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METHODS PAPER

Early belowground interactions in lupin–wheat intercropping assessed by a simple root phenotyping approach

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Abstract

Background and aims Legume-cereal intercropping is a common strategy to enhance agricultural sustainability. Many plant-plant interactions occur in the underground but remain poorly understood due to methodological challenges. In the current study, we developed an indoor rhizobox-based simple and rapid root phenotyping approach to study early interactions between roots of white lupin seedlings cultivated as a sole crop or in intercropping with bread wheat.

Methods A rhizobox-based root phenotyping approach assessed the belowground structure of white lupin in the two conditions during early growth stages. The method focused on spatial root metrics and a rapid graphic indicator of root overlap between neighbour plants, termed Root Merge (RM) index. Root traits that could promote facilitative processes were also recorded.

Results Lupin modified its root architecture spatially in response to intercropping rather than simply altering total root production. The RM index clearly separated the two lupin cropping systems. Lupin in pure stand showed an avoidance behaviour between neighbours (RM = 0.17) indicating spatial compartmentalization, whereas in intercropping large lupin–wheat root intermingling was evident (RM = 0.69), suggesting mechanisms for nutrient-availability facilitation. Lupin aboveground and belowground growth was not inhibited by the great spatial soil exploration of wheat in intercropping. Lupin nodulation significantly increased in intercropping compared to pure stand, highlighting facilitative interaction through enhanced biological nitrogen fixation.

Conclusions The root visual parameters proved to be useful indicators of phenotypic plasticity in response to intercropping. The method could be easily extended to other legume-cereal combinations or to intercropping involving other plant types.

Keywords Root phenotyping · Belowground interactions · Intercropping · Rhizobox · Competition · Facilitation

Introduction

Modern agriculture predominantly relies on monoculture and the extensive use of fertilizers. While this approach undeniably increased yields and farmer profits, it has also had significantly negative impacts on the environment and biodiversity

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(Steffen et al. 2015; Bourke et al. 2021). Intercropping is an ancient farming system consisting in growing two or more crops on the same field still widely practiced by smallholders in Asia, Africa and South America (Yang et al. 2021).

In recent decades, intercropping has gained interest also in other regions as a possible way to improve the sustainability of crop production. Several studies and meta-analyses have shown greater yield and yield stability of intercropping compared to monocultures (Bedoussac et al. 2015; Raseduzzaman and Jensen 2017; Martin-Guay et al. 2018; Annicchiarico et al. 2019; Yang et al. 2021). An efficient use of resources among companion crops can reduce mineral fertilizer inputs and the subsequent environmental pollution caused by agriculture (Jensen et al. 2020). Intercropping is associated with a wide range of ecosystem services, such as improved soil biophysical quality and fertility, and better pest and weed control (Cong et al. 2015; Gu et al. 2021; Lopes et al. 2016; Lu et al. 2025).

Spatial and temporal variation of crop associations result in different types of intercropping, which are usually categorized as: (i) mixed intercropping, when the crop species are randomly mixed with no pre-ordinate pattern in the space; (ii) row intercropping, when two or more crops are grown in separate, alternate rows; (iii) strip cropping, when two or more crops are grown side by side in long and narrow multi-row strips; and (iv) relay intercropping, when the crops are not sown and harvested at the same time and their cycles overlap only partly (Homulle et al. 2022; Juventia et al. 2022).

Different mechanisms of interaction can occur between intercropped species, which were summarized by the '4C approach' in Justes et al. (2021), namely, *competition*, *complementarity*, *cooperation* and *compensation*. *Cooperation* was used in that study as a synonym for *facilitation* commonly used in ecology (Barry et al. 2019). Plant-plant interactions between species are more pronounced (in space and/or time) in mixed intercropping and row intercropping than in the other two types of intercropping. While interspecific interactions are widely studied on the plants' aerial part (e.g. Bedoussac and Justes 2011; Annicchiarico et al. 2021; Bybee-Finley et al. 2023), greater knowledge is necessary about belowground interactions between intercropped species, to shed light on the nature of root-root interactions and the contributing root traits (Homulle et al. 2022).

Root interactions in intercropping certainly involve at least three of the broad categories of interaction previously mentioned, namely, competition, occurring when one component of the mixture hinders the performance of the companion; complementarity, when there is a resource partitioning between components through niche differentiation (either in space, time or in chemical form) resulting in a more efficient resource use; and facilitation that occurs when a species benefits the companion by improving its growing condition (as it occurs in nutrient-release based abiotic facilitation) (Barry et al. 2019; Yang et al. 2021; Homulle et al. 2022). These three mechanisms are not mutually exclusive but instead they often overlap or can shift one into the other during crop growth also in response to input availability (Boudsocq et al. 2022). As outlined in Fig. 1, belowground niche differentiation is observed when intercropped species have contrasting root architectures such as deep and shallow roots (Brooker et al. 2015), whereas facilitation by nutrient enrichment implies root interaction to favour plant-to-plant exchanges, although nitrogen (N) uptake by the recipient species can also occur from decomposing plant material or through transfer by mycorrhizal network (Homulle et al. 2022). Thus, root interactions encompass physical, chemical and biological processes occurring simultaneously (Barry et al. 2019).

Plants possess mechanisms for self-/non-self-recognition (Chen et al. 2012; Falik et al. 2003) and adjust themselves to belowground competition through root plasticity. Root 'decisions' are therefore very important for maximizing the 'team benefits' (Hauggaard-Nielsen and Jensen 2005; Homulle et al. 2022). Root spatial segregation (i.e. avoidance behaviour between neighbouring roots) is generally associated with competition pressure (Schenk et al. 1999; Schmid et al. 2015) whereas extensive root intermingling between companion crops is in many cases associated to a nutrient-based facilitation (Li et al. 2006; Zhang et al. 2020, 2013; Zhu et al. 2023). Maize roots avoided those of intercropped wheat but grew toward the legume neighbour (Weidlich et al. 2018). Root spatial rearrangement in intercropping can be a relatively fast developing process based on kin recognition rather than on the effect of neighbour on resources availability (Garlick et al. 2021)

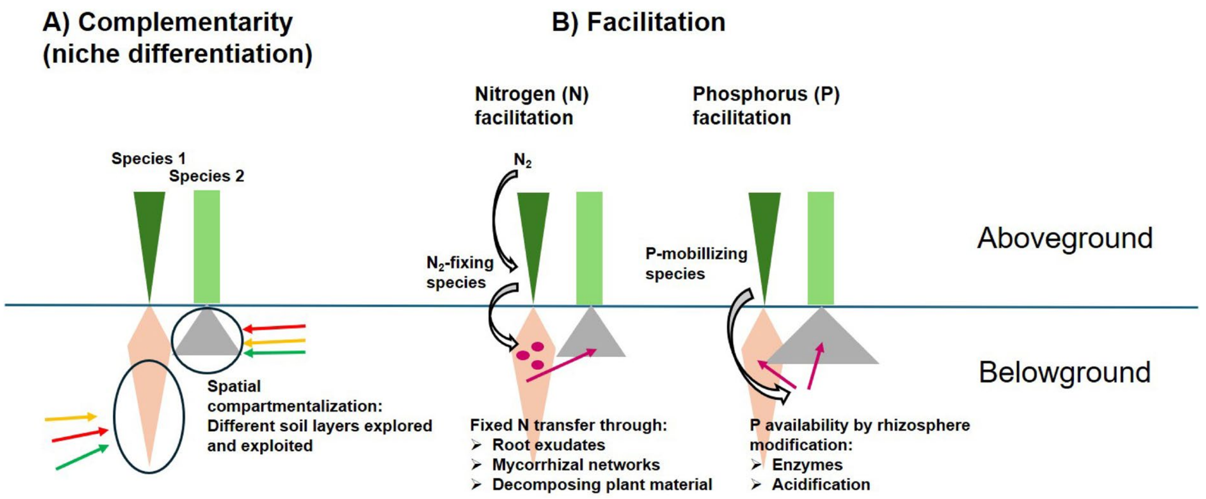


Fig. 1 Schematic representation of belowground interactions between intercropped species: **A** complementarity by spatial niche differentiation, where intercropped species can uptake and exploit resources from separate soil volumes; **B** direct facilitation. Nitrogen facilitation entails rhizobial fixation of atmospheric N_2 and transfer to the non-nitrogen-fixing species through different ways (see the Figure), not necessarily requiring close proximity between intercropped roots. Phosphorus facilitation involves secretion or exudation of compounds by the P-mobilising species in the rhizosphere to mobilise soil organic P and insoluble inorganic P, which become available to both species: close proximity between intercropped roots is required. Figure inspired by Fig. 2 in Yang et al. (2021) and Fig. 2 in Homulle et al. (2022)

and root intermingling seems rather a cause than a consequence of facilitation. Resource partitioning by niche differentiation can also involve changes in roots spatial arrangement. Faba bean intercropped with wheat grew a deeper root system and developed more nodules in deep soil compared to the pure stand (Bargaz et al. 2016). Barley intercropped with pea also developed a deeper root system compared to sole cropping and altered the pattern of lateral expansion (Ghaley et al. 2005). Zhang et al. (2014) showed that polyculture over-yielding was largely caused by spatial niche complementarity between neighbouring roots. Under low phosphorus (P) availability tomato roots overextended in the direction of soybean (Zhang et al. 2024). Teste et al. (2020) provided the first visual evidence that the presence of cluster roots of the tree species *Banksia attenuata* significantly primed companion species root proliferation and this physical interaction led to a P-acquisition benefit for roots growing close to cluster roots.

Because of the key role that the root spatial arrangement plays in belowground plant interactions, it could be worth devising a simple, visual method for assessing the components of root spatial structure (e.g. covered area, explored volume, shape of the root system, density), to be used as root metrics in intercropping root phenotyping. Among architectural traits, aggregate metrics describing the overall geometry of root systems, such as convex hull, aspect ratio, roundness, and solidity, offer valuable insights into root spatial organization (Rangarajan and Lynch 2021). The convex hull (CH) is defined as the smallest convex polygon enclosing the root system and represents the maximum extent of root exploration, thereby indicating the total soil area potentially influenced by roots. Variations in CH within intercropping systems can signal dominance or antagonistic suppression by companion crops. Solidity measures the root density within the CH. Low solidity indicates roots spreading widely and deeply but leaving significant gaps, whereas high solidity reflects densely packed roots that thoroughly explore available space (Topp et al. 2013). The aspect ratio, calculated as the ratio of the major axis to the minor axis of an ellipse fitted around the root system, reflects the directional bias of root growth. An increase of the aspect ratios may suggest a shift to elongated, vertical growth. Roundness, on the other hand, measures root system symmetry. Higher roundness values indicate isotropic growth patterns, often associated with evenly distributed resources, contrasting with root segregation strategies. Root system geometric measures underscore different rooting patterns in competitive environments, moving beyond conventional frameworks of gross size reduction to fine scale variation of root spatial distribution (Belter and Cahill 2015; Rangarajan and Lynch 2021).

Facilitative interactions among companion plants play a pivotal role in enhancing ecosystem functioning, and legume-cereal intercropping is very popular in this sense and paradigmatic of the possible advantages intercropping can bring compared to sole crops (Bedoussac et al. 2015). Improvement of nutrient availability favoured by legumes (and their symbiotic rhizobia) in intercropping include biological N fixation and transfer to non-legume companions, (P) release from organic compounds through extracellular enzymes, and dissolution of inorganic P fixed in soil by rhizosphere acidification (Hauggaard-Nielsen and Jensen 2005; Zhu et al. 2023). While enhanced inorganic P availability is the consequence of a direct facilitative interaction, the N cycle in intercropping involves in succession competitive (depletion of soil mineral N by the demanding and non-N-fixing cereal), complementary (self-production of fixed N for the legume *vs* use of soil N for the cereal) and facilitative (increase of available N derived from fixation and transfer to the cereal) interactions (Jensen et al. 2020). In legume intercropping rhizobial nodule density and spatial distribution is a trait than can be observed macroscopically and is indicative of underlying facilitation and resource partitioning (Bargaz et al. 2016; Zhao et al. 2020).

White lupin (*Lupinus albus* L.) is a legume species known to form specialized root assemblies termed as cluster roots (or proteoid roots), induced mainly by P deficiency, which release organic acids and protons and favour the solubilization of P (Neumann et al. 1999). In intercropping with lupin, companion species can thus benefit directly from nutrients solubilized by cluster root exudates.

With a raising demand for healthy and nutritious plant-based foods, white lupin has great potential interest because of its elevated and high-quality protein content, useful nutritional and functional characteristics, and suitability for sustainable production (Lucas et al. 2015). Unlike pea-cereal and faba bean-cereal intercropping that have been widely investigated, white lupin-cereal intercropping is a rather novel practice, and studies are still scant (Dourmap et al. 2025). Nonetheless, intercropping white lupin with cereals has a high potential of development to overcome lupin limits such as marked yield variability and low competitive ability against weeds (Carton et al. 2020).

Plant phenotyping is the activity of measuring and analysing observable plant characteristics (traits) and it is a basic process in crop improvement programs. A trait is a specific characteristic of an individual and its expression is determined by genes, environmental factors or by a combination of both. For what previously mentioned, phenotyping belowground traits and their plasticity in intercropping is important to reveal mechanisms underpinning intercrop performance and select the best match of species and/or cultivars to enhance ecosystem functions (Yu et al. 2021). It is also important to screen useful genetic resources for target breeding. Modern varieties selected for pure stands, are not necessarily the best suited for intercropping, and novel breeding programs are envisaged to address the specific challenges of crop mixtures (Annicchiarico et al. 2019; Bourke et al. 2021). While shoot traits can be easily observed and scored, addressing a root ideotype for intercropping is at its beginning (Yu et al. 2021). Phenotyping roots in intercropping adds the extra task of discerning roots belonging to different species and individuals to the methodological, economic and labour constraints typical of root research. Root separation methods either based on manual (Li et al. 2006) or on spectroscopic techniques (Hadir et al. 2024) are labour, financial and computational demanding. Non-destructive methods such as minirhizotron require a colour contrast between species (Andersen et al. 2014; Hassan et al. 2021). Isotope labelling tracers can be used to assess root distribution in intercropping (Han et al. 2022), but individual root phenes which are important for breeding cannot be retrieved. Phenotyping indoor methods that make use of clear pots known as rhizotrons or rhizoboxes allow the direct visualization of root dynamics and spatial structure with high temporal and spatial resolution and have been successfully used in intercrop root research (Bargaz et al. 2016; Schwerdtner and Spohn 2022). In the current study, we used indoor rhizoboxes to grow white lupin both as sole crop and intercropped with bread wheat (*Triticum aestivum* L.), evaluating the two cropping systems in separate boxes, with the overall aim of a better understanding of mechanisms occurring on roots of lupin seedlings in the two growing conditions.

Attention was given to traits that could be manageable proxies of belowground interactions, with a focus on competition, complementarity (niche partitioning) and facilitative interactions. The spatial distribution and architecture of roots, along with traits that could promote facilitative processes, were analysed and compared under the two conditions. In practice, this was implemented by recording simple and relatively high-throughput characters either retrieved during the plant growth, such as visible root length, or easily recorded upon completion of the experiment and opening of the rhizoboxes, such as the projected root area, some root shape parameters, a root merge index that quantifies root overlap between neighbouring plants, and counts of rhizobial nodules and cluster roots per plant.

Materials and methods

Rhizoboxes

For the experiment in-house assembled rizoboxes were used, with components easily available in craft shops. The box structure was made of rectangular hollow aluminium profiles and transparent PVC sheets held together with hexagonal screw bolts and nuts. The rhizobox dimensions were 25 cm width × 50 cm depth × 2 cm thickness.

Plant growth

The rhizoboxes were filled with a substrate made of mixture of field-soil previously air-dried and sieved at 2 mm, and siliceous river sand (60:40 w/w). The soil had the following physical–chemical properties: a clay-loam texture according to USDA (41% sand, 35% silt, and 24% clay), pH in water 7.3, 2.05% carbon (C), 0.21% N, 6.9 g kg^{-1} $CaCO_3$, 77 mg kg^{-1} extractable P, and 1755 mg kg^{-1} exchangeable K. The air-dried substrate was poured into the rhizoboxes from the top with the aid of a funnel to spread it uniformly and avoiding compaction. A bulk density of 1.325 ± 0.043 g cm^{-3} was obtained in all rhizoboxes, which were subsequently irrigated to 90% of field capacity of the substrate, by supplying water very slowly from the top to ensure uniform infiltration. A fertilizer (Dünger® 70, L. Gobbi s.r.l., Campo Ligure, GE, Italy) was supplied with the irrigation water at a rate of 75 kg P_2O_5 ha^{-1}. It was a powder ternary compound completely soluble in water with 10% N, 45% P_2O_5, and 10% K_2O plus microelements (B, Co, Cu, Fe, Mn, Mo, Zn). After irrigation, rhizoboxes were left to settle for 24 h allowing water to distribute through the profile. Two pre-germinated (with radicle length of approximately 5 mm) seeds were transplanted per rhizobox placing them close to the observation sheet (front face) at 1 cm depth and at equal distance of ~ 7.6 cm from each side and between themselves. Both faces of each rhizobox were shed from light with a black polyethylene foil and a sheet of polystyrene 0.5 cm thick. The top of the soil was covered with a thin layer of vermiculite to reduce evaporation. Two cropping systems were compared for white lupin, namely, white lupin in pure stand (LP) and white lupin-bread wheat intercropping (LIC), each with four replications (rhizoboxes) in a randomised complete block design. In each rhizobox, two plants of the white lupin cultivar Arsenio were grown and evaluated in LP, and one plant of Arsenio and one plant of the bread wheat cultivar Monviso in LIC. Prior to sowing, all white lupin seeds were inoculated with the rhizobium inoculant Vitalianz R Lupin (Cérience, Beaufort-en-Anjou, France). The rhizoboxes were maintained in a growth chamber under artificial lighting for 12 h light day^{-1} at the temperature of 22/23 °C (dark/light) using eight Combo lamps (C-LED, Bologna, Italy) of 300 W each, placed in two rows at 1.6 m from the ground. The rhizoboxes were placed inclined at 30° from the vertical on a wooden rack to encourage roots to grow against the observation face facing the ground, and irrigated twice per week replenishing water losses determined by weighting. Plants were grown for three weeks, when the primary root reached the base of the rhizobox. The position of the experimental blocks in the growth chamber, and that of individual rhizoboxes within blocks, were re-randomised twice a week in correspondence with irrigation, to limit any possible effects of the box position within the chamber on to the plant growth.

Visible root length tracing and shoot measurement

Twice a week, prior to irrigation of each rhizobox visible root length in each rhizobox was traced with a permanent marker on a transparent PVC sheet

(0.18 mm thickness) attached to the rhizobox at fixed marked position and marked with a permanent segment of 2 cm to be used as a scale for calibration. For each box individual root systems were traced on separate sheets and by different marker colour for each plant to facilitate root length analysis per plant. At each root tracing, sheets were scanned (600 dpi) for subsequent image analysis. In this way, a subsequent tracing was appended onto the previous one. Every time the roots were traced, the number of fully expanded leaves and shoot height per plant were recorded.

Rhizobox opening end recovery of the whole root system

At the end of the experiment, shoots were cut at the ground level and oven dried at 60 °C for four days for dry biomass determination. Before opening the rhizoboxes, bolts were loosened and the rhizoboxes were gently immersed in water for a few seconds to detach any roots glued through mucilage to the transparent face. Thereafter, rhizoboxes were taken out of water and unscrewed keeping upward the sheet that was facing the ceiling while the rhizoboxes were resting in the rack. To expose the whole root system at the end of the experiment, the pinboard method was used (Thangthong et al. 2018), which consists in framing the whole root system on a rigid dense grid of nails. The pinboard was made of marine plywood (23 cm × 50 cm × 1 cm thickness) nailed in a grid of 1.5 cm × 1.5 cm with 495 (15 × 33) steel nails (1.6 mm diameter, 30 mm length). The pinboard was overlaid with a sheet of black polyurethane foam (50 cm × 50 cm) gently pressed between each row of the grid to have it pierced by the nails and well-stretched over the board. The function of the foam was to provide a black contrast against roots (which were white or yellowish) to render any image-analysis easier, as well as to easily lift up the whole root system for scanning or for taking pictures. Once the pinboard was inserted and firmly pressed on the substrate, the rhizobox was turned face up and the front sheet (the one towards which the roots tended to grow) was slowly removed. The substrate between nail rows was gently pressed with the aid of a cylindrical steel rod so that the roots were kept in place by the nails. The substrate was washed away from the root system of both plants with a gentle nozzle spraying water from the bottom to the top of the board kept at an angle of about 30° until the entire root system was exposed. After that, the pinboard was laid on the table and topped with a rigid plexiglass sheet of 2 mm covered with a transparent PVC sheet. For each plant, the position of the collar, and the tip of the main roots were marked on the sheet using a permanent marker with a different colour for the two plants. Thereafter, a simple polygon, one for each plant, was traced, whose vertexes were the marked points, and that delimited the surface (projected on the pinboard) explored by the root system. Images of the roots on the pinboard were taken with a Sony ILCE-6400 camera.

Root Image analysis

The root visible length was measured by image analysis importing scans in the software *WinRhizo Arabidopis* (Reagents Instruments, CA). The visible root length per plant of lupin in pure stand was calculated as the average root length of the two plants in the rhizobox.

The root system projected area of each plant, defined as the area of the polygon delimiting the external perimeter of the whole root system, was calculated with a semi-automatic procedure using the freeware software ImageJ (http://imagej.net) involving the digitalization of the polygons with the function polyline and selecting the tools for calculating area and shape parameters. The following shape parameters were computed on the polygons:

$$Aspect\ ratio = \frac{Major\ axis}{Minor\ axis}$$

$$Roundness = 4\frac{Root\ projected\ Area}{\pi Major\ axis}$$

$$Solidity = \frac{Root\ projected\ Area}{Convex\ hull}$$

where *Major axis* and *Minor axis* are the axes of an imaginary ellipse encompassing the root system projected area (Root projected Area), and *Convex hull* is the volume encompassing the smallest convex set of points containing the entire root system (Atkinson et al. 2015.)

A novel index of root interaction named Root Merge (RM) was defined to quantify the degree of overlap between the root system projected area of two neighbouring plants. The RM was computed as the ratio between the surface explored by the root system of a lupin plant that is shared (overlaps) with the root system of the other plant in the rhizobox (either lupin or wheat) and the total lupin root system projected area. The index was calculated for lupin in pure stand and intercropped with wheat:

$$RM = \frac{Area\ of\ overlap\ between\ root\ systems}{Individual\ root\ system\ projected\ area}$$

The *Area of overlap between root systems* is the area of the intersection between the two polygons delimiting the root systems of the two plants in the rhizobox. The index ranges between 0 (absence of overlap) and 1 (total overlap) and can be indicative of root avoidance when it approaches zero or of large root intermingling when it is close to one (Fig. 2).

Count of visible nodules and cluster roots

Nodules formed by N-fixing symbiotic rhizobia on white lupin roots were counted manually using root

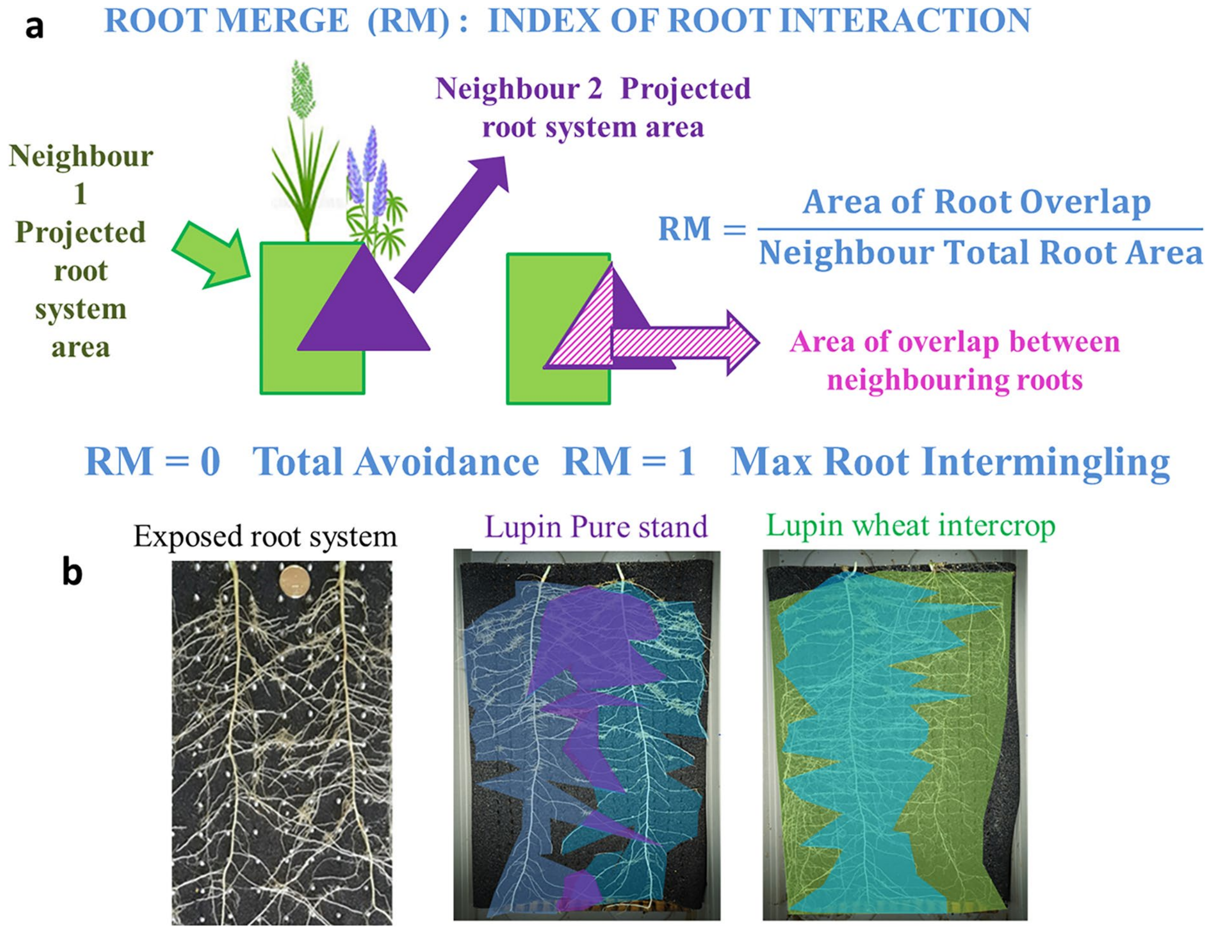


Fig. 2 **A** Graphical representation of the Root Merge (RM) index, calculated as the ratio between the overlapping area of neighbouring plant root systems and the projected area of an individual root system. **b** From left to right: sample images of the root systems of two lupin plants framed on the pinboard; manual tracing of the projected root system area (shaded in blue), and the overlapping area (shaded in purple) between the lupin plants; manual tracing of the projected root system area of intercropped lupin (light blue) and wheat (green)

images and, therefore, only visible nodules on one side of the root system could be quantified. The number of nodules per plant in pure stand was computed as the average number of nodules visible on the two plants per rhizobox.

The total of cluster roots per lupin plant was also recorded manually counting the number of visible cluster roots on root images, averaging the numbers recorded in the two lupin plants in the pure stand.

Statistical analysis

We conducted an analysis of variance (ANOVA) for all recorded root and shoot variables. We compared lupin pure stand (LP) and lupin intercropped with wheat (LIC) for the number of fully expanded leaves, plant height, Root Merge index, number of nodules, and number of cluster roots. Although the main interest in this investigation was comparing the behaviour of white lupin in the two growing conditions, for the remaining traits the values recorded on wheat were also included in the analyses. We created a dummy variable ('Cropping system') with three levels, namely, pure-stand lupin (LP), intercropped lupin (LIC), and intercropped wheat (WIC), and compared their mean values for visible root length, root system projected area, and root shape parameters (aspect ratio, roundness, and solidity). The ANOVA assessed the effect of species, cropping system and measurement date and their interaction on visible root length development (cm day^{-1}), separating mean values through least significant difference test. In all other ANOVAs, means were separated at $P<0.05$ through the Tukey post-hoc test or, when necessary, through the Welch *t*-test for unequal variance and sample size. All the analyses were carried out within the R environment for statistical analysis (R Core Team 2021).

For each parameter, a phenotypic plasticity index (PPI) was calculated to assess the ability of root shape parameters to discriminate between cropping systems. This index was defined as the absolute value of the percentage difference between the mean of the values measured in the intercrop system and the mean of the values measured in the pure stand.

$$PPI = abs\frac{(X_{IC} - X_{PS})}{X_{PS}} \times 100$$

where:

X_{IC} the mean trait value in intercropping

X_{PS} the mean trait value in pure stand

We calculated the plasticity index using the bootstrap method in the R package *boot* package v1.3–27, computing 1000 iterations to ensure robust estimates.

Results

After the three weeks of growth, lupin reached the phenological stage of five fully expanded leaves, and wheat plants had five fully expanded leaves and had entered the tillering stage (one visible tiller per plant). No significant differences in shoot traits were observed in lupin between cropping systems (data not reported). The plants reached the bottom of the container in approximately 19 days without differences between species and cropping systems. All lupin plants grew a substantial part of their primary root against the transparent window. The first order lateral roots were visible about 10 days after transplanting and by the third (and last) week of the experiment some cluster roots were visible on the sides and along the primary root.

During the root tracing along the experiment, wheat roots were discernible from those of lupin due to: i) the smaller diameter, ii) the whiter translucid colour; iii) the presence of root hairs; and iv) the presence of a dark shadow around the root possibly due to the presence of the rhizosheath. Visible root length in time is depicted in Fig. 3. At the end of the experiment, visible root length ranged from an average of 129.0 cm in lupin pure stand to 183.5 cm in wheat Root length development was significantly affected by the cropping system and time ($P<0.01$) with a trend ($P=0.07$) for an interaction between date and cropping system.

Wheat visible root length was always greater than that of lupin but there was a sharp increase in wheat root length during the third and last week of the experiment when wheat had greater value than lupin in either growing condition. The lowest root length was measured in the lupin pure stand,

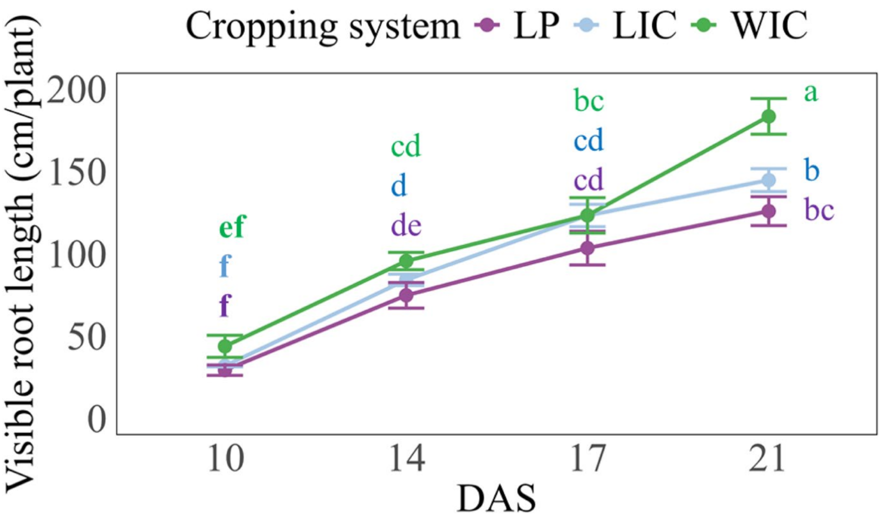


Fig. 3 Visible root length over time (DAS = days after sowing) for different cropping systems in a rhizobox experiment, namely, lupin in pure stand (LP, solid purple line), lupin intercropped with wheat (LIC, solid blue line), and wheat intercropped with lupin (WIC solid green line). Means are overlay by standard error bars. Different letters above the lines indicate significant differences ($P < 0.05$) across cropping system-date combinations according to the least significant difference test

although differences with intercropped lupin did not reach statistical significance (Fig. 3).

When the rhizoboxes were opened, the entire root system was easily exposed over the pinboard, and the roots adhered to the foam coating the pinboard due to its roughness. Individual plants could be separated following the root trajectory and detecting root tips in crowded areas. Tracing the root projected area took only a few minutes per box (less than 10 min on average). Once the whole root system is exposed on the pinboard, many root traits can be observed, such as nodule number and size, the number and position of cluster roots, as well as the overlap between adjacent plants. Figure 4 shows sample pictures and examples of root details that can be observed or measured on the images. The merging behaviour of intercropped wheat and lupin is clearly imaged (Fig. 4a), lupin nodules are easy to detect (Fig. 4b), as well as the large intermingling between cluster (lupin) and non-cluster (wheat) roots (Fig. 4c).

Wheat developed a much larger area than lupin in both conditions (Fig. 5a) with a root system almost totally encompassing that of the intercropped lupin (Fig. 4c).

Root shape parameters displayed some significant differences. The aspect ratio of lupin in pure stand was remarkably higher than that of both species in intercropping (Fig. 5b); white lupin plants assumed a rather elongated, vertical growth when growing with a co-specific neighbour, and much less so when growing with a wheat neighbour.

Wheat had greater root area roundness than lupin in either condition (Fig. 5c), indicative of a more symmetric, evenly distributed root growth and confirming the ubiquitous appearance of the cereal root system (Fig. 4a, c). Intercropped lupin had a clear tendency of greater roundness than lupin in pure stand (Fig. 5c). In fact, the difference in roundness between intercropped and pure stand lupin would have been significant ($P < 0.05$) according to Tukey's test if the two lupin conditions were compared alone.

Lupin in pure stand had low solidity (Fig. 5d), meaning that its root system likely presented large gaps in its distribution, whereas wheat in intercropping densely colonized the explored soil volume, as indicated by its high solidity value (Fig. 5d). Intercropped lupin had intermediate solidity compared to lupin pure stand and wheat.

The bootstrap computation of the phenotypic plasticity index for lupin across the two growing conditions (Fig. 6) indicated that roundness and aspect ratio were the traits most responsive (in absolute terms) to intercropping, thus supporting the ANOVA differences between lupin cropping systems described in Fig. 5, where roundness was shown to increase, and aspect ratio to decrease in intercropping compared to pure stand.

The most outstanding difference between the two growing conditions of lupin was in the overlapping extent of the root system of lupin plants in pure stand compared to that of lupin plants when intercropped with wheat. The computed RM index to estimate such overlapping clearly separated lupin in pure stand

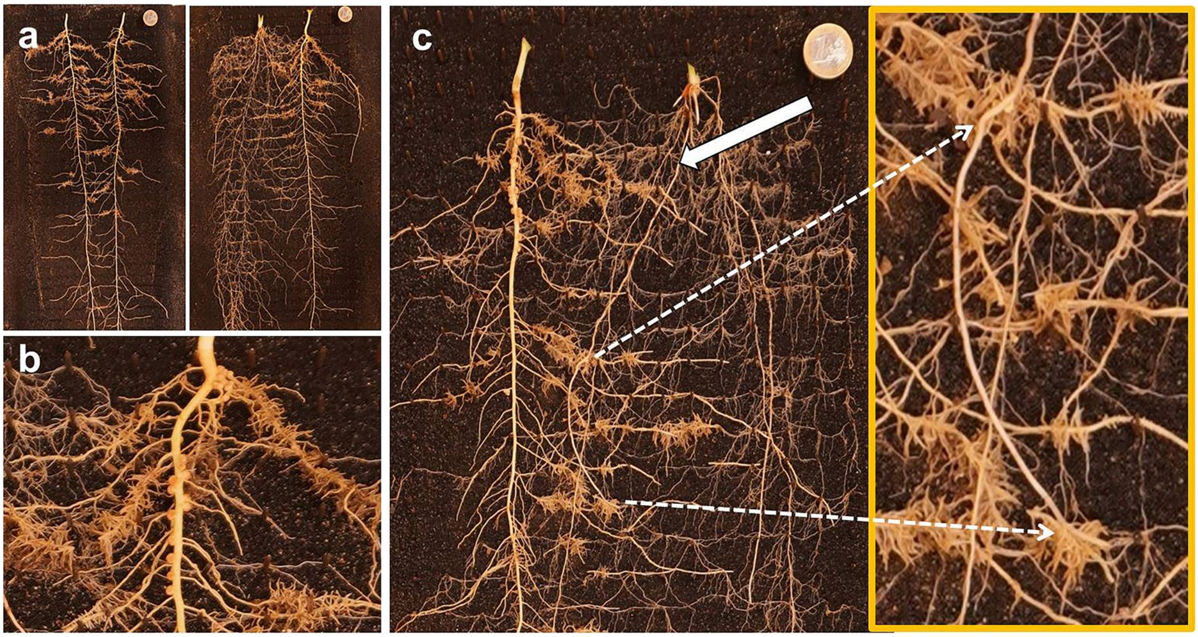


Fig. 4 **A** Whole root system of lupin in a rhizobox after root framing on the pinboard. Left: lupin pure stand. Right: lupin and wheat intercropped plants. **b** Detail of root nodules visible along the main axis of intercropped lupin. **c** Wheat nodal root growing in close proximity to lupin cluster roots

(RM = 0.17) from lupin in intercropping (RM = 0.69) (Fig. 7a). A substantial part of the lupin root area in intercropping was overlapped by wheat, but this did not depress lupin shoot or root growth. Oppositely, there was a minimum overlap between lupin plants in pure stand suggesting an avoidance behaviour, which was very consistent among replicates as shown by the low standard deviation (Fig. 7a).

Lupin developed more rhizobial nodules on roots when intercropped with wheat than when grown in pure stand (Fig. 7b), whereas the lupin cluster root count showed no significant differences between cropping systems (Fig. 7c).

Discussion

In the context of global challenges such as climate change and resource scarcity, intercropping is increasingly recognized as a sustainable strategy. Understanding belowground interactions in intercropping is essential for maximizing system productivity and ecosystem benefits by optimizing traits matching between companion crops (Yu et al 2021). However, studying root interactions remains a challenge, and common methods are often time-consuming, invasive, and limited in scalability. There is a growing need to gather more information on genetic variability and plasticity in intercropping systems. This is particularly important because crop breeding has historically focused on monocultures, overlooking traits related to competitive ability and facilitation in mixed cropping systems, which is critical for understanding the genetic basis of adaptability in breeding programs. These challenges highlight the need for high-throughput techniques to study root diversity and intra- and inter-specific interactions in intercropping. Such methods should enable the capture of plant traits that confer superior adaptability to intercropping, while remaining relatively fast and simple.

We investigated root interactions in rhizoboxes by assessing some rapid indicators of root architecture/plasticity in intercropping together with traditional root traits such as visible root length.

The rhizoboxes used in this study provided sufficient space for sowing operations and root growth, while ensuring adequate aeration and drainage, thus avoiding waterlogging—a common issue in shallow

Fig. 5 Mean value (and relative standard deviations) of the root system projected area (**a**) and three root shape parameters, namely, Aspect ratio (**b**), area Roundness (**c**) and Solidity (**d**), for lupin pure stand (LP), intercropped lupin (LIC) and intercropped wheat (WIC) in a rhizobox experiment. Different letters above the bars indicate significant differences between means ($P < 0.05$) according to the Tukey's post-hoc test

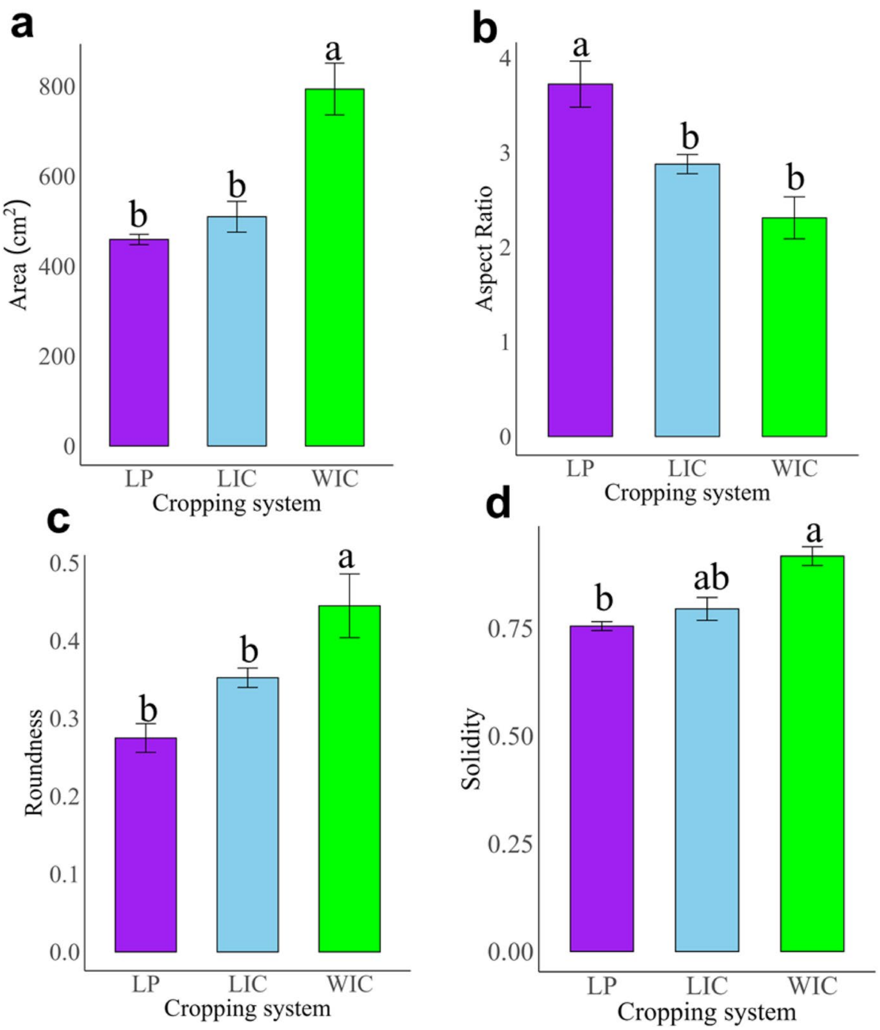
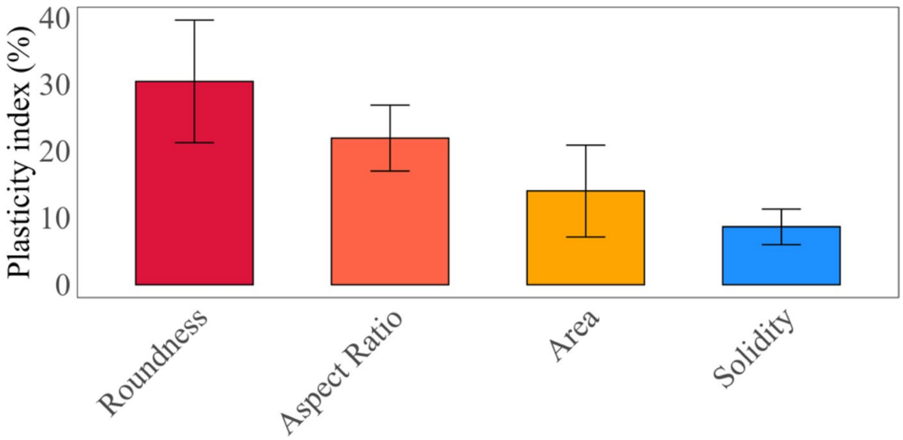


Fig.6 Mean values (and standard deviations) of the plasticity index calculated for the root system projected area (Area) and three root shape parameters, namely, Aspect ratio, area Roundness, and Solidity for white lupin grown either as pure stand or intercropped with wheat in a rhizobox experiment



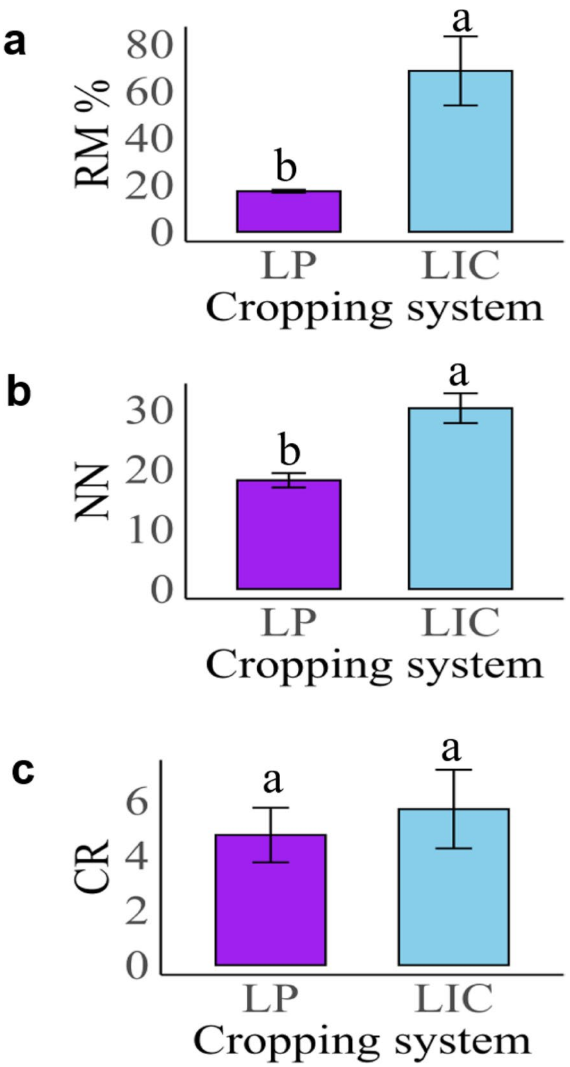


Fig. 7 Mean values (and standard deviations) of the Root Merge index (RM %: a), number of rhizobial nodules per plant (NN: b), and number of cluster roots per plant (CR: c) of white lupin grown in two cropping systems (Cropsys), namely, pure stand (LP) and intercropped with wheat (LIC). Different letters above the bars indicate significant differences between means ($P < 0.05$) according to the analysis of variance

containers or repacked soils (Passioura 2006). There are no standardized guidelines regarding rhizobox dimensions, which can vary significantly (e.g. Nagel et al. 2015; Figueroa-Bustos et al. 2018). The current height of rhizoboxes allowed roots to grow for three weeks before reaching the bottom of the container and distorting root architecture (Poorter et al. 2012). This time window corresponds to a juvenile stage in the field (beginning of tillering for cereals and five leaves for lupin). Even during this short phase, neighbouring roots interacted between each other, and we could observe sizable differences between species and cropping systems. Phenotyping root competition at early stage of growth is very important because root competition in crop mixture begins before, and can be more intense, than aboveground competition (Sobkowicz 2005).

Wheat achieved approximately twice the visible root length of lupin by the end of the experiment, filling the container space more extensively. Differences in root length density between legumes and small grain cereals are well known (Osborne et al. 2020) and the current results are consistent with the documented vigorous root growth of cereals compared to legumes in intercropping (Mariotti et al. 2009; Sobkowicz 2005). In soybean-wheat intercropping, wheat developed 46% and 74% more root length than soybean at high and low P availability, respectively (Bargaz et al. 2017). Wheat roots outgrew faba bean and maize, especially during early stages of growth (Li et al. 2006). In our study, there was no evidence of niche pre-emption, as both species reached the container bottom simultaneously (approximately 19 days after sowing) at a descending velocity of ~ 2.5 cm day^{-1}. Although substantial genetic variability in seminal root length can be found in wheat (Chen et al 2020; Watt et al 2013), the descent rate for wheat was slower than what reported elsewhere (Smit and Groenwold 2005). This suggests that the presence of lupin may influence the wheat root allocation, potentially favouring lateral root investment—a hypothesis that warrants further investigation as the lack of a wheat monoculture in this experiment (our focus being the legume crop) hinders inference on wheat.

To track root interaction changes over time, we recorded visible root length development. While most intercropping studies rely on a single final harvest, repeated measurements of crop growth over time provide deeper insights into competition, since plant-plant interactions evolve due to interplay between growing organs, reciprocal influence and resource availability modifications (Andersen 2005). The dramatic increase in wheat root length during the third week, coinciding with intense lupin cluster root formation and the appearance of active rhizobial nodules (cluster roots and pink nodules began to be visible through the transparent box window in that week), suggests potential abiotic facilitation for P and N uptake. We hypothesize that at this stage nutrients release from lupin roots through abiotic facilitation primed the growth of wheat roots which in fact grew in close proximity of lupin cluster roots. Besides N facilitation, P release from lupin roots (e.g., via rhizosphere acidification) likely drove this wheat root response, mirroring chickpea-wheat systems where P mobilization enhanced wheat root proliferation to the point of drifting the system toward antagonistic competition (Zhang and Li 2003). Root contact can be associated with P facilitation through the presence of cluster roots, resulting in stimulated root production in the companion species (Teste et al. 2020). These studies align with our hypothesis but a comparison with wheat grown as a pure stand, which is currently unavailable in our study, remains essential to validate these findings.

Facilitative interactions emerged as a key driver of belowground dynamics. The increased nodule formation in intercropped lupin compared to pure stand represents a clear indicator of facilitative interactions in the crop association. Nitrogen transfer from legumes to cereals occurs even during early stages of growth (Jensen 1996). Increased nodule density implies greater N fixation and larger N-self-sufficiency for the legume, which, in turn, decreases competition for soil mineral N. As a feedback in the process, the non-legume species might also be able to favour the N fixation by the legume (Homulle et al. 2022). Resource partitioning—whereby each species accesses different resource pools (wheat utilizing soil mineral N while lupin fixes atmospheric N_2)—cannot be ruled out without adequate methodologies such as isotopic tracing to quantify N transfer (Jamont et al. 2013). Indirect evidence of complementarity between species is given by the fact that the aggressive wheat root phenotype did not inhibit lupin growth. It is likely that direct facilitation and resource partitioning may be operating simultaneously in the system, though additional physiological measurements are needed to separate these effects. Unexpectedly, there was no significant differences in cluster root production between lupin cropping systems. Cluster roots, which dramatically increase root absorbing area and rhizosphere acidification capacity, play a key role in nutrient scavenging and foraging capacity. Even in the absence of a strong competitor for nutrients such as wheat lupin invested a similar amount of resources in cluster roots formation.

Cluster roots represent a C cost, which is usually compensated by increased photosynthesis and nodulation (Thuynsma et al 2014; Pueyo et al. 2021; Wang et al. 2019). Without data of tissue P, although in the presence of a sub-optimal basic P fertilization in the experiment, it is difficult to ascertain whether this investment conferred a physiological advantage, but the lack of biomass gain in lupin pure stand indicates that this strategy may not always be adaptive, at least during early stages of growth.

Differences in root aspect ratio further highlighted variation in root growth strategies between pure stand and intercropped lupin, supporting the hypothesis that lupin in pure stand prioritizes vertical rather than lateral root development and tends to avoid its neighbour. Root segregation is generally associated with competition pressure (Schenk et al. 1999; Schmid et al. 2015). However, while root segregation has been linked to reduced intra- and inter-specific competition and improved resource use efficiency through spatial niche partitioning (e.g. Cabal et al. 2024; Holzapfel and Alpert 2003), our findings did not support the possibility that this avoidant behaviour constitutes a cooperative adaptive response. Specifically, lupin exhibited neither biomass advantages nor increased root exploration despite segregation—a result that challenges the assumption of automatic benefits from spatial niche differentiation through a complementary use of resources (Laclau et al. 2013). Similarly, in a large grassland biodiversity experiment, belowground resource partitioning did not enhance resource uptake or biomass production. Instead, mechanisms such as species facilitation, species-specific biotic feedback, and aboveground resource partitioning emerged as the primary drivers of improved ecosystem functioning associated with greater biodiversity in plant mixtures (Jesch et al. 2018).

The projected root area was little responsive to intercropping. This suggests that classical root parameters measured in rhizobox studies, such as root length, projected area or convex hull, may not be the best descriptors of root interactions in intercropping as they may overlook the spatial dynamics occurring between neighbours. While they are key traits for measuring root foraging and water uptake capacity, the spatial allocation of roots away from, or towards the neighbouring plant is equally important in intercropping, and measures of such spatial allocation may be relevant in root phenotyping protocols.

Root spatial descriptors, such as the Root Merge (RM) index and the root shape indicators, can provide a deeper understanding of root system interactions. The results showed that lupin modifies its root architecture spatially in response to intercropping rather than simply altering total root production. The novel RM index clearly separated the two cropping systems, with lupin in pure stand showing reduced overlap between neighbouring root zones, thus indicating spatial compartmentalization. In contrast, the RM index was much higher in intercropping, where the wheat root zone largely encompassed that of lupin. Extensive root intermingling between wheat and legumes is not uncommon and was associated with symmetric facilitation for calcium, iron and zinc uptake in wheat-chickpea intercrop (Li et al. 2014). Free root intermingling between wheat and faba bean increased N transfer three-fold compared to when a mesh barrier prevented root contact but still allowed N exchange between the plants (Xiao et al. 2004). As previously mentioned, root intermingling can also facilitate P availability (Teste et al. 2020). One limitation of the RM index is that it only measures overlap between root zones. To properly assess facilitation, the index should be coupled with net measures of competitive pressure. In this study, however, the combination of RM with lupin shoot biomass and root size (length and area) provided evidence of facilitation rather than antagonistic competition between lupin and wheat.

The current experiment focused on a white lupin–wheat case study because of the raising interest for this legume crop but the same procedure could be easily extended to other legume-cereal combinations or, even, to intercropping involving other plant species. Rhizobox dimensions could be modified to accommodate plants with larger roots (Isaac et al. 2012) but the simple phenotyping procedure proposed would remain relevant. Root interactions in weed science or in ecological studies could be addressed through this rhizobox approach, provided sufficient morphological differences exist between species to distinguish them. This methodology works well in legume-cereal intercropping where roots of the two crops are easily discernible for morphological traits, but it becomes more difficult in the case of mixtures between Poaceae species. Quantification of traits such as rhizobial nodules or cluster roots as indicators of facilitation obviously apply only to intercropping with legumes, the former, and with lupins and a few more species, the latter. Given the importance and prevalence of legume-based intercropping, the current setup can provide a framework for a preliminary evaluation of the legume genetic variation aiding the identification of the most responsive genotypes for further in-depth studies of belowground processes.

The adopted rhizobox experimental layout can be adapted to study root interactions simulating mixed intercropping or row intercropping patterns. It can also be extended to simulate strip intercropping but only with reference to the edges of the strips where interactions between non-conspecific neighbours can occur (Gao et al. 2010). On the other hand, the established benefits of strip intercropping mainly rely on biodiversity enhancement rather than on crop interactions (Juventia et al. 2022). Standard rhizoboxes are spatially restrained for manageability, making this system unsuitable for relay intercropping, which requires much longer growth periods. Time constraint due to the rhizobox size is in fact a possible limit of the method, as it does not allow, for instance, assessing any niche differentiation between neighbours through time (Homulle et al. 2022).

As the root spatial configuration is retained upon rhizobox opening, this methodology might enable site-specific root exudate collection, facilitating the study of belowground chemical signalling. Moreover, rapid root trait scoring methods, such as those used in shovelomics (Trachsel et al. 2011), could be integrated in rhizobox studies, further enhancing system throughput and information. In conclusion, our results contribute to addressing the quest for rapid root phenotyping approaches specifically suitable for intercropping, expanding the current knowledge on white lupin–wheat belowground interactions and opening ground for broader applications across diverse cropping systems.

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Data availability Data will be made available on request.

Declarations

Competing interest The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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