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RESEARCH ARTICLE

Intercropping alters rhizosphere chemistry but not early root distribution in a low phosphorus field

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Summary

Background and aims Intercropping represents an opportunity to diversify root system architectures and rhizosphere processes, optimising soil resource use, especially for nutrients with low availability. This study assessed belowground interactions in faba bean (*Vicia faba*) and barley (*Hordeum vulgare*) intercropping under marginal field conditions during early growth.

Methods Root distribution was measured to 40 cm depth, and high-resolution bio-chemical imaging visualized in situ soil pH, phosphatase activity, and P concentrations. Planar optodes, zymography, and diffusive gradients in thin films (DGT) were successively deployed in the rhizospheres of each species.

Results Soil heterogeneity (e.g., pH, phosphatase activity) strongly influenced rhizosphere processes, with acidification and alkalization observed around barley roots and varying phosphatase release. Intercropped barley showed two-fold greater pH changes but lower phosphatase activity than sole barley. Faba bean acidified its rhizosphere by only 0.10-0.17 pH units in both systems but increased its phosphatase release five-fold when intercropped. Root distribution along the soil profile was not significantly affected by intercropping at this stage.

Conclusion Our findings highlight the potential of intercropping to modulate root-driven soil processes during early growth. Extending this approach across crop development stages and intercrop systems may deliver further insights into belowground

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crop interactions and their relationships with nutrient acquisition in resource-limited soils.

Keywords Bere barley · Phosphatase activity · Interspecific interactions · Soil heterogeneity · Soil pH · Planar root length

Introduction

Crop diversity is emerging as a key target in the pursuit of sustainable and resilient farming systems. Intercropping is the practice of growing several crop species together in the same field (Willey 1979; Vandermeer 1992) and delivers greater diversity to the system. This offers potential benefits such as enhanced yields (overyielding) and greater yield resilience compared with sole crops (Hinsinger et al. 2011; Bedoussac et al. 2015; Jensen et al. 2020). Among the most studied intercropping systems are those combining cereals and legumes. These have been observed to increase the use of resources such as light, water and nutrients compared with sole crops (Yin et al. 2020; Maitra et al. 2021; Justes et al. 2021). While positive effects have often been attributed to the atmospheric nitrogen (N) fixing capacity of the legume (Rodriguez et al. 2020; Bekele 2021), evidence suggests that belowground interactions between intercropped root systems may play a crucial role in enhancing soil nutrient uptake (Duchene et al. 2017; Homulle et al. 2022).

Roots of intercropped species may explore and take up nutrients from different parts of the soil profile as a result of contrasting root system architectures (Zhang et al. 2024). For example, many legumes tend to have a taproot and grow rapidly towards deep soil layers (Abdelhamid 2010; Burridge et al. 2020), while cereals may explore relatively shallow soil layers (Maqbool et al. 2022). This would lead to a deeper and more even distribution of roots in the system, allowing for a greater exploitation of a larger soil volume (Homulle et al. 2022).

Plant roots can also release exudates, serving a range of functions to support plant performance under stress conditions such as nutrient limitation. The released exudates directly impact the soil chemistry in the rhizosphere by inducing various chemical reactions that modify nutrient availability and microbial activity (Jones et al. 2004). This ability to chemically alter the rhizosphere is often associated with phosphorus (P) deficiency, in which root-induced acidification or alkalization and release of organic anions may improve plant-availability by dissolving or desorbing sparingly available P forms (Jones 1998; Dakora and Phillips 2002). With regards to cereal-legume intercropping, cereals have been shown to both increase (Devau et al. 2010) or decrease (Bhattacharyya et al. 2003; Li et al. 2008) the rhizosphere pH, whereas legumes are more likely to acidify their rhizosphere due to the imbalance of cation over anion uptake while actively fixing atmospheric N (Bolan et al. 1991; Hinsinger et al. 2003; Sun et al. 2020). Additionally, plant roots can also promote mineralization of organic P forms through the release various phosphatase enzymes (Nannipieri et al. 2011), and recent evidence suggest these may function in-tandem with organic anions (Clarholm et al. 2015; Richardson et al. 2025), opening the possibility of synergistic chemical interactions in the rhizosphere of intercropped root systems (Giles et al. 2018). Intercropping can alter rhizosphere chemistry through changes in root exudation, nutrient uptake patterns, and microbial activity, which in turn affect the concentrations of compounds such as organic acids, inorganic N, and P in the soil solution (Zhang et al. 2019; Lo Presti et al. 2021; Kumar et al. 2022). However, reported effects are inconsistent: some studies observe increased mobilization of P due to enhanced exudation of organic acids, while others report reduced nutrient availability or no significant changes in rhizosphere P concentrations. These contrasting results may partly arise from the strong spatial heterogeneity of rhizosphere processes, particularly in intercropping systems where different root species interact within millimetres. Characterizing rhizosphere chemistry at fine spatial scales may help identify localized chemical gradients around individual roots and clarify interspecific root interactions.

Species interactions in intercropping may involve modification of root growth and activity, including root distribution patterns (i.e. architectural plasticity) and rhizosphere processes. For example, species have been observed to grow deeper roots or altered the growth and development of different types of roots (Li et al. 2006; Miyazawa et al. 2010; Streit et al. 2019). Such alterations of root distribution may be a major determinant for legume-cereal intercropping

benefits, whether driven by modification of the legume, the cereal, or both. Similarly, intercropping may alter patterns of root exudation intensity and distribution (Bargaz et al. 2017), potentially inducing local regions of increased acidification/alkalization or enzyme activity in the rhizosphere (Betencourt et al. 2012; Li et al. 2016). However, much of the understanding has been derived from destructive sampling (e.g. soil coring) providing a time-limited snapshot, or from controlled conditions that likely impact root growth and activity dynamics compared with field conditions.

Studying belowground interactions in the field is difficult and therefore rarely done. To analyse rhizosphere processes, studies frequently employ destructive sampling of rhizosphere soil, often pooled before analysis (Betencourt et al. 2012; Zhu et al. 2022a, 2022b). However, rhizosphere processes might be highly dependent on soil fine-scale local heterogeneity in chemical, biological and physical properties (Xue et al. 2016; van der Bom et al. 2023). Methods are now available to visualize biochemical changes around roots, such as optodes (soil pH changes) (Blossfeld and Gansert 2007; Merl et al. 2022), zymography (phosphatase activity) (Spohn et al. 2013a) and diffusive gradients in thin films (DGT, phosphorus fluxes) (Santner et al. 2015). To date, these have primarily been applied in laboratory studies, but there is opportunity to combine them in field conditions (Vetterlein et al. 2021; Bilyera et al. 2022), where they provide accurate information about root-soil interactions and the spatial heterogeneity of rhizosphere processes (van der Bom et al. 2022). Furthermore, they may help clarify how belowground interactions between intercropped root systems can enhance soil nutrient uptake in intercropping systems.

Here, we explored belowground interactions between intercropped faba bean (*Vicia faba*) and barley (*Hordeum vulgare*) under low P field conditions. We constructed custom root windows to assess root growth over time, and successively deployed planar optodes, zymography and diffusive gradients in thin films (DGT) to visualize soil pH, acid phosphatase activity and P concentrations around roots. We hypothesized that 1) the distribution of roots in the soil profile will contrast between the two species, with a deep root system for faba bean as opposed to shallower roots for barley; 2) intercropping will increase the difference in root distribution; 3) species will determine rhizosphere pH, phosphatase activity and P availability, with faba bean having greater rhizosphere changes (pH and phosphatase) than barley; and 4) intercropping will produce greater rhizosphere changes than sole crops.

Material and methods

Site description

The study was conducted in 2024 at the experimental farm of the University of Copenhagen (Taastrup, Denmark, 55°40 N, 12°18E), on an arable Luvisol (WRB IWG 2014) with a sandy clay loam texture. The climate is temperate with an average annual rainfall of 646 mm and an average temperature of 9.3 °C (Petersen et al. 2023). The field was cropped with winter wheat in the preceding year. The overall crop rotation is approximately 50% spring barley, 35% winter wheat and 15% mixed crops (rapeseed, faba bean, clover). The N and P fertilization levels were usually 13% and 48% below the typical Danish recommendations for malting barley on this soil type. The experimental field had previously been identified as P limited for crop growth, based on soil analyses, fertilisation records and apparent deficiency symptoms. The soil had an Olsen P concentration of 13 mg kg^{-1} (Table 1), considered 'very low' in the Danish fertiliser-P recommendation system (Jordan-Meille et al. 2012).

Soil samples were collected from the plough layer (0–20 cm) of the experimental field, air dried, passed through a 2 mm sieve, and then analysed using standard methods (either in-house or at AGROLAB Agrar und Umwelt GmbH, Sarstedt, Germany). Briefly, total C and N concentrations were measured with an elemental analyser (Flash, 2000, ThermoFisher Scientific); soil pH was measured in a 0.01 M $CaCl_2$ solution at a 1:5 soil:solution ratio; soil texture was analysed following ISO 11277:2002–08; CEC following ISO 13536: 1997–04; Olsen P and exchangeable potassium (K), magnesium (Mg) and copper (Cu) according to Sørensen and Bülow-Olsen (1994); and oxalate-extractable P, Al, and Fe according to Schwertmann (1964), using inductively coupled plasma atomic-emission spectroscopy (ICP–OES).

Table 1 Summary of selected topsoil properties. Data are mean ± standard deviation

pH ($CaCl_2$)	Clay (g kg^{-1})	Silt (g kg^{-1})	Sand (g kg^{-1})	Total C (g kg^{-1})	Total N (g kg^{-1})	Ca (cmol + kg^{-1})	Mg (cmol + kg^{-1})	K (cmol + kg^{-1})	Na (cmol + kg^{-1})	Potential CEC (cmol + kg^{-1})
5.3 ± 0.0	149.3 ± 11.0	146.0 ± 7.9	686.7 ± 18.6	12.5 ± 0.3	1.3 ± 0.1	7.2 ± 0.6	0.6 ± 0.0	0.3 ± 0.0	0.1 ± 0.0	13.3 ± 0.8

Olsen P (mg kg^{-1})	Exch. K (mg kg^{-1})	Exch. Mg (mg kg^{-1})	Exch. Cu (mg kg^{-1})	Al_{ox} (mg kg^{-1})	Fe_{ox} (mg kg^{-1})	P_{ox} (mg kg^{-1})
13.3 ± 5.4	90.5 ± 0.5	69.7 ± 8.7	27.7 ± 2.6	577 ± 28	3623 ± 517	225 ± 56

CEC, cation exchange capacity; Exch, exchangeable, extracted with ammonium acetate; ox, oxalate-extractable

Experimental design

The field experiment was set up in a randomized complete block design with three replicates. Each block contained three plots measuring 1.5 × 12 m, separated by a 4 m wide buffer zone. The plots corresponded to three different cropping systems: 1) sole crop barley, 2) sole crop faba bean, and 3) intercropped barley and faba bean. The faba bean cultivar was 'Stella' and the barley cultivar was 'Bere', a Scottish landrace that has previously been observed to sustain uptake of several nutrients under marginal conditions (George et al. 2014; Schmidt et al. 2019, 2023; Cope et al. 2020; Martin et al. 2023).

Before sowing, 1.5 × 1 × 1.5 m pits were excavated at the head of each plot and one custom root window (60 × 50 cm) was installed per pit to facilitate access to soil and roots (Fig. 1a and b). The root windows consisted of an aluminium frame that was attached to a wooden structure (built in place to stabilize each pit). The aluminium frame held three main components: 1) a plastic foil in contact with soil to maintain the integrity of the soil profile while opening the window, 2) a transparent acrylic glass plate for root observations, 3) two PVC boards to ensure dark conditions. The windows were positioned such that the top of each frame was aligned with the soil surface. Bolted arms provided the possibility to adjust the pressure of the window against the soil profile. After installing the windows, a narrow space between the window and the soil profile was filled with 2 mm sieved topsoil to ensure proper and homogeneous contact between the root window and the soil profile, and to facilitate application of the in-situ methods.

Barley and faba bean were sown on 18 April 2024 with a target planting density in sole crop of 325 plants m^{-2} for barley and 40 plants m^{-2} for faba bean. Intercropping plots were seeded with a 50/50 replacement design. Seeds were manually placed at the head of each plot to ensure a proper plant stand next to each window, such that roots would be visible through the acrylic glass. All plots were treated with herbicides 19 days after seeding (1.0 L ha^{-1} Stomp, 1.0 L ha^{-1} Fighter 480 and Mavrik). The sole barley, sole faba bean and intercropping plots were fertilized with 120.0 and 60 kg N ha^{-1} each (50% nitrate and 50% ammonium), and all plots received 70 kg K ha^{-1} and 12 kg S ha^{-1} (with a combination of Patentkali

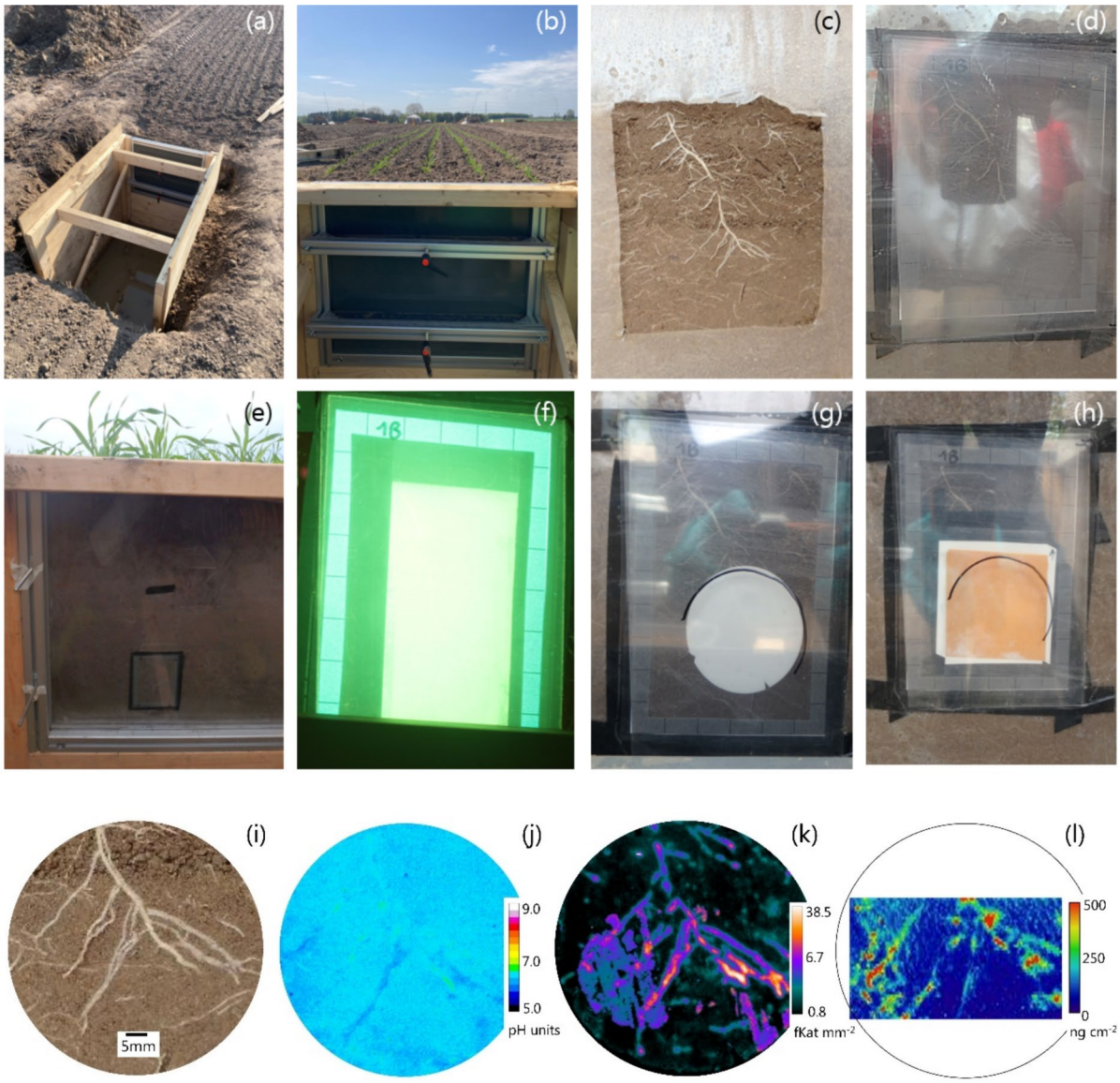


Fig. 1 Temporary stabilized pits **a** and root windows **b** at the experiment facility at the University of Copenhagen (Taastrup, Denmark). The crop shown in this figure is sole barley. The region of interest was chosen according to root visibility and general quality **c**. A template was used to define the region of interest and as scale **d**. For each new sampling campaign, a new region of interest was defined (and the one from the previous campaign was closed again by taping the plastic foil **e**. Planar pH optodes **f**, zymography **g** and Kapton DGT **h** were then consecutively applied within the same region of interest, resulting in 2D visualization around roots **i** of pH **j**, phosphatase activity **k** and P concentrations **l**

and Extran), in accordance with the Danish agronomic recommendations.

Root growth and distribution

Early root growth over time was recorded through root length measurements across six consecutive dates. The measurements started as soon as roots were visible through the root windows (27 days after sowing) and were conducted until the faba bean started flowering (57 days after sowing). On each date, a transparent sheet was placed against the acrylic glass of each window and all visible roots were traced on the sheet. For the intercropped species,

roots of each crop were traced on a separate sheet. Faba bean roots and barley roots could easily be distinguished from each other by tracing their growth from first initiation at the seed. After tracing, each transparent sheet was scanned with a flatbed scanner (600 dpi, Epson Expression 12000XL). The images were analysed with RhizoVision Explorer version 2.0.3 (Seethepalli and York 2021) to determine root length along 10-cm intervals. These data are reported as *planar* root length density (pRLD), considering the two-dimensional nature of observation windows, serving as a proxy for root presence at the soil-profile interface rather than a volumetric root length density (Wacker et al. 2024).

Bio-chemical imaging

Three sampling campaigns were conducted between 36 and 56 days after sowing, in which three bio-chemical imaging techniques were successively deployed on the same region of interest. Each sampling campaign took three days. Planar optodes were applied on d1, zymography on d2, and DGT on d3. This sequence was considered optimal, taking into account each methodology and their potential influence on physical and chemical integrity of the zone of interest (see discussion). For each campaign, regions of interest were selected on each root window based on the number of visible roots, root tips and the quality of the surrounding soil (no holes, biopores etc., Fig. 1c). A plastic template was taped to the acrylic glass window for each region, serving as a frame of reference for application of the imaging techniques and as a size calibration for image processing (Fig. 1d). For the intercropping system, regions of interest were selected for each species.

Soil pH around the root was visualized with planar optodes (Merl et al. 2022). The sensor foils for pH planar optodes were prepared as described by (Merl and Koren 2020) and were cut into 5×8 cm pieces. Before application in the field, the sensor foil was soaked in 0.1 M phosphate buffer solution (pH = 7, ionic strength = 0.377 M adjusted with NaCl) for one hour in a container covered with aluminium foil to exclude light. The sensor foil was carefully rinsed and then taped to the acrylic glass window at the region of interest, with the sensitive side facing the soil profile. The window was then pushed against the soil profile and good contact was ensured by adjusting the pressure on the acrylic glass. The optode set-up was installed in the pit with a fixed distance between the camera and the sensor foil. A black opaque cloth was used to cover the pits and ensure dark conditions. The image acquisition was conducted 1 h after the sensor foil application. Images were taken with a DSLR camera (EOS, Cannon 1300D) with a yellow 455 nm long-pass filter (GG455 SCHOTT, 52 mm×2 mm) and an additional plastic filter (#10 medium yellow; LEEfilters.com) attached in front of the lens. The camera settings were shutter speed 1″, aperture f/3.5, and ISO 200. A 405 nm UV LED (r-s components, Copenhagen, Denmark) with a 390 nm band-pass filter (HFB-3925 HOYA, U.Q.G Optics, Cambridge, UK) was used to excite the fluorescence signal. The resolution of the pH optodes was 120 µm. For each optode image, soil temperature was measured with a soil thermometer to facilitate calibration of the optode sensor foils at the same temperature as in the field. To calibrate the optode measurements, a sensor foil was soaked in 0.1 M phosphate buffer solution (ionic strength = 0.377 M adjusted with NaCl) after which it was consecutively adjusted to pH 3, 5.5, 6, 6.5, 7, 7.5, 8, 8.5, 9, 12. The sensor foil was imaged for each pH after stabilization, using the same set-up as for the experimental images. From the calibration images, ratio images were obtained in ImageJ, by dividing the blue channel by the red channel. The grey scale values from the ratio images were plotted against the measured pH values and a calibration curve was fitted with a sigmoid function (Supplementary Fig. 1). The parameters derived from the sigmoidal fit were used to convert grey values in the experimental images to pH values.

Acid phosphatase activity was imaged with zymography following (Razavi et al. 2016). The time-dependent phosphatase activity is estimated from a fluorescent signal released after enzymatic cleavage of the fluorophore substrate 4-methylumbelliferyl-phosphate (4-MUF-phosphate) (Spohn et al. 2013a). A polyamide membrane (0.45 µm pore size, 50 mm diameter, Whatman™, GE Healthcare, Buckinghamshire, UK) was soaked for 15 min in 10 mM of the substrate 4-MUF-phosphate diluted in 0.1 M 2-(N-morpholino)ethanesulfonic acid (MES) buffer. This buffer was adjusted to pH 6.5, which corresponded approximately to the soil pH in the bulk soil, as measured by optodes (Guber and Kravchenko 2024). The membrane was then air dried for 3 min

and taped to the root window within the region of interest. After closing the window, good contact between the membrane and the soil was ensured by adjusting the pressure on the acrylic glass. After 1 h, the membrane was removed from the window with tweezers, cleaned with a brush to remove soil particles and stored between two plastic foils covered by aluminium foil while being transported back to the laboratory. The membranes were photographed under dark conditions and UV light within 5 h of sampling. The DLSR camera (EOS, Cannon 4000D) had a 100 mm lens and the following settings: shutter speed 2″, aperture f/8, and ISO 200. The resolution of the zymograms resulted in 13 µm. For the calibration, pieces of membrane were cut into 1 × 1 cm squares, their dry mass was recorded, and they were then soaked for 1 h in eleven different concentrations (0, 10, 25, 50, 100, 200, 400, 800, 1200, 1600 and 2000 µM) of methylumbelliferone dissolved in a 0.1 mM MES buffer. Two replicated membranes were included for each concentration. After soaking, the membranes were left to dry for 3 min and the wet mass was weighed to calculate the volume of the MUF solutions absorbed by the membrane. The calibration membranes were then imaged under UV light under similar conditions to the experimental membranes. Specifically, we ensured constant time lap between sampling and imaging for the experimental and the calibration membranes; as such, any decrease in fluorescence on zymogram has been taken into account in the calibration. The grey scale values were measured from the calibration images with ImageJ and plotted against the different concentrations. A calibration curve was fitted with an exponential function (Supplementary Fig. 2). Experimental images were transformed to 8-bit grayscale images in ImageJ and calibrated to acid phosphatase activity as fKat mm^{-2} using the exponential fit.

Labile P was sampled with DGT and mapped with LA-ICP-MS. Kapton (3 M Low Static Polyimide Film Tape 7419) was used as a non-hydrogel based DGT binding layer (BL) for phosphorus, following (Moens et al. 2024). This novel BL is quick and easy to prepare, easy to handle and does not shrink upon drying, making it advantageous compared with the traditional gel-based design, particularly for larger scale investigations under field conditions. Briefly, the polyimide tape was homogeneously covered with titanium dioxide-based Metsorb (HMRP5, Graver Technologies), taped with the back to a plastic foil, and covered with a protective polyethersulfone membrane (PES, 0.45 µm pore size, 0.14 mm thickness). The DGTs (5 × 5 cm) were deployed by taping them to the inside of the acrylic glass after which the root window was closed while ensuring good contact. The DGTs were deployed for 24 h based on preliminary tests that demonstrated a suitable accumulation of labile P without saturating the BL. Before deployment, soil moisture was adjusted to ensure equivalent conditions than in the field. After deployment, the protective membranes were removed from the BLs and each BL was stored individually in a closed plastic box prior to analysis with LA-ICP-MS. For calibration, DGTs were prepared as described by (Moens et al. 2024). Duplicate BLs were exposed for 24 h to six different solutions with contrasting P concentrations, were determined by ICP-MS after elution (Supplementary Fig. 3). The BLs were attached on glass plates using double-sided tape. The P distribution on each DGT was analysed with Laser Ablation Inductively Coupled Plasma Mass Spectrometry (LA-ICP-MS). The LA instrument was the Analyte Excite^{+} excimer laser ablation system (Teledyne), equipped with a 2-Volume HelEx II Cell and ARIS (Aerosol Rapid Introduction System, Teledyne) for fast wash-out. The elemental analysis was done with a Triple Quadrupole ICP-MS (8900, Agilent). The binding layers were ablated using line scans with 20 Hz repetition rate, squared laser beam of 80 µm, a fluence of 3.2 J cm^{-2}, and a scan speed of 800 µm s^{-1}. The interline distance was 400 µm. The pixels were extrapolated laterally to provide a visual image where the pixel size was 80 by 400 µm.. The ICP-MS monitored ^{31}P and ^{49}Ti in MS/MS mode with mass-shift (+16) using oxygen cell gas to reduce polyatomic interferences and measure P with high sensitivity. The certified reference samples NIST614 and NIST612 were measured at the start and end of each sample to correct for daily sensitivity and drift during analysis. The LA-ICP-MS data processing software Iolite (v4.8.0) was used for image reconstruction and calibration and images were exported to ImageJ (v1.54p) for final editing. Signal intensities were converted to elemental surface concentrations using an external calibration function derived from the analysis of matrix-matched DGT calibration standards. The DGT results were then interpreted as surface loadings.

Image analysis

Images with soil pH, phosphatase activity and P concentrations were obtained for each region of interest (Fig. 1i-l). We selected suitable roots on each region of interest. When several roots were suitable on the same region of interest, they were analysed as pseudo-replicates. For the DGT images, we observed variation in quality and visibility and of the soil-root-interface (as commonly observed for this method (Wagner et al. 2020)), therefore, only those images that demonstrated a clear visible soil-root-interface were analysed.

The image analysis consisted of extracting data from three lines orthogonal to roots in ImageJ. For pH and phosphatase activity, the lines were between 2 and 10 mm from the root tip. For P concentrations, the lines were positioned where a hotspot was visible along the root. The pH, phosphatase activity or P concentration were extracted from each pixel, after which a five-point floating average was calculated for each line. As part of the data analysis, the middle of each analysed root was defined as a distance from the root axis of 0 mm (Fig. 2). A four-parameter logistic curve was then fitted for the pH, phosphatase activity or P concentration as:

$$y = \alpha 1 + \frac{\alpha 2 - \alpha 1}{1 + e^{-\beta(x - x_0)}}$$

where y is the pH, phosphatase activity or P concentration and x is the distance from the root axis. The parameters α 1 and α 2 are the two asymptotes, and β is the slope around the inflection point. We extracted the pH or phosphatase activity from the fitted function at three points: A) in the middle of the root (x = 0); B) at the interface between the root and the soil; and C) at the interface between the rhizosphere and the bulk soil. We measured the visible width of each root in ImageJ, and calculated the mean diameter (n = 3). The location of the root-soil interface (point B) was then determined by subtracting half the mean root diameter from the middle of the root. The interface between the rhizosphere and the bulk soil (point C) was defined as the point where the pH, phosphatase activity or P concentration was 1% different from the asymptote. From these data, the *rhizosphere extent* was calculated as the distance (mm) between B (root surface) and C (bulk soil); and the *rhizosphere effect* was calculated as the difference in pH, phosphatase activity or P concentration between B and C. For barley roots, we observed that the rhizosphere pH could be either higher or lower than that of the bulk soil, therefore barley roots were categorized into 'acidifying roots' or 'alkalizing roots' (Fig. 2).

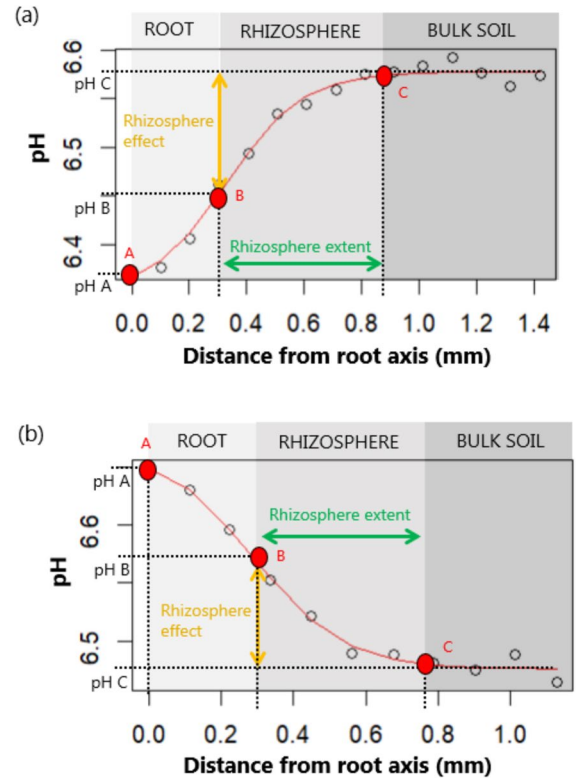


Fig. 2 Example of the measured variables (rhizosphere effect and extent) on the sigmoid fit (red line) obtained from the lines drawn orthogonally to the roots. The middle of the root is set at the distance 0 (point A). The interface between the root and the soil is determined based on the root diameter (point B). The interface between the rhizosphere and the bulk soil is defined where pH or phosphatase activity was at 1% different from the asymptote (point C). **a** is an example of acidifying barley root (pHC > pHA) and **b** is an example of alkalizing barley root (pHC < pHA). The same was done for phosphatase activity and P concentration

Aboveground biomass

Aboveground biomass was sampled 57 days after seeding, corresponding to the end of the third imaging campaign. Barley and faba bean plants were sampled separately from a surface area of 40 × 40 cm in each plot. All biomass samples were dried at 70 °C

for 72 h after which their dry weight was determined. The aboveground biomass is reported on a per plant basis, taking into account the plant density. Plant nutrient concentrations were analysed by YARA Analytical Services (UK) using Dumas combustion (total N) and microwave accelerated digestion in nitric acid, followed by determination with ICP-OES (all other nutrients). Root C concentration was determined with an organic elemental analyser (Flash, 2000, ThermoFisher Scientific).

Statistics

All statistical analyses were performed with R software (version 4.3.1) (R Core Team 2020). P-values below the threshold of 0.05 were considered significant. Linear mixed models were fitted with the three replicated blocks as random factors. The effects of the species (barley and faba bean), the cropping system (sole crop and intercropping) and their interaction (fixed effects) were tested on the aboveground biomass per plant and nutrient concentrations. The effects of the cropping system (sole barley, sole faba bean and intercropping), the species, the sampling date and their interaction (fixed effects) were tested on the root length density. The effects of the root type (barley alkalizing, barley acidifying and faba bean roots), the cropping system (sole crop and intercropping) and their interaction (fixed effects) were tested on the pH and phosphatase activity (i) in the middle of the root, (ii) in the surrounding bulk soil, (iii) rhizosphere effect and (iv) rhizosphere extent. For all the linear mixed models and analyses of variance, the *lme4* and *car* packages were used. The normality of the residuals and the homogeneity of the variances were verified graphically. When necessary, logarithmic or square root transformations were applied. Post hoc Tukey tests were used to establish the significance of the differences between each category. A simple ordination of variables related to pH and phosphatase activity changes was conducted using principal component analysis (PCA) with the *vegan* and *factoextra* packages.

Results

Plant aboveground biomass and nutrient concentrations

The faba bean aboveground biomass and N concentrations were significantly greater than those of barley ($P < 0.001$; Table 2). Intercropped plants tended to have greater biomass and N concentrations than plants growing as a sole crop, but this effect of cropping system was not significant ($P = 0.4$, Supplementary Table 1). In contrast, the cropping system significantly impacted plant P concentrations depending on the species ($P < 0.05$). Intercropped faba bean had smaller P concentrations than faba bean in sole crop, whereas P concentrations were similar for sole and intercropped barley (below 3.9 g P kg^{-1}).

Table 2 Aboveground biomass and nutrient concentrations in aboveground plant parts of faba bean and barley in two cropping systems (intercropping and sole crops), 57 days after sowing. Data are mean ± standard deviation (n = 3). Lowercase letters indicate significant differences between species and cropping systems when significant

Cropping system	Species	Aboveground biomass (g $plant^{-1}$)	Carbon (g kg^{-1})	Nitrogen (g kg^{-1})	Phosphorus (g kg^{-1})	Potassium (g kg^{-1})	Magnesium (g kg^{-1})	Calcium (g kg^{-1})
Sole crop	Barley	0.76 ± 0.18 b	408.1 ± 7.9	31.3 ± 3.9 c	3.5 ± 0.2 b	23.5 ± 1.1 b	1.5 ± 0.2 b	8.5 ± 0.9 b
Sole crop	Faba bean	2.51 ± 0.39 a	414.8 ± 16.3	50.2 ± 2.5 a	5.1 ± 0.6 a	29.3 ± 0.3 a	2.7 ± 0.3 a	17.1 ± 1.4 a
Intercropping	Barley	0.80 ± 0.49 b	407.0 ± 2.3	33.7 ± 4.3 c	3.9 ± 0.7 b	24.6 ± 2.2 b	1.6 ± 0.1 b	10.1 ± 0.9 b
Intercropping	Faba bean	2.92 ± 0.72 a	408.2 ± 3.3	44.3 ± 1.0 b	4.0 ± 0.6 b	25.5 ± 4.1 a	2.7 ± 0.2 a	16.7 ± 3.4 a

Cropping system	Species	Sulfur (g kg^{-1})	Zinc (mg kg^{-1})	Iron (mg kg^{-1})	Molybdenum (mg kg^{-1})	Copper (mg kg^{-1})	Boron (mg kg^{-1})	Manganese (mg kg^{-1})
Sole crop	Barley	3.8 ± 0.6 a	66.9 ± 9.4	571.0 ± 253.8	1.5 ± 0.8	11.5 ± 1.2 b	7.7 ± 0.7	68.7 ± 11.0 b
Sole crop	Faba bean	3.0 ± 0.5 b	78.7 ± 13.7	541.7 ± 37.4	0.7 ± 0.3	22.2 ± 3.6 a	38.6 ± 3.4	102.6 ± 26.7 a
Intercropping	Barley	4.7 ± 0.2 a	78.0 ± 9.6	464.3 ± 50.4	1.0 ± 0.3	12.7 ± 2.4 b	7.5 ± 0,1	71.8 ± 7.5 b
Intercropping	Faba bean	3.3 ± 0.6 b	80.0 ± 5.8	509.3 ± 115.3	1.0 ± 0.5	19.8 ± 6.0 a	34.8 ± 3.7	112.5 ± 31.9 a

Root growth and distribution

The *planar* root length density increased with time as roots grew between mid-May until mid-June. In general, root length density was significantly greater for barley than for faba bean, both when comparing the crops growing in sole crops ($P < 0.01$, Fig. 3a) and when comparing the crops growing in the intercropping system ($P < 0.001$, Fig. 3b). The rate of increase in visible *planar* root length density slowed in intercropped faba bean from 39 to 57 days after sowing, in contrast to the intercropped barley as well as the sole cropped barley and faba bean.

The distribution pattern of roots along the profile depth was similar between systems and species, with the greatest density observed for the 10–20 cm depth soil layer, from 33 days after sowing and onward. At 57 days after sowing, barley started to show more roots in the deepest layer (30–40 cm) compared with faba bean, both in sole crop and within intercropping. However, this difference between barley and faba bean was not significant.

pH changes

The planar optodes revealed both acidification and alkalization in the rhizosphere of barley roots from the same plant (Fig. 1j; 4a and b). The pH in the middle of the barley roots was around 6.4 and 6.6 for acidifying and alkalizing roots (Fig. 4a, b and d). The pH of the bulk soil around barley acidifying roots (around 6.6) was slightly, but significantly, greater than the bulk soil around alkalizing roots (<6.5, Fig. 4e). Furthermore, the alkalizing effect was

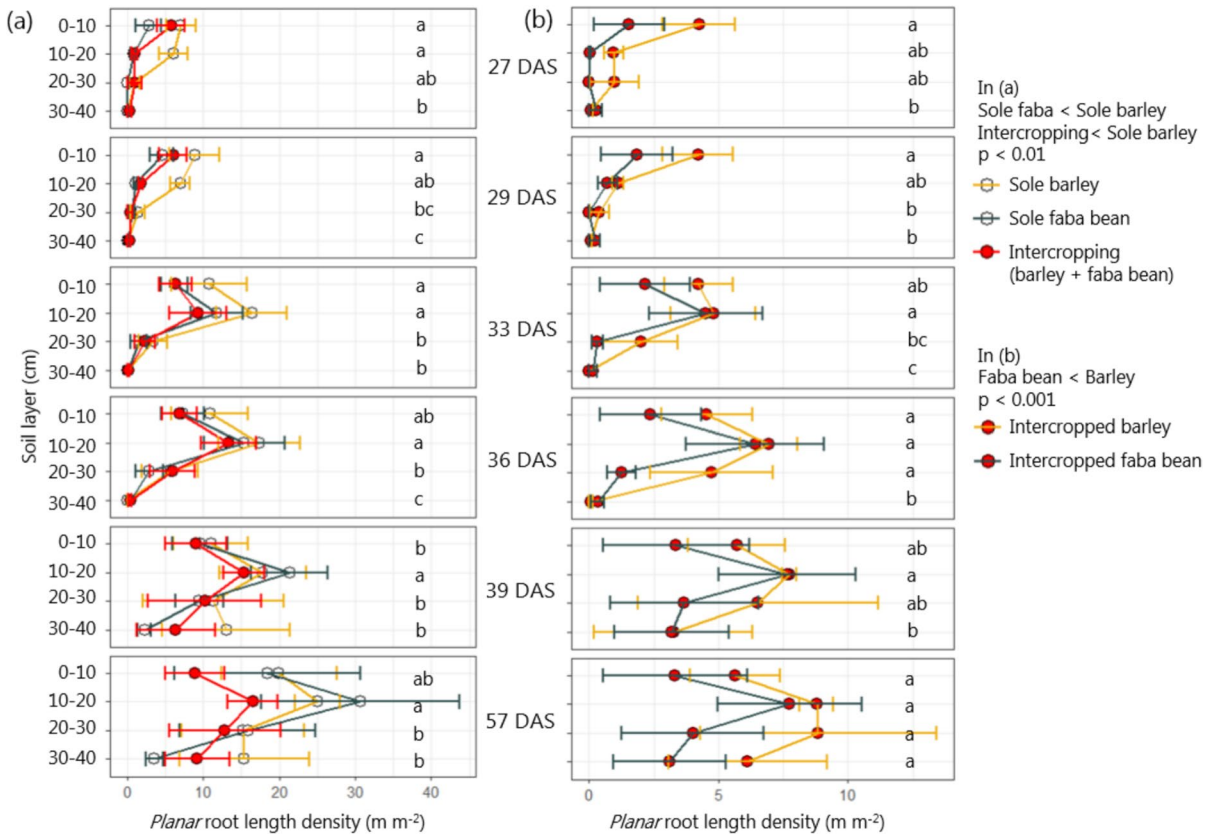


Fig. 3 *Planar* root length density of faba bean and barley from 27 to 57 days after sowing (DAS), in three cropping systems (sole barley, sole faba bean and barley/faba bean intercropping, down to 40 cm soil depth. **a**) Root length distribution for the three cropping systems. **b**) Root length distribution of barley and faba bean grown in the intercropping system. Data are mean ± standard deviation (n = 3). Lowercase letters indicate significant differences between soil layers ($P < 0.001$). Significant differences between cropping systems **a** and species **b** are written on the right side of the figure

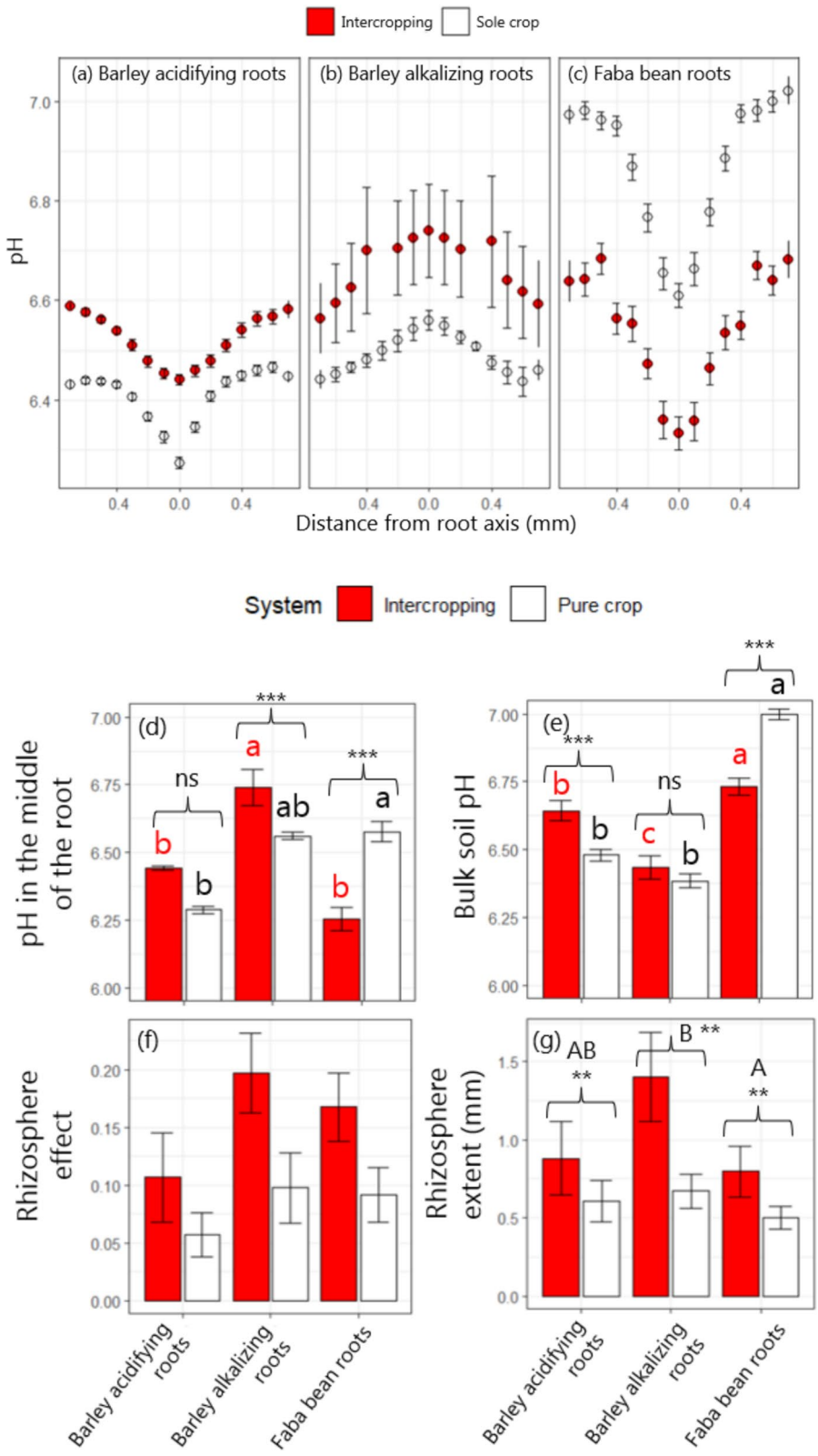


Fig. 4 pH according to the distance to the root axis of barley acidifying roots **a**, barley alkalizing roots **b** and faba bean roots **c**, pH in the middle of the root **d** and in the bulk soil surrounding the root **e**, rhizosphere effect **f** and rhizosphere extent **g** related to pH in two cropping systems (faba bean/barley intercropping and sole crops). Data are mean values and error bars represent standard deviation. *** and ** represent significant differences between cropping system ($P < 0.001$ and $P < 0.01$). Uppercase letters represent significant differences between root types ($P < 0.05$). Lowercase letters represent significant differences between root types within cropping system ($P < 0.001$)

similar in relative effect (Fig. 4f) and extent (Fig. 4g) as the acidifying effect.

Faba bean roots acidified the rhizosphere (Fig. 4c) and had a pH in the middle of the root of > 6.5 in sole crop (Fig. 4d). The bulk soil around faba bean roots had a higher pH (up to 7.0) than those observed for the barley roots ($P < 0.001$, Fig. 4e). The rhizosphere effect and extent (0.5 mm) were in the same range as barley roots (Fig. 4f and g).

Intercropping consistently increased the rhizosphere effect (Fig. 4f) and extent (Fig. 4g) of pH changes, for all types of roots (i.e. barley acidifying, barley alkalizing and faba bean roots) compared with sole crops ($P < 0.01$, Supplementary Table 4). Furthermore, for the intercropped barley, the effect of alkalization was relatively greater than the effect of acidification, being two- fold greater in effect and 1.5-fold in extent.

The bulk soil pH measured with planar optodes ranged between 6.3–6.7 (Fig. 4b). This value was more than 1 unit higher than the measurement with an electrode on water saturated soil 5.3 ± 0.0 (Table 1).

Phosphatase activity changes

The phosphatase activity in the middle of the root of sole barley (6.9 fKat mm^{-2}) was greater than that of sole faba bean (< 3 fKat mm^{-2}, Fig. 5a-e). The variations in phosphatase activity in the middle of the roots were similar to those in the bulk soil surrounding roots (Fig. 5f). The rhizosphere extent was heterogeneous between species (Fig. 5h), reaching as far as 24 mm for faba bean roots in sole crop and 2–5 mm for barley roots (Fig. 5f).

Cropping system and crop species had an interactive effect on the rhizosphere effect (Fig. 5g) and extent (Fig. 5h) of phosphatase activity ($P < 0.01$, Supplementary Table 4). Intercropped barley roots had a lower rhizosphere effect than barley roots in sole crop, whereas it was the opposite for faba bean. The rhizosphere extent of sole faba bean was greater (24) than that of intercropped faba bean (15 mm), whereas the rhizosphere extent of barley roots was similar between the two cropping systems.

Phosphorus availability around roots

The DGT method was successful for a limited number of samples. However, it showed qualitatively greater P concentrations around faba bean roots than barley roots (Fig. 6). For illustration purposes, the P concentration in the middle of the root segments in Fig. 6 was as much as 1000 ng cm^{-2} for the faba bean and up to 600 ng cm^{-2} for the barley root. In addition, the DGT images highlighted the patchiness of P availability in the soil.

Relationships between variables related to pH and phosphatase activity changes

The first axis of the PCA captured 38.4% of the variation related to pH and phosphatase activity among root types and cropping systems (Supplementary Fig. 4). The rhizosphere effect of phosphatase activity was associated with an increase in the phosphatase activity in the middle of the root and in the bulk soil along this axis. The relationship between phosphatase activity in the rhizosphere and in the surrounding bulk soil was confirmed with linear regressions for all root types and cropping systems (Fig. 7) and was mainly represented by the intercropped faba bean roots. The second axis of the PCA (20.4% of the total variation) grouped the roots according to the rhizosphere extent of phosphatase activity.

Discussion

This study explored belowground interactions in legume-cereal intercropping as an opportunity to optimize the use of scarce soil resources. Previous studies have mostly focused on aboveground performance, destructive soil sampling, or controlled conditions. In contrast, we integrated customized root windows in the field, similar to (Vetterlein et al. 2021; Bilyera et al. 2022). We deployed this setup in a faba bean—barley intercropping system, allowing root growth and development to be monitored over time alongside deployment of in-situ biochemical imaging techniques that have commonly been used in controlled settings (e.g. rhizoboxes). Under marginal field conditions, these methods revealed laterally resolved changes in rhizosphere chemistry. The approach offers a starting point for future field-based investigations of root–soil and root-root interactions in intercropping systems. In addition, our study used a Scottish barley landrace (Bere) with known adaptation to

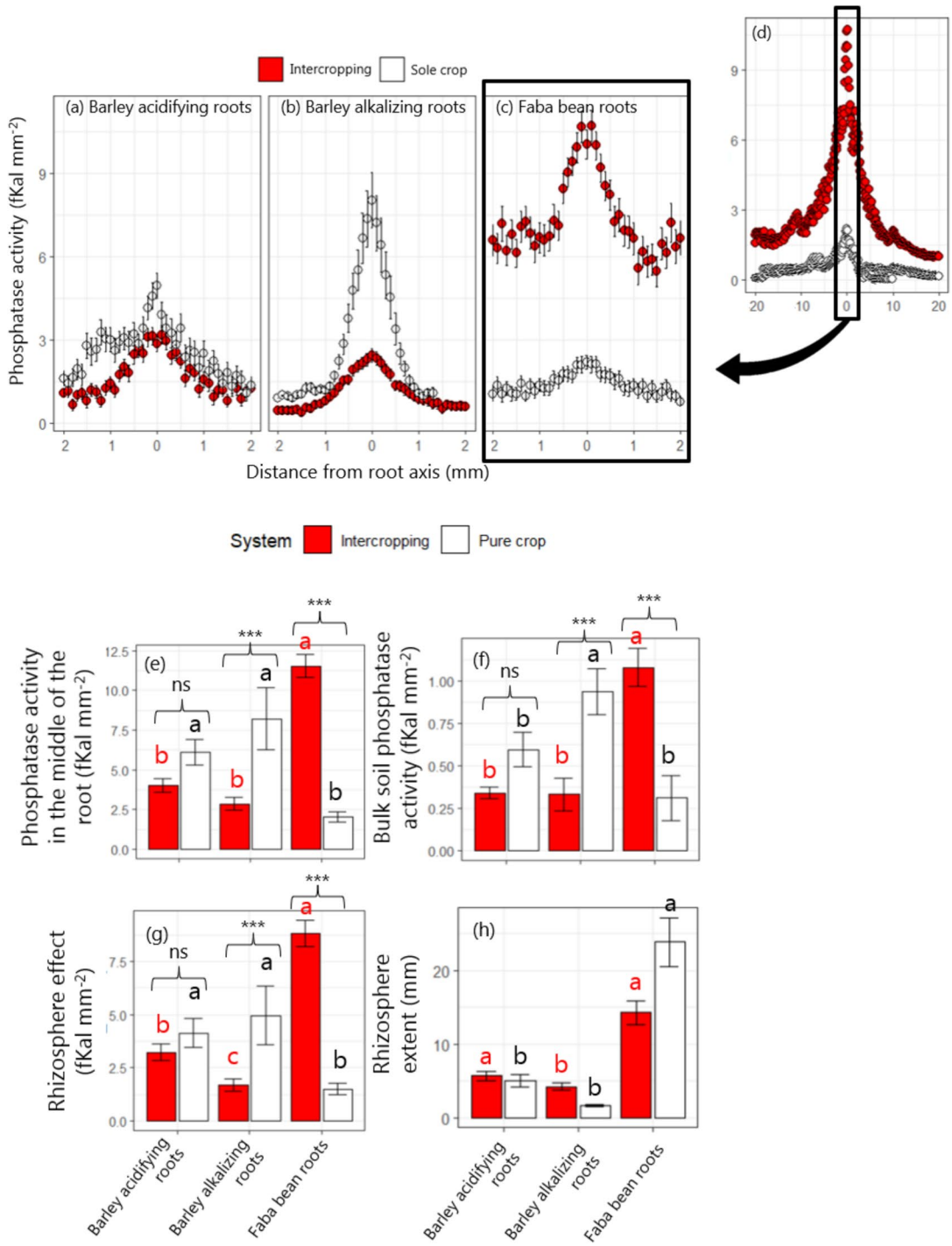
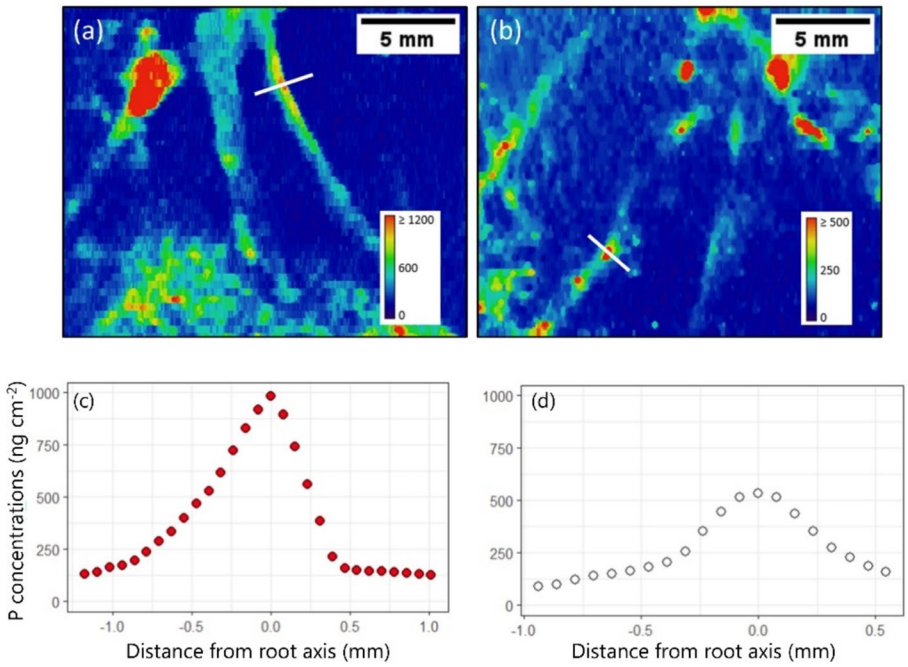


Fig. 5 Phosphatase activity according to the distance to the root axis of barley acidifying roots **a**, barley alkalizing roots **b** and faba bean roots **c**, in the middle of the root **e** and in the bulk soil surrounding the root **f**, rhizosphere effect **g** and rhizosphere extent **h** related to phosphatase activity in two cropping systems (faba bean/barley intercropping and sole crops). For visualization simplification, Fig. 5a-c show only the central ±2 mm around roots, whereas rhizosphere extent values in Fig. 5 h were calculated from the full fitted profiles (example shown in Fig. 5 d). Data are mean values and error bars represent standard deviation. *** Represent significant differences between cropping system ($P < 0.001$). Lowercase letters represent significant differences between root types within cropping system ($P < 0.001$)



Bulk soil P concentration (ng cm^{-2})	217.7	126.4
Rhizosphere effect (ng cm^{-2})	850.3	409.1
Rhizosphere extent (mm)	1.63	0.98

Fig. 6 P concentrations (ng cm^{-2}) around intercropped faba bean (**a** and **c**) and sole barley (**b** and **d**) roots. Fig **c** and **d** correspond to the extracted data from the white line shown on a and **b**

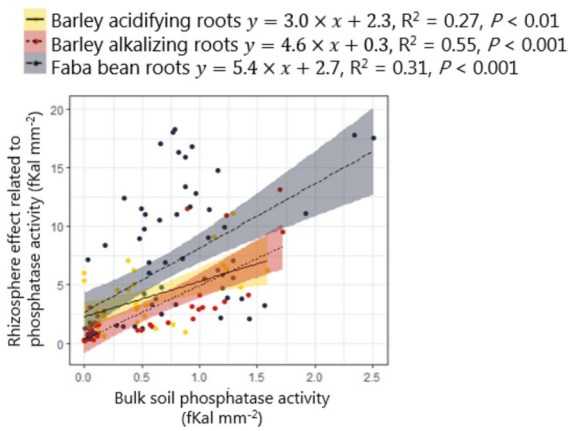


Fig. 7 Rhizosphere effect (phosphatase activity) in the bulk soil surrounding the roots. The colours refer to the type of roots (Bere barley alkalizing roots, Bere barley acidifying roots and faba bean roots)

marginal soils, which may exhibit distinct root distribution and/or rhizosphere dynamics.

Root distributions

In the intercropping system, the visible *planar* root length steadily increased over time until 39 days after sowing, after which it started to level off. This levelling off was primarily driven by the slowed increase of *planar* root length of faba bean growth (Fig. 3b), whereas *planar* root length of intercropped barley roots and sole faba bean roots continued increasing. These different root growth dynamics ultimately resulted in similar overall root lengths among all treatments, hence, differences in root distribution patterns were not significant, as opposed to our hypotheses. Contrastingly, previous observations down to 60 cm demonstrated that

intercropped faba bean and wheat roots reached deeper soil layers than their sole crop stands (Streit et al. 2019). In our study, root measurements were first limited to the field of view of the root windows (40 cm depth), therefore it is possible that roots of intercropped faba bean continued growth in deeper layers. Second, the root distribution was assessed as *planar* root length, a 2D method that limits the visualization to the interface with the soil profile (Wacker et al. 2024). Third, our experiment occurred at early growth stages, which may be too early to see root architectural plasticity in response to intercropping. In part, root growth and distributions are driven by competitive dynamics with neighbouring plants (Homulle et al. 2022), in which root-root interactions may trigger relative (plastic) changes (Hodge 2009). In our experiment, competition from the barley crop may have induced the intercropped faba bean to preferentially grow roots in deeper layers. Here, species interactions will initially have been small during early growth, but then gradually increased in intensity as root systems became larger, leading to the observed levelling off of faba bean growth in the top 40 cm.

Soil heterogeneity as a driver of pH and phosphatase activity in the rhizosphere

On average, the soil pH was one unit higher when measured with optodes compared with the standard bulk soil method (a 1:5 $CaCl_2$ solution) with an electrode. It is often observed that optode measurements do not correspond to the standard method with $CaCl_2$ solution (Merl et al. 2022, 2023), as the underlying mechanisms differ between each method (optical sensor, electrode in water solution and electrode in $CaCl_2$ solution). Firstly, the $CaCl_2$ solution of the bulk soil measurement will result in a greater electrolyte concentration compared with measuring soil pH in a water solution, commonly resulting in a lower pH value of 0.5 pH units or more (Thomas 1996; Kissel et al. 2009). Secondly, optical sensors measure the concentration of a pH dependent indicator, while the electrodes measure a proton activity. Thirdly, electrodes measure soil pH on a bulk sample of saturated soil, under constant stirring, resulting in a homogenized system that is far removed from local, heterogeneous soil conditions. Lastly, the optode method reads the pH off the sensor foil a few mm away from the soil contact surface. Specific assumptions and limitations of each method have further been discussed by Janata (1987) and Merl et al. (2022). While neither optical nor electrochemical sensors can measure pH precisely (Janata 1987), in the current study, the application of pH optodes allowed identification of pH heterogeneity at a relatively fine lateral scale (the optode measured bulk soil pH ranged between 6.4 and 7.0), and identification of changes around roots relative to the background pH.

For barley, the rhizosphere effect of pH changes was relatively small. This small change was consistent with previous observations (Ye et al. 2018), and this was expected in this soil with near-neutral pH as measured by the planar optodes (Barrow et al. 2024). Barley roots induced both acidification and alkalization in their rhizosphere, with these effects being observed for roots from the same root system, and even along the axis of the same root. Rhizosphere pH changes can be explained by several factors, including the cation–anion exchange balance between root and soil, the production of CO_2 through root respiration, the release of low molecular weight organic anions by plants and microorganisms, and the induction of redox processes by root activity (Hinsinger et al. 2003; Custos et al. 2020). The cation–anion balance in particular has a strong relationship with rhizosphere pH, because roots compensate for an unbalanced charge in the cell through the release (or absorption) of H^+ or OH^- (Hinsinger et al. 2003; Neumann and Römheld 2012). For example, excess nitrate (NO_3^-) uptake tends to result in an increase of the rhizosphere pH through root absorption of H^+ (or excretion of OH^-), whereas excess ammonium ($NH4^+$) uptake tends to do the opposite (Nye 1981). Therefore, the observation of both acidification and alkalization may be explained by an uneven uptake of cations and anions along the roots. Presumably, this would have been partially driven by a heterogeneous distribution of N, considering the amounts of N taken up by the plant are relatively large compared with those of other nutrients. Therefore, visualizing local N forms, e.g. with novel planar optodes (Merl et al. 2024; Kalinichev and Koren 2024) may provide further understanding.

P availability was patchy and heterogeneous, and as expected P availability was generally low. Indeed, barley was P deficient; as a reference, a P

concentration of 4 mg P kg^{-1} or lower is considered to indicate critical P deficiency for barley at this vegetative stage of growth (Reuter and Robinson 1997). Plant P deficiency has often been linked to increased root release of low molecular weight organic anions together with protons (H^+), which can induce local regions of acidification and may increase plant P availability by desorbing inorganic P from soil minerals, complexing metal cations, or dissolving mineral and precipitated forms of P (Neumann and Römheld 2012; Barrow 2017). However, in the current study, the effects on rhizosphere pH seem to be primarily dependent on species (Ye et al. 2018; Ma et al. 2021) and the local pH of the bulk soil (Barrow et al. 2024) rather than on P availability. In our study, the relatively small effect of rhizosphere pH changes was consistent with previous results on barley (Ye et al. 2018), and expected in this soil with near-neutral pH (Barrow et al. 2024).

The acid phosphatase activity depended on the background levels in the surrounding bulk soil. Specifically, the rhizosphere effect of acid phosphatase activity was larger when the phosphatase activity in the bulk soil was greater. Among the several factors impacting phosphatase activity, organic P and total N are major factors explaining variation in phosphatase activity (Margalef et al. 2017). In addition, soil phosphatase activity is tightly linked to microbial activity through different mechanisms (Richardson and Simpson 2011). For example, under sparsely available P conditions, competition between plants and soil microorganisms for P may trigger roots to release more phosphatase (Marschner et al. 2011). Therefore, local soil heterogeneity in our soil (patchiness of nutrient availability and microbial communities) could be responsible for identical variations in acid phosphatase activity in the surrounding bulk soil and in the rhizosphere.

Changes in phosphatase activity in barley rhizosphere were similar for acidifying and alkalizing roots. Thus, pH changes did not significantly modify the activity of phosphatase already present in the soil, as shown in other setups (George et al. 2007). Despite the low pH changes observed in our experiment, we deduce that changes in phosphatase activity measured in the rhizospheres were primarily driven by phosphatase release, whether by root exudation or by root associated microbes in the rhizosphere (Hinsinger et al. 2015).

The rhizosphere extents of phosphatase activity were exceptionally large for faba bean (up to 24 mm) and barley (2—5 mm) compared with the rhizosphere extents of pH changes (<2 mm) and compared with commonly reported ranges (1—4 mm) (Nuruzzaman et al. 2006; Kuzyakov and Razavi 2019). One explanation for the difference between faba bean and barley roots may a strong exudation of C and N into soil by faba bean roots. Faba bean is known for substantial exudation rates (Hinsinger et al. 2003; Nuruzzaman et al. 2006), which would have then stimulated the microorganisms to produce phosphatase (Spohn et al. 2013b; Na et al. 2022). A more speculative explanation could be the effect of the hyphosphere on organic P mineralization. For instance, arbuscular mycorrhizal fungi have shown a greater role than root hairs of maize for priming the rhizosphere microbial community and enhancing rhizosphere organic P mineralization (Zhou et al. 2022). In our experiment, important mycorrhization of faba bean roots could have enforced the rhizosphere microbial recruitment and thus the mineralization of organic P. A last possibility is that the large rhizosphere extents were an artifact of the zymography method, in which diffusion of substrates and fluorescent reaction products with the membrane-soil system may have broadened the apparent activity (Guber et al. 2018). However, these diffusions should affect all samples similarly and thus cannot explain the contrast between faba bean and barley roots. Further, the larger rhizosphere extents of phosphatase activity around faba bean roots was also consistent with the large rhizosphere extent of P availability, which was twice as large around faba bean roots as that around barley roots (Fig. 6). Altogether, the difference of rhizosphere extents between pH and phosphatase activity changes highlights the need of defining rhizosphere soil depending on the specific conditions and processes of interest.

Differences in rhizosphere changes between two species

The P concentrations and phosphatase activity were twofold greater in the faba bean rhizosphere than in the barley rhizosphere. Our results were in agreement with other intercropping studies that reported greater P availability in the rhizosphere of legumes than cereals (Betencourt et al. 2012; Zhu et al. 2022b). The release of phosphatase activity is an adaptive mechanism in response to low P conditions (Tadano and

Sakai 1991; Nuruzzaman et al. 2006), enabling plants to increase the availability of organic P through mineralization. Accordingly, greater phosphatase release has previously been observed to coincide with greater P availability in legume rhizospheres (Nuruzzaman et al. 2006; Hummel et al. 2021). The difference of P availability in the rhizosphere between the two species could thus be due to the difference in phosphatase release between faba bean and barley rhizospheres.

Barley acidifying roots and faba bean roots showed a similar rhizosphere effect and extent with regard to pH changes. Both species were expected to release organic acids (Gahoonia et al. 2000; Jing-xiu et al. 2014; Bolan et al. 2025). However, a greater acidification was expected in the rhizosphere of faba bean, given exudation rates tend to be greater for legumes than cereals (Nuruzzaman et al. 2006; Li et al. 2008; Bei et al. 2021) and owing to the legumes ability to fix atmospheric N (Bolan et al. 1991; Hinsinger et al. 2003). Reliance on N fixation changes the cation–anion balance because reduced uptake of NO_3^- increases the excess charge in the cell. This is compensated by excretion of H^+ (Haynes 1990), resulting in acidification of the legume rhizosphere (Bolan et al. 1991). The relatively low acidification in the rhizosphere of faba bean may be explained by the low P status of the soil, considering N fixation is a P demanding process (Peoples et al. 2009). However, the plant P concentrations suggest that faba bean was not P deficient (Reuter and Robinson 1997). An alternative explanation would be that in the current system, N fixation by the legume may have been reduced due to the availability of soil mineral N. The presence of mineral N in the soil tends to inhibit both nodule formation and nitrogenase activity (Sprent et al. 1988). This is also supported by the absence of visible nodules through the root windows during field work, even in the sole faba bean plot where no N fertilization was applied. However, our measurements occurred at early development stages, therefore nodulation and N fixation by faba bean may still increase in later stages, as both faba bean and barley roots scavenge for soil mineral N.

Impact of intercropping on pH and phosphatase activity changes in the rhizosphere

For faba bean, the intensity of phosphatase release was significantly greater in intercropping than in sole crops, whereas the opposite was true for barley. This was in agreement with previous studies that showed the rhizosphere extent and effect can shift depending on crop management (Jia et al. 2024). In pot experiments, phosphatase activity of maize and grass pea has been observed to increase when intercropped compared with sole crop (Zhu et al. 2022a, 2022b). The increased phosphatase release in intercropping could be due to changed competition by the presence of a different neighbour (Hodge 2009; Cahill et al. 2010). Specifically, changed competition for P or other factors (e.g. light, water.) may have induced an increase or decrease of root activities that affect P availability and acquisition (Li et al. 2021). An alternative explanation could be that contrasting biomass production between the two cropping systems altered the allocation of C to the roots and ultimately increased root exudation (Wen et al. 2022). However, this is unlikely considering biomass between intercropped and sole plants were similar.

Intercropping increased the rhizosphere effect and extent related to pH changes for all root types. This was in agreement with previous results showing increased atmospheric N fixation in intercropped legumes under low P conditions (Betencourt et al. 2012) and thus stronger rhizosphere acidification in intercropping than in sole crops. However, this change of pH around faba bean roots (1.5-fold) was much less than the change in phosphatase activity (six-fold). Increased phosphatase activity is thus likely to contribute mainly to P uptake. For barley, higher changes may help compensation for the decrease in phosphatase release in intercropping compared with sole crop.

Methodological considerations

The deployment of bio-imaging techniques in the field allowed the study of an agroecosystem under natural climatic conditions, with realistic soil volume available for plant root growth. However, working in field conditions also leads to methodological challenges compared with controlled conditions. For example, good contact between the probes and soil was difficult to obtain, but we managed by adding plastic layers between the acrylic glass and the different probes. In the field, light is difficult to control. The PVC boards were closed during

deployment of DGTs and zymography membranes, but had to be removed when imaging the pH optodes. Layering several blackout fabrics over the pits then ensured total dark conditions. Temperature has a strong control of soil acidity generation (e.g. after fertilization (Tao et al. 2025)). Using calibrations with different temperatures in the laboratory allowed to adjust for temperature variations in the field.

The sequential deployment of optodes, zymography and DGT was considered optimal, considering that the optode foils should not chemically interact with soil P, whereas the zymography membranes soaked in acid buffer may modify the soil pH, and the DGT gels are more likely to damage soil integrity in and around the region of interest when removed. The deployed sequence follows the previous approach by Bilyera et al. (2022) who ruled out significant influence, though we acknowledge that sequential application of methods on the same location may potentially lead to biases if one method influences the results of the subsequent one(s). Extra care was therefore taken to minimize any unknown effects (e.g. by rinsing of the optodes, and by ensuring good contact with soil and constant soil moisture content). Nonetheless, maintaining physical integrity of the soil surface, and completing a successful sequential deployment, remained challenging. Within this, time-requirement (labour) for in-field deployment places an upper bound on the number of samples that can be deployed at any given time, and time-lag between deployment and analyses (visualization) precludes the flexibility to rapidly obtain additional samples 'on demand' if needed. The latter is particularly challenging for the DGT method which requires a time-consuming LA-ICP-MS analysis. In the current study, this was partially countered by an experimental design that deployed a large number of (pseudo-)replicates, in an effort to obtain sufficient successful data, yet ultimately resulted in insufficient replication and thus only qualitative data for rhizosphere P availability. This should be taken into account when designing similar experimental set-ups.

In intercropping, the presence of roots from both species on the root window was visually verified, and their proximity to neighboring roots appeared uniform in the region of interest. However, it was not possible to predict if other roots were hiding behind the visible soil surface. This limited the rigorous selection of region of interest and of roots, and it may be anticipated that the risk of 'invisible roots' becomes larger when deploying these methods in high root density areas and/or later crop growth stages, potentially limiting the opportunity to study bio-chemical gradients as a function of distance from the roots. Despite the methodological challenges, our work provides a demonstration of compelling approaches for advancing in situ biochemical imaging in complex systems, such as intercropping. Visualizing bio-chemical changes in shared rhizosphere between two species over the crop growth could provide novel insights on the interspecific root-root interactions.

Conclusion

This study highlighted biochemical changes in Bere barley and faba bean rhizospheres under field conditions. Occurrence of both alkalization and acidification in the rhizosphere of barley roots demonstrates the ability of roots to influence their rhizosphere pH as a response to local differences in soil conditions (e.g. nutrient forms and availability) at a fine spatial scale. Our study further illustrates that intercropping can shift belowground interactions between plant and soil, demonstrated by the increased phosphatase release by faba bean and pH changes around both barley and faba bean roots. The shift in biochemical changes in intercropping compared with sole crops was not accompanied by a change in *planar* root length density and distribution in the soil profile. However, as this study focused on early-stage development under field conditions, further research is needed to investigate longer-term dynamics, species-specific interactions, and the scalability of these effects across diverse agroecosystems. Combining *in-situ* imaging techniques with measurements of nutrient dynamics and gene expression in the rhizosphere microbiome at different crop development stages may offer deeper insight into the temporal dynamics and functional roles of belowground interactions in intercropping systems.

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Author contributions LS: Conceptualization, Methodology, Investigation, Formal analysis, Visualization, Writing – original draft, Writing – review and editing; MB: Investigation, Formal analysis, Writing – review and editing; TM: Methodology, Investigation, Writing – review and editing; CM: Methodology, Formal analysis, Visualization, Writing – review and editing; CZ: Investigation, Writing – review and editing; TG: Conceptualization, Writing – review and editing; KK: Methodology, Writing – review and editing; DBD: Conceptualization, Writing – review and editing; FVDB: Conceptualization, Methodology, Writing – review and editing.

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Data availability All data analysed during this study are included in this published article and its Supplementary Information. Raw datasets generated during the study are available from the corresponding authors on reasonable request.

Declarations

Competing interests The authors have no relevant financial or non-financial interests to disclose.

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