2) the effects of the soil layer, cropping system, P treatment, and their interactions (fixed factors) on root length density. The normality of the residuals and the homogeneity of the variances were verified visually from residual plots. If necessary, logarithmic or square root transformations were applied. Post hoc Tukey honest significance difference (HSD) tests were used to establish the significance of the differences between each category, with a threshold value of 0.05.

3 | Results

We observed clear interactions among cultivar and cropping systems ($p < 0.001$, Table S1). For Anneli, the aboveground biomass per plant was similar between pure stand and mixture, but for Feedway it was lower in mixture (Figure 2). Accordingly, Feedway and Anneli had an equivalent aboveground biomass per plant in pure stand, but the aboveground biomass of Feedway was lower than that of Anneli in mixture. This was also reflected in the P uptake, with Anneli taking up significantly more P when growing in mixture compared with growing in pure stand ($p < 0.001$), while P uptake by Feedway decreased in the mixture ($p < 0.001$). Subsequently, Feedway tended to take up less P (from soil and from the hotspot) than Anneli when the two were grown in mixture together, and this difference was significant for the high P treatment and the low P treatment ($p < 0.05$). Further, the mixture as a whole took up less P from the hotspots than each of the pure stand cultivars (Figure 3).

The high P treatment increased the aboveground biomass per plant ($p < 0.001$, Figure 2) and P uptake ($p < 0.01$, Figure 3) of

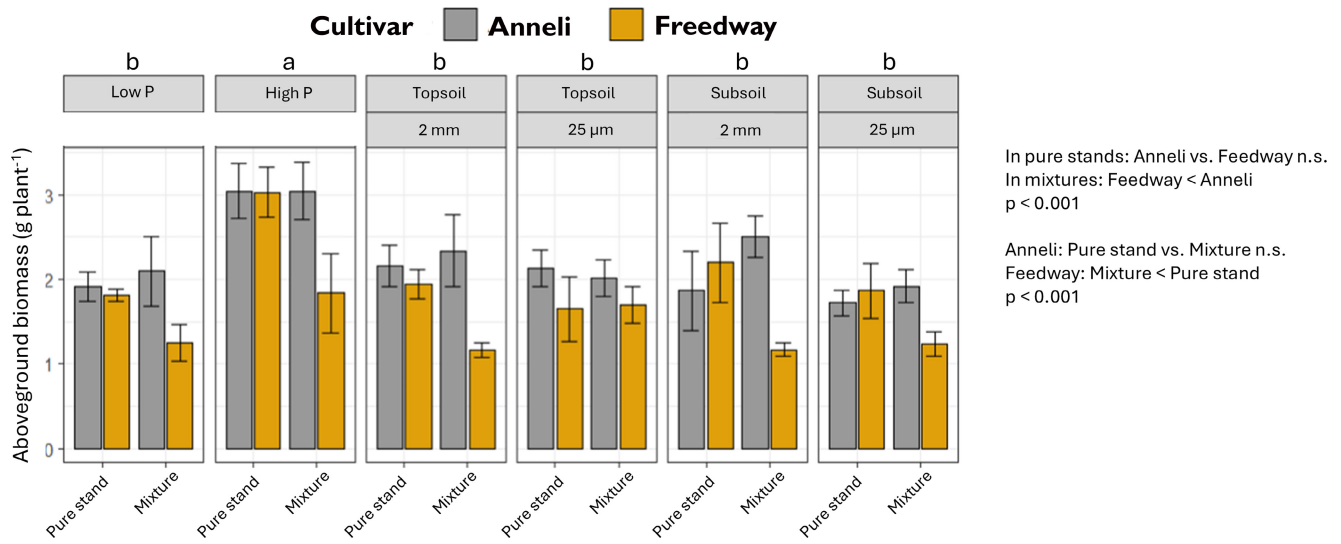


FIGURE 2 | Aboveground biomass of two barley cultivars grown in pure stand or as a mixture. Plants were grown under four P placements: Homogeneous low or high P, a hotspot placed at 5 cm depth (topsoil), and a hotspot placed at 35 cm depth (subsoil). The hotspots were placed in mesh bags of either 2 mm or 25 µm mesh size. Data are mean values $\pm$ standard errors ($n = 4$). Lowercase letters indicate significant differences between P treatments ($p < 0.001$). Significant interactions between cultivars and cropping systems are written on the right of the figure.

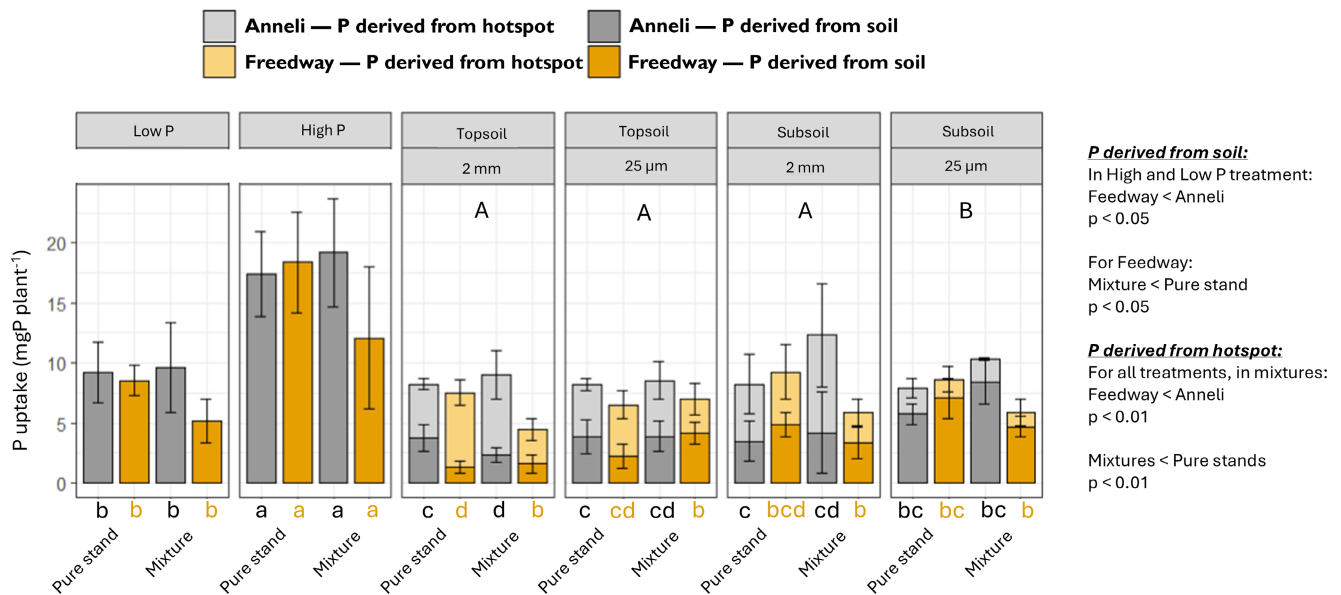


FIGURE 3 | Uptake per plant of supplied P from the hotspot (light shades) and of P derived from soil (dark shades) of two barley cultivars grown in pure stand or as a mixture. Plants were grown under four P placements [homogeneous low or high P, a hotspot placed at 5 cm depth (topsoil) and a hotspot placed at 35 cm depth (subsoil)]. The hotspots were placed in mesh bags of either 2 mm or 25 µm mesh size. Data are mean values $\pm$ standard errors ($n = 4$). For P derived from hotspot, uppercase letters indicate significant differences between P treatments ($p < 0.001$). Significant differences with regard to the interaction between cultivars and cropping systems ($p < 0.001$) and between systems ($p < 0.01$) are written on the right of the graph. For P derived from soil, lowercase letters below the bars indicate significant differences between treatments within each cultivar and system ($p < 0.05$). Significant differences between cultivars within treatments and between systems within cultivars ($p < 0.05$) are written on the right of the figure.

both cultivars, regardless of whether in pure stand or in mixture. In contrast, uptake in the hotspot treatments was not significantly different from the low-P treatment ($p = 0.1$, Figure 3). However, the ^{33}P data showed that plants took up P from the fertilizer in both hotspot treatments. Further, the date at which radioactivity was first detected in the aboveground biomass was treatment-dependent, from day 4 after seeding for the topsoil hotspot to 23 days after seeding for the subsoil hotspot (Table S2).

The RYT ranged from 0.8 to 1.0 among the different P treatments (Figure 4), whereas aggressivity ranged from 0 to 0.2. In the mixture, Anneli was generally more competitive than Feedway, and this competitive ability was slightly greater when the hotspot was in the subsoil.

On average across all treatments, root biomass and root length density were greatest in the top 0–10 cm layer, after which they

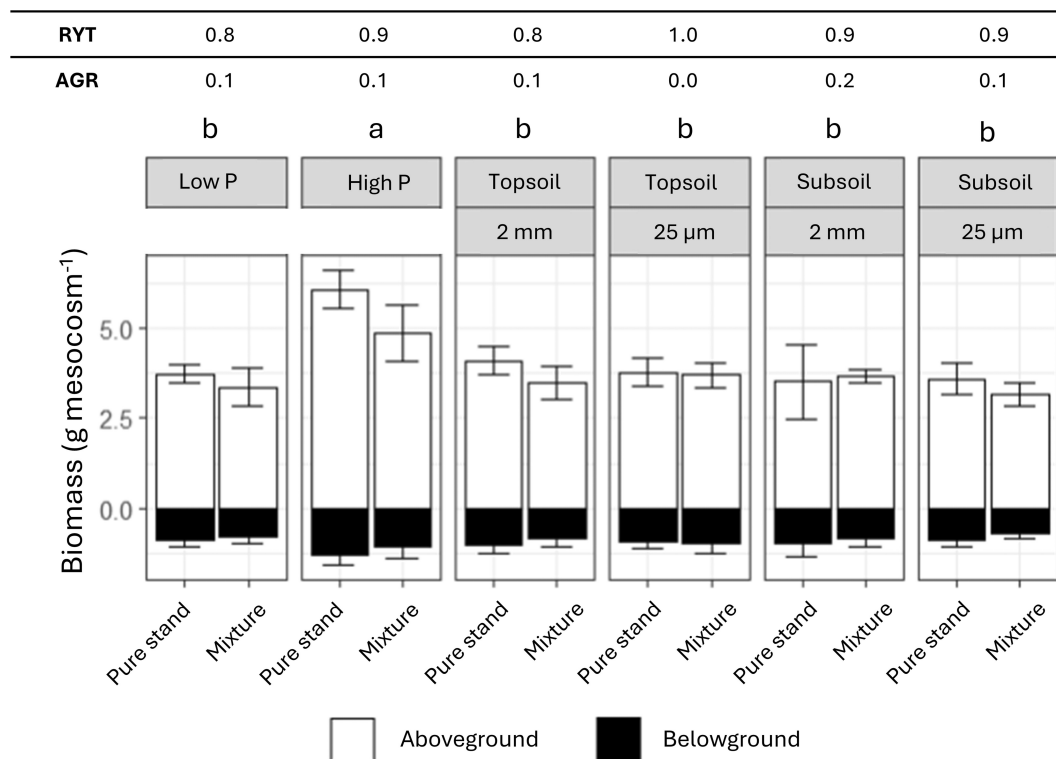


FIGURE 4 | Plant aboveground and belowground biomass of two barley cultivars grown in pure stand or as a mixture. Plants were grown under four P placements [homogeneous low or high P, a hotspot placed at 5 cm depth (topsoil) and a hotspot placed at 35 cm depth (subsoil)]. The hotspots were placed in mesh bags of either 2 mm or 25 µm mesh size. Data are mean values ± standard errors ($n = 4$). On top of the graph are shown the relative yield total (RYT) and aggressivity of Anneli with respect to Feedway (AGR), calculated based on aboveground biomass.

tended to decline with increasing depth ($p < 0.001$; Figures S3 and S5). However, there was variation among the treatments and cultivars. In the high P treatment, Feedway had a root length density greater than 35 m cm^{-3} at 0–10 cm depth, while the root length density of Anneli in this layer was only 16 m cm^{-3} , and root length density of the mixture was in-between (Figure 5). Where root length density rapidly declined with depth for Feedway, it initially increased for Anneli. The mixture tended to follow a pattern similar to that of Anneli. Somewhat similarly, under low P conditions, Anneli tended to have a small root length density in the top 0–10 cm layer, which rapidly increased with depth. While the mixture also tended to increase with depth, this increase was very small.

Both cultivars significantly increased their specific root length in response to P scarcity especially in the mixture ($p < 0.01$; Figure S2). No root proliferation, either in length (Figure 5) or in biomass (Figure S3), was observed in the layers where the P hotspot was placed (0–10 and 30–40 cm).

The percent of AMF root colonization was lower in the mixtures than in the pure stands for all P treatments ($p < 0.001$; Figure 6a). For all cropping systems, the percent AMF root colonization was lower under high P conditions than when a P hotspot was placed in the mesocosm. The both hotspot treatments had a lower AMF colonization than the low P treatment ($p < 0.001$). The AMF contribution to fertilizer P uptake was significantly greater when the hotspot was in the topsoil (90%) than in the subsoil (45%) ($p < 0.001$; Figure 6b).

4 | Discussion

This study explored the opportunity to design crop mixtures from contrasting cultivars, to optimize the use of spatially heterogeneous resources. Soil nutrient heterogeneity, especially for P, is widespread in modern agriculture due to fertilizer application, tillage, and soil biochemical processes (Robinson 1997; Adamchuk et al. 2010) and is likely to become increasingly common with crop and soil management scenarios, shifting to future systems with lower-input and heterogeneity. The cultivars in this study are in recent use in Northern Europe, and therefore acted as a model for potential farmer choices for mixing contrasting cultivars. While previous studies have focused on aboveground effects, our study expands this research by quantifying belowground interactions and their effect on the uptake of P from contrasting locations.

4.1 | Phosphorus Uptake Patterns From Different Placements

Total P uptake increased in the high P treatment but not when a P hotspot was added, relative to the low P treatment. The difference between the hotspot and the high P treatment was threefold: (1) the amount of P applied, (2) its location and (3) the volume of P-enriched soil. For the first, each hotspot contained 100 mg of P, whereas the high P treatment received 990 mg P per mesocosm. However, 100 mg of P should be expected to also elicit a crop response, considering the

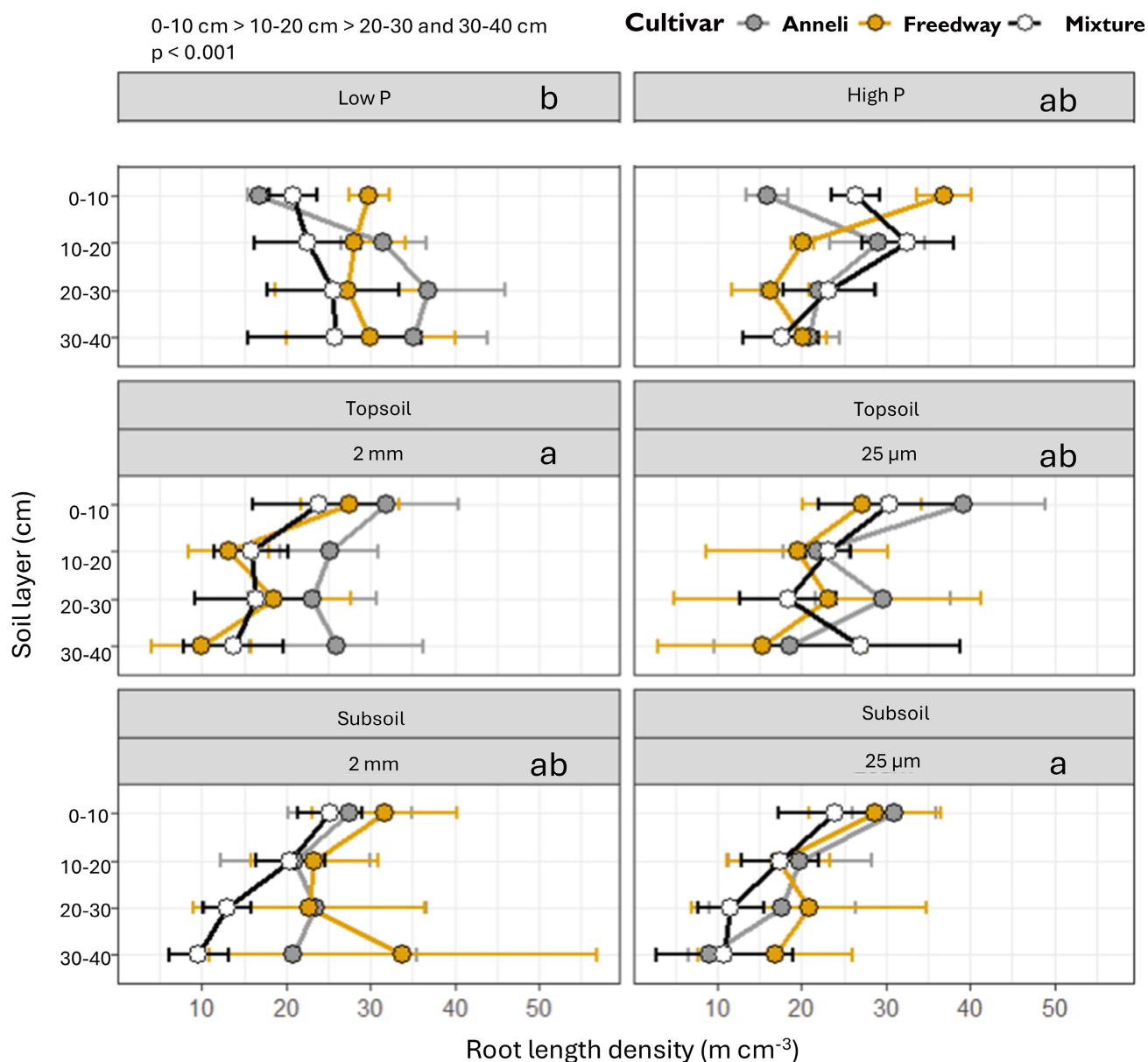


FIGURE 5 | Distribution of root length density of two barley cultivars grown in pure stand or as a mixture. Plants were grown under four P placements [homogeneous low or high P, a hotspot placed at 5 cm depth (topsoil) and a hotspot placed at 35 cm depth (subsoil)]. The hotspots were placed in mesh bags of either 2 mm or 25 μm mesh size. Data are mean values $\pm$ standard errors ($n=4$). Lowercase letters indicate significant differences between treatments ($p < 0.05$). Significant differences between soil layers ($p < 0.001$) are written above the graph.

experimental methodology ensured P was limiting (Raymond et al. 2018) and plant P concentrations indicated that plants were P deficient (Reuter and Robinson 1997). Further, the concentrated placement should locally saturate soil sorption sites such that relatively greater amounts of P remain available (Nkebiwe et al. 2016; Freiling et al. 2022). This is further supported by the high Olsen-P concentration in the hotspot. For the second, the availability of P throughout the profile in the high-P treatment would have allowed young roots to encounter P as they grew, whereas placement as a hotspot required roots to first grow in the low-P environment until they encountered the hotspot, scavenging only minimal P in the process. This was shown previously for wheat and sorghum, in which the ability of the root system to take up P early was of major importance to overall crop growth (van der Bom, Williams,

Borrell, et al. 2023). Therefore, placement of P out of reach from the young root system may have delayed the uptake of P by the plants, with this early P deficit limiting the crop's ability to achieve its growth potential (Grant et al. 2001). For the third, the small volume of the P hotspot may have limited the ability of roots to exploit the applied P fertilizer. This is further supported by the fact that we did not observe any signs of root proliferation, as is commonly observed under these conditions (Drew 1975; Drew and Saker 1978), meaning only few roots had developed around the hotspot.

Although we did not find evidence for root proliferation, we detected radioactivity as early as 4 days after seeding for the topsoil hotspot and 18 days for the subsoil hotspot (for Freedway, Table S2). Therefore, roots must have been able to extract small amounts of

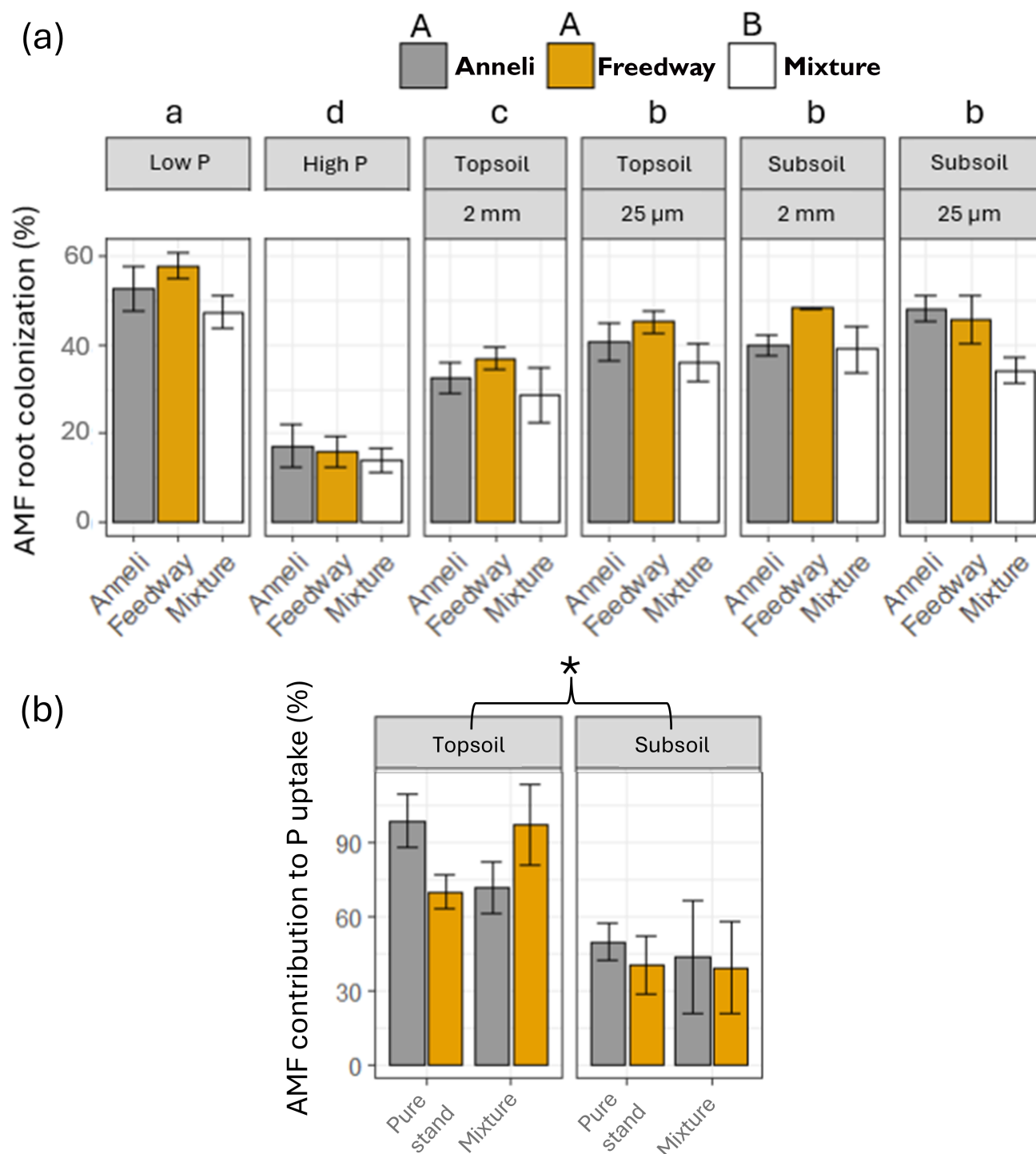


FIGURE 6 | Percent AMF root colonization (a) and AMF contribution to P uptake (b) of two barley cultivars grown in pure stand or as a mixture. Plants were grown under four P placements [homogeneous low or high P, a hotspot placed at 5 cm depth (topsoil) and a hotspot placed at 35 cm depth (subsoil)]. The hotspots were placed in mesh bags of either 2 mm or 25 μ m mesh size. Data are mean values $\pm$ standard errors ($n=4$). Lowercase letters indicate significant differences between treatments ($p<0.001$). Uppercase letters indicate differences between cropping systems ($p<0.001$). * indicates significant differences between the location of the hotspot ($p<0.001$).

P before the onset of proliferation. This fits with the understanding that root tips play a particularly important role in total P uptake (Smith 2002), as well as that root physiological responses to nutrient-rich hotspots (e.g., improved P uptake) can occur before morphological changes take place (e.g., the initiation of new roots) (van Vuuren et al. 1996; Hodge 2004). Secondly, AMF could have played a significant role in hotspot-P uptake. Indeed, AMF root

colonization increased with P scarcity (high P < hotspot < low P), in agreement with previous findings (Wang et al. 2017), leading to higher aboveground crop performance.

The total P uptake in the hotspot treatments was similar to that in the low-P control. This suggests that the plants were not able to access or utilize the P from the hotspots effectively, even though

radioactivity was detectable in the aboveground biomass. In addition, when a hotspot was present, the specific activity of ^{33}P demonstrated that plant uptake of soil P was partly replaced by that of hotspot-P. We deduce that plants adjusted their P uptake patterns depending on the placement of P (hotspot vs. low-P treatment, topsoil vs. subsoil) without altering their total P uptake. When the hotspot was located in the topsoil, the AMF contribution to P uptake was greater (90%) compared to when it was located in the subsoil (45%, Figure 6b). We propose that this discrepancy was due to low AMF hyphal growth rates and slow AMF infection of roots growing into the subsoil. In this scenario, early root growth in the topsoil and greater quantity and quality of resources available to AMF would have favored AMF infection in this layer (Rillig and Field 2003). As AMF hyphal growth rates are typically slower than roots (Schütz et al. 2022), this would then result in a proportionally lower AMF contribution to P uptake from the subsoil—at least within the timeframe of the current experiment. Alternatively, roots may have accessed the P tracer if P diffused from the mesh bag into the bulk soil, but this seems unlikely given the methodology incorporated a buffer soil volume to prevent this.

4.2 | Competition in Cultivar Mixtures

Mixing of two phenotypically different barley cultivars did not increase aboveground biomass and P uptake compared with the pure stand. Specifically, Feedway consistently underperformed when mixed with Anneli, evidenced by its lower aboveground biomass and lower P uptake. Combined with the lower competitive ability (RYT/AGR), these data suggest that Feedway must have had a poorer ability to forage and take up P, resulting in overall system domination by Anneli. This pattern was consistent among all treatments, even though it was expected that varietal differences in root distribution would lead to preferential P uptake depending on its placement (e.g., topsoil vs. subsoil). However, where previous experiments observed clear differences between Anneli and Feedway in terms of rooting depth (Germon et al. 2023), these differences were relatively small in the current experiment. Further, the overall poor mixture performance was in contrast to common observations that indicate yield and yield stability benefits across a range of environments (Reiss and Drinkwater 2018; Huang et al. 2024). However, these analyses typically include other mechanisms such as disease resistance, where the current experiment specifically focused on the ability of plants to exploit (heterogeneous) soil P. On the other hand, there was a slight tendency for Anneli to increase its biomass in mixture. This tendency, although not significant, may be interpreted as an early indication of a potential compensation effect (Döring and Elsalahy 2022), but the current growth period would have to be extended for this to be conclusive. Therefore, our data both highlights the potential relevance of considering belowground processes and root architecture, but also the challenges posed by root plasticity for designing complementary mixtures.

Root length density and specific root length at depth were greatest for the low P treatment. This was consistent with deeper roots found in wheat mixtures under competitive conditions (Fang et al. 2014). A higher specific root length usually indicates thinner roots, necessary for exploration and improvement of P

uptake under low P conditions (Freschet et al. 2015). In the low P treatment, root plasticity (i.e., change of specific root length in mixture compared to pure stands) was not accompanied by improved aboveground biomass or P uptake in the mixture compared to the pure stands, confirming plants need to scavenge for P.

The limited benefits of root modifications in mixtures could be explained by the role of AMF colonization in the competitive dynamics between the two cultivars. AMF colonization was consistently lower in mixtures compared to pure stands. An explanation could be that plants in mixtures may have reduced C allocation for AMF root colonization. The literature shows varying effects of coexisting plants on the root mycorrhizal colonization of each other, according to the species (Chen et al. 2005). In the present study, the reduction in AMF colonization probably occurred equally for the two cultivars, as the relative contribution of AMF to P uptake was similar for both Feedway and Anneli. A shared AMF hyphal network between the two cultivars could have amplified competition for limited resources rather than mitigating it, potentially explaining the poorer performance of mixtures (Mikkelsen et al. 2008; Merrild et al. 2013). These observations underscore the complex interplay between root distribution, cultivar competition, and AMF associations, and highlight the need for studies evaluating the underlying mechanisms involved in this reduction in AMF root colonization in mixtures. Such studies should include more fine-scale measurements, including the external mycelium of AMF and the composition of AMF species colonizing the roots.

5 | Conclusion

The novelty of this study lies in its integrated exploration of crop mixtures under contrasting P availability, focusing on belowground traits, such as root distribution, AMF interactions, and competitive dynamics. Most previous research has emphasized aboveground factors such as pest and disease resistance. A key finding is the reduced performance of one cultivar in mixture, which limited the potential benefits of the mixture for biomass accumulation and P uptake. Nonetheless, mixtures had greater specific root length at depth relative to the pure stands, highlighting potential for greater soil exploration for limiting resources. Our results further suggest a potential role of AMF colonization in the competitive dynamics between cultivars in the mixture. Together, our findings underscore the complexity of belowground interactions in crop mixtures and point to a need for further studies to understand the underlying mechanisms and refine our understanding of how these factors interact under different environmental conditions.

Author Contributions

Lorène Siegwart: conceptualization, methodology, investigation, formal analysis, visualization, writing – original draft, writing – review and editing. **Dorette Müller-Stöver:** conceptualization, methodology, writing – review and editing. **Dorte Bodin Dresbøll:** conceptualization, methodology, writing – review and editing. **John Larsen:** formal analysis, writing – review and editing. **Tino Colombi:** conceptualization, methodology, writing – review and editing. **Thomas Keller:** conceptualization, methodology, writing – review and editing. **Frederik**

van der Bom: conceptualization, methodology, writing – review and editing.

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Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

Data sharing is not applicable to this article as all newly created data is already contained within this article.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section.