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Traits affecting early season nitrogen uptake in nine legume species

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Abstract

Legume crops are known to have low soil N uptake early in their life cycle, which can weaken their ability to compete with other species, such as weeds or other crops in intercropping systems. However, there is limited knowledge on the main traits involved in soil N uptake during early growth and for a range of species. The objective of this research was to identify the main traits explaining the variability among legume species in soil N uptake and to study the effect of the soil mineral N supply on the legume strategy for the use of available N sources during early growth. Nine legume species were grown in rhizotrons with or without N supply. Root expansion, shoot and root biomass, nodule establishment, N_2 fixation and mineral soil N uptake were measured. A large interspecific variability was observed for all traits affecting soil N uptake. Root lateral expansion and early biomass in relation to seed mass were the major traits influencing soil N uptake regardless of the level of soil N availability. Fenugreek, lentil, alfalfa, and common vetch could be considered weak competitors for soil N due to their low plant biomass and low lateral root expansion. Conversely, peanut, pea, chickpea and soybean had a greater soil N uptake. Faba bean was separated from other species having a higher nodule biomass, a higher N_2 fixation and a lower seed reserve depletion. Faba bean was able to simultaneously fix N_2 and take up soil N. This work has identified traits of seed mass, shoot and root biomass, root lateral

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expansion, N_2 fixation and seed reserve depletion that allowing classification of legume species regarding their soil N uptake ability during early growth.

Keywords: Agriculture, Plant biology

1. Introduction

Legumes are known to be less efficient at recovering soil inorganic N during the growing season compared to non-legume crops including those in the Poacae or Brassicaceae family (Thorup-Kristensen, 1994). This low soil N uptake may be a key factor explaining their low ability to reduce N leaching as catch crops (Thorup-Kristensen, 2001), their low ability to compete against weeds (Corre-Hellou et al., 2011) and compete with non-legumes when intercropped (Naudin et al., 2010).

Early plant growth is a crucial phase for the establishment of relative competitive ability with other species (weeds or crops) (Bellostas et al., 2003; Fayaud et al., 2014). Small differences in early-season seedling growth can result in large differences in size and resources capture ability later in the season (Andersen et al., 2007). Therefore plant strategies resulting in greater early season crop growth and N acquisition can be critical for determining competitive interactions between crops and other species. During early growth legumes species are expected to have low soil N uptake. Nevertheless, key factors explaining this early low soil N uptake ability of legumes are not known. During early growth, legumes may rely mainly on soil N uptake similar to non-legume species because legumes have not had time to initiate nodulation and develop effective nodules infected with nitrogen fixing bacteria. Moreover, grain legumes can rely also on seed N reserve for a significant time due to the large seeds with a relatively high nitrogen content (Herdina and Silsbury, 1990). This can result in low use of external N sources.

Few studies have examined the variability among legume species in early season soil N uptake (Hamblin and Hamblin, 1985). Early season soil N uptake may differ among legume species because of differences in root expansion, early plant growth and symbiotic N_2 fixation (nodules establishment and activity).

Although the ability of roots to quickly and effectively occupy the soil volume during crop establishment can be a major trait related to the competitiveness for soil N, species differences in this trait are not well documented (Jamont et al., 2013). A greater root occupancy of the soil volume should allow for greater N uptake, resulting in less available N for other species (Dunbabin, 2007). Thus, the root depth penetration and lateral expansion may be key traits affecting the competitive ability of legumes for soil N during early growth. Moreover, species that have a greater initial growth are often more competitive for soil resources at the beginning of the crop life cycle (Andersen et al., 2007).

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During the early growth phase, legumes may access three different sources of N: the seed reserve, soil N, and in a lower extent, atmospheric N_2. In similar agronomic conditions, the relative contributions of each of these sources to the plant growth may differ across species. The seed mass can explain differences among species in the level of shoot biomass during early growth (Gross, 1984; Fayaud et al., 2014). A species able to rely on biological N_2 fixation for N nutrition earlier than others may have reduced N uptake from the soil. Nodule establishment and activity can differ across species according to seed size (Dobert and Blevins, 1993), type of emergence (hypogeal or epigeal) (Sprent and Thomas, 1984) and depletion of seed N reserves, all which interact with the mineral soil N availability (Voisin et al., 2010).

Although some studies have shown differences among legume crops in root development (Hamblin and Hamblin, 1985; Liu et al., 2011) and N_2 fixation (Herridge et al., 2008; Saia et al., 2016), there have been few legume species studied, and the simultaneous analysis of different traits, correlations between several traits, and consequences on soil N uptake during early growth have not been investigated.

The objectives of this research were i) to identify the main traits explaining the variability among legume species in soil N uptake during early growth and ii) to study the effect of the soil mineral N supply on the legume strategy for the use of available N sources. A greenhouse experiment was undertaken to monitor the early growth stages of nine legumes species sown in rhizotrons either at a low or high level of soil mineral N.

2. Materials and methods

2.1. Plant material and experimental design

Nine divergent legume species (Table 1) were selected for evaluation based on their expected contrasting traits related to early growth and N acquisition. Peanut (*Arachis hypogaea* L.), fenugreek (*Trigonella foenum-graecum* L.), faba bean (*Vicia faba* L.), winter lentil (*Lens culinaris* L.), alfalfa (*Medicago sativa* L.), pea (*Pisum sativum* L.), chickpea (*Cicer arietinum* L.), soybean (*Glycine max* L.) and common vetch (*Vicia. sativa* L.) were grown with or without mineral nitrogen in a factorial completely randomized design experiment. The experiment was conducted under natural light in a greenhouse from March 27th to April 30th, 2014 in western France (Angers; 47.5°N, 0.6°W). The mean temperature and humidity during the experiment were 20.1 ± SD 0.9 °C and 41.8 ± SD 10.3%, respectively. This experiment was carried out using transparent rhizotrons (Jamont et al., 2013) with inner dimensions of 50 × 20 × 3 cm that were covered with black polyethylene plastic sheeting. The rhizotrons were inclined at 45° so that the roots would grow towards the underside. Each rhizotron was filled with 4.1 kg of a

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Table 1. List of studied species.

Latin name	Common name	Variety	Emergence type	Initial seed dry matter (g) (isdm)	Seed N content (%sN)	Phenological Stages at 35 DAG[3]	Rhizobium strain
Medicago sativa L.	Alfalfa	Cannelle	Epigeal	0.002	6.68	13	*Sinorhizobium meliloti* (2011)[2]
Trigonella foenum-graecum L.	Fenugreek	Wild genotype	Epigeal	0.012	5.44	14	*Sinorhizobium* sp. (MSDJ3538)[2]
Lens culinaris L.	Winter lentil	ANICIA	Hypogeal	0.024	4.68	19	*Rh.leguminosarum* bv *viciae* (P221)[2]
Vicia sativa L.	Common vetch	Nacre	Hypogeal	0.051	5.19	39	*Rh.leguminosarum* bv *viciae* (P221)[2]
Glycine max L.	Soybean	Protina	Epigeal	0.177	8.17	13	*Bradyrhizobium japonicum* (G49)[2]
Pisum sativum L.	Pea	Standal	Hypogeal	0.273	3.97	18/37	*Rh.leguminosarum* bv *viciae* (P221)[2]
Cicer arietinum L.	Chickpea	Principé	Hypogeal	0.364	4.16	23	*Mesorhizobium ciceri* (UMPCa7)[1]
Arachis hypogaea L.	Peanut	TE3	Epigeal	0.407	5.69	15/22	*Bradyrhizobium tropici* (CB756)[1]
Vicia faba L.	Faba bean	Divine	Hypogeal	0.539	4.97	16	*Rhizobium leguminosarum* bv *viciae* (FH34)[2]

Sources of Rhizobium strains:

[1] INRA, UMR Eco&Sols (Montpellier SupAgro – CIRAD – INRA – IRD), France.

[2] UMR 1347 – Agroecologie, Pôle MERS, Microbiologie Environnementale et Risque Sanitaire, Center INRA, Dijon, France.

[3] According to BBCH scale of (JKI, 2010) at sampling date (35 days after seed germination).

mixture of sandy fresh soil (25%) and dry sand (75%). The soil was sieved (7 mm) prior to being homogeneously mixed with dry sand. The sand was previously washed and dried at 105 °C for 48 h. The final composition of the soil sand mixture was 2.9% clay, 6% loam, 90.6% sand and 0.4% organic matter, with 7 mg NO_3^- N kg^{-1} dry soil and the pH of the mixture was 6.9. The phosphorus (P Olsen) and potassium (K) contents were 30.3 and 78.7 mg kg^{-1} of dry soil, respectively. 150 ml of a N-free nutrient solution was added to each rhizotron at the following concentrations (mmol l^{-1}): 1.24 KCl, 0.34 KH_2PO_4, 0.24 $MgSO_4$ $7H_2O$, 0.18 $CaCl_2$ and 0.04 $FeCl_3$, with a controlled pH of 6.8. In half of the rhizotrons, the soil was supplemented with a solution of KNO_3 to reach a concentration of 36 mg NO_3^- N kg^{-1} dry soil. The nutrient solutions were mixed with the soil before filling the rhizotrons.

The legume seeds were weighed and selected to reduce the variation in the individual seed mass to less than ± 3.5% (except alfalfa, with a variation of ± 7%, because of the high seed dry matter (DM) heterogeneity). The seeds were first germinated on filter paper until the radicle emerged from the seed (BBCH stage

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05) (JKI, 2010), and one seed was sown per rhizotron before being inoculated with an appropriate strain of rhizobia (Table 1) at an estimated rate of 108 cells per plant to allow for N_2 fixation (Naudin et al., 2011); for each legume species, the seeds were sown in four rhizotrons without N (0N treatment) and four rhizotrons with a N supply (N treatment). After the seedling emergence, a 1-cm thick layer of sand (100 g) was added to the surface of the rhizotron to limit water evaporation from the soil. During the experiment, the soil humidity was kept at field capacity.

Common wheat (*Triticum aestivum* L, cv Renan) and oilseed rape (*Brassica napus* L., cv Boheme), known for their contrasting abilities to acquire N (Moreau et al., 2013), were grown as controls to compare the ability of legume species with non-legume species to deplete soil N.

Plants of each legume species were also grown simultaneously in N-free sand to determine the ^{15}N abundance of plants relying only on N_2 fixation (β value) and thus estimate the N_2 fixation using the natural abundance method (Amarger et al., 1979). The pots were supplied once per week with 100 ml of N-free nutrient solution with a composition of (mmol l^{-1}) 0.54 KCl, 0.17 KH_2PO_4, 0.12 $MgSO_4$ $7H_2O$, 0.09 $CaCl_2$, and 0.02 $FeCl_3$ with a controlled pH of 6.8 and maintained at field capacity.

2.2. Sampling and data collection

The root depth and lateral expansion were monitored using a grid of 5 mm × 5 mm squares printed on a transparent plastic sheet fixed on the underside of the rhizotron. Three times a week for five weeks, the root presence in the grid squares was marked to denote vertical and horizontal root distribution.

The plants were harvested 35 days after germination (DAG) while still in early growth phase. At 35 days, legumes should rely mainly on seed N and soil N. A little nitrogenase activity was reported to be evident in grain legumes approximately 20 days after sowing at 20 °C (Herdina and Silsbury, 1990), depending on the mineral N level. The root systems were removed from the rhizotron and washed with demineralized water. The roots and nodules were sorted, and the nodules were placed on a white sheet to be photographed with a Canon EOS 350D digital camera (lens: SIGMA 50 mm F2.8 DG). The pictures were analysed with ImageJ version 1.40 g (National Institutes of Health, USA; http://rsb.info.nih.gov/ij/) to obtain the total number of nodules. The seed residues were collected to quantify the remaining seed DM. Plants grown in N-free sand were harvested at the same time and sorted into shoot and root samples. After oven-drying (48 h at 70 °C), the dry matter of the roots, shoots, nodules and seed residues were determined. All of the samples were ground to fine powder for total N and ^{15}N:^{14}N ratio measurements in the original seeds, nodulated roots, shoots,

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and seed residues using a CHN analyser (EA3000, Euro Vector, Milan, Italy) and a mass spectrometer (IsoPrime, Elementar, Hanau, Germany).

At each sowing and harvest date, soil samples were collected for all species (legumes, wheat and oilseed rape), and the soil inorganic N content was measured using a segmented flow analysis (SKALAR SA3000), which enables the determination of the nitrate content by KCl extraction according to the international standard ISO 14256–2.

2.3. Calculations and statistics

The part of the plant N derived from the seed was calculated using the amount of N from the seed (QN seed) consumed during the experiment by each plantlet. QN seed is the difference between the seed N content at sowing and that at harvest. Exogenous N is the difference between the N accumulated in the plant and the QN seed. The relative contributions of the exogenous sources of N (air and soil) were then determined.

The part of the plant exogenous N derived from the air (%Ndfa) was estimated using the ^{15}N natural abundance method (Amarger et al., 1979). Common wheat was used as a non-fixing reference crop Eq. (1),

$$\%Ndfa = 100 \times [(\delta^{15}Nwheat - \delta^{15}Nlegume) / (\delta^{15}Nwheat - \beta)] \quad (1)$$

where $\delta^{15}Nlegume$ and $\delta^{15}Nwheat$ express the natural ^{15}N abundances of legume and of wheat, respectively. β is the isotopic fractionation factor measured from each species of legumes grown in N-free sand.

The $\delta^{15}N$ of the whole crop was calculated using Eq. (2):

$$\delta^{15}Nwhole\ crop = [(\delta^{15}Nshoot \times shoot\ DW) + (\delta^{15}Nroot \times root\ DW)] / (shootDW + rootDW) \quad (2)$$

The values of $\delta\ ^{15}N$ for legumes and common wheat were corrected considering the N derived from the seed as another source of N for the plant (Jensen et al., 1985), Eq. (3):

$$\delta^{15}N\ corrected = [(\delta^{15}N\ crop - ((QN\ seed / QN\ crop) \times \delta^{15}N\ seed))] / [(1-(QN\ seed\ borne / QN\ crop))] \quad (3)$$

where $\delta^{15}N$ seed expresses the natural ^{15}N abundance of the original seeds.

The contribution of the soil N was calculated as the difference between the exogenous N and the N derived from air.

The rates of vertical and lateral root expansion (mm day^{-1}) were calculated by linear regression analysis. The effect of species on the shoot and root dry matter, root depth penetration and lateral expansion rates, nodule establishment, N

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acquisition and remaining soil N was tested by a one-way analysis of variance (type III sum of squares; $\alpha = 0.05$). The normality of the residues and homoscedasticity were tested using Pearson's and Levene's tests, respectively ($\alpha = 0.05$). The data were log transformed using Ln(x) for the root, shoot DM, plant DM, root:shoot ratio and soil N uptake and log(x) for the nodule number. Means were compared using Tukey's HSD test (Honest Significant Differences; $\alpha = 0.05$) to determine whether a main effect was significant. All statistical analyses were performed using R Commander package in R software, version 3.1.2 (RCore, 2014).

Principal component analysis (PCA) was used to analyse the correlations between the seed, shoot and root traits and N accumulation. PCA was implemented using the package FactoMineR (Lê et al., 2008) in R software. Each original variable was standardized to have equal weights in the multivariate analysis. The principal components (PC) were derived by calculating independent axes to explain the total variance in the dataset. An ascending hierarchical clustering algorithm was also applied to the data using the same standardized variables as in the PCA. The function hclust was implemented using the Euclidian distance to calculate the dissimilarity matrix among species using Ward's minimum variance method as the clustering algorithm. This type of analysis provides information on the relative distance among clusters and species. A separate PCA analysis was performed for each N treatment.

3. Results

3.1. Growth and N acquisition at a low mineral N level

Shoot and root biomass – In the 0N treatment, the biomass varied among species ($p < 0.0001$), with a coefficient of variation higher than 0.7 (Table 2). The shoot biomass varied from 0.008 (alfalfa) to 0.47 (faba bean) g per plant ($p < 0.0001$), and the root:shoot ratio ranged between 0.40 (pea) and 0.87 (alfalfa) ($p < 0.0001$). For all root parameters (root depth penetration rate, lateral expansion rate and root biomass), there were significant differences among legumes (Tables 2 and 3, $p < 0.0001$). The rate of depth penetration of the taproot (linear regressions, average $R^2 = 0.96$) varied from 7.1 in alfalfa to 22.6 mm day^{-1} in soybean. The rate of the root lateral expansion (linear regressions, average $R^2 = 0.86$) varied from 1.3 in fenugreek to 10.4 mm day^{-1} in chickpea. The ranking of species differed according to the root parameters, and different types of root exploration were observed. Lentil and alfalfa both had slow lateral and vertical root expansions, whereas soybean and chickpea had rapid lateral and vertical root expansions. Common vetch had a rapid vertical root penetration but a medium lateral root expansion. Fenugreek had a medium rate of vertical root penetration and a very slow lateral root expansion. A greater variability was observed for lateral expansion (CV = 0.55) than depth

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penetration (CV = 0.35). For species in which the rate of depth penetration is greater than 15 mm day^{-1} (peanut, faba bean, pea, chickpea, common vetch, soybean), the lateral expansion varied from 5.2 (common vetch) to 10.4 (chickpea) mm day^{-1}.

Nodule number and biomass – In the 0N treatments, all species began to produce nodules but not all of them showed developed functioning nodules. There were large differences in the number of nodules among species ($p < 0.0001$, Table 3). The greatest number of nodules was observed in faba bean and common vetch, with 48.8 and 60.5 nodules per plant, respectively, whereas nodule number was very low for alfalfa (1.5), fenugreek (4.3) and chickpea (4.3). The mean biomass per nodule also varied very significantly ($p < 0.0001$). For example, the common vetch and faba bean roots both had numerous nodules, but the mean biomass per nodule was low in the common vetch (0.07 mg $nodule^{-1}$), whereas it was high in faba bean (0.36 mg $nodule^{-1}$).

N accumulation in the plant and N sources – Without a mineral N supply, the N accumulation differed largely among legume species ($p < 0.0001$, Fig. 1a). Faba bean accumulated much more N in the shoots and roots than any other legume species (approximately 36 mg pl^{-1}), whereas the least N accumulated were observed in fenugreek, alfalfa and lentil with less than 3 mg pl^{-1}.

For most species, more than 50% of the plant N was derived from the seed (peanut, faba bean, lentil, chickpea, pea and soybean), whereas in alfalfa, only 6% of the plant N was derived from the seed (Fig. 1b).

Faba bean, alfalfa, fenugreek, soybean and common vetch began to fix atmospheric N_2; in these species, the percentage of N derived from the atmosphere in plant N ranged from approximately 6% (common vetch) to 20% (faba bean). The other species, peanut, chickpea, lentil and pea, relied on only the seed N and mineral soil N. Although the nodules had been initiated, biological N_2 fixation was not effective yet.

Mineral soil N depletion by all legumes was lower than that of the non-fixing control plants, wheat and oilseed rape. However, the amount of nitrate remaining in the soil after 35 DAG varied between legume species from a high of 5.07 and 7.94 mg kg^{-1} of dry soil for soybean and fenugreek, respectively (Fig. 1c) compared to a low of 3.46 and 1.10 mg kg^{-1} for wheat and oilseed rape, respectively (data not shown).

Relationships between seed traits, early growth and N acquisition – The seed, shoot and root traits, N accumulation, soil N uptake and remaining soil N of species grown without N supply were included in a principal component analysis (Fig. 2a). The first two PCs comprised 72% of the total variability. The first component (PC1, 55% of the variability) consisted mostly of the level of soil N

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Table 2. Shoot and root dry matter, root: shoot ratio and initial seed dry matter for nine legume species grown without N supply. Values are means (n = 4, except for fenugreek, where n = 3). CV: coefficient of variation. *P* values (analysis of variance ANOVA Type III, sum of squares, $\alpha = 0.05$). ***, **, *, indicate significant differences among species at $p < 0.001$, $p < 0.005$, $p < 0.01$, respectively. Means on the same line with different letters are significantly different ($P < 0.05$).

	Alfalfa	Fenugreek	Lentil	Common vetch	Soybean	Pea	Chickpea	Peanut	Faba bean	CV	*P* value
Shoot dry matter (Sdm) (g pl^{-1})	0.008 **d**	0.038 **c**	0.046 **c**	0.159 **b**	0.413 **a**	0.406 **a**	0.412 **a**	0.398 **a**	0.427 **a**	0.73	$1.02 \times 10^{-15***}$
Root dry matter (Rdm) (g pl^{-1})	0.007 **e**	0.024 **d**	0.028 **d**	0.065 **c**	0.219 **ab**	0.166 **b**	0.331 **a**	0.277 **ab**	0.292 **ab**	0.82	$2.04 \times 10^{-15***}$
Root:shoot ratio (R:S)	0.87 **a**	0.63 **bc**	0.62 **bc**	0.41 **c**	0.53 **bc**	0.40 **c**	0.80 **ab**	0.69 **ab**	0.68 **ab**	0.25	$8.00 \times 10^{-06***}$
Initial seed dry matter (isdm) (g)	0.002	0.012	0.024	0.051	0.177	0.273	0.364	0.407	0.539		

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Table 3. Root exploration and nodules establishment for nine legume species grown without N supply. Values are means (n = 4, except for fenugreek, where n = 3). CV: coefficient of variation. *P* values (analysis of variance ANOVA Type III, sum of squares, $\alpha = 0.05$). ***, **, *, indicate significant differences among species at $p < 0.001$, $p < 0.005$, $p < 0.01$, respectively. Means on the same line with different letters are significantly different ($P < 0.05$).

	Alfalfa	Fenugreek	Lentil	Common vetch	Soybean	Pea	Chickpea	Peanut	Faba bean	CV	*P* value
Root lateral expansion rate (Rlr) (mm day^{-1})	1.8 **d**	1.3 **d**	3.5 **cd**	5.2 **bcd**	8.5 **ab**	8.2 **abc**	10.4 **a**	10.1 **a**	6.9 **abc**	0.55	$6.59 \times 10^{-07***}$
Root depth penetration rate (Rdr) (mm day^{-1})	7.1 **c**	12.8 **bc**	8.2 **c**	21.0 **a**	22.6 **a**	18.6 **ab**	20.4 **ab**	16.5 **ab**	18.5 **ab**	0.35	$1.56 \times 10^{-06***}$
Nodule number (Nnb) (per plant)	1.5 **c**	4.3 **bc**	21.5 **ab**	60.5 **a**	8.3 **abc**	17.3 **ab**	4.3 **bc**	43.5 **a**	48.8 **a**	0.95	$2.07 \times 10^{-05***}$
Nodules dry matter (Ndm) (mg pl^{-1})	0.03 **b**	0.62 **b**	0.60 **b**	4.42 **b**	1.84 **b**	0.76 **b**	0.28 **b**	10.13 **ab**	17.71 **a**	1.50	$7.98 \times 10^{-05***}$

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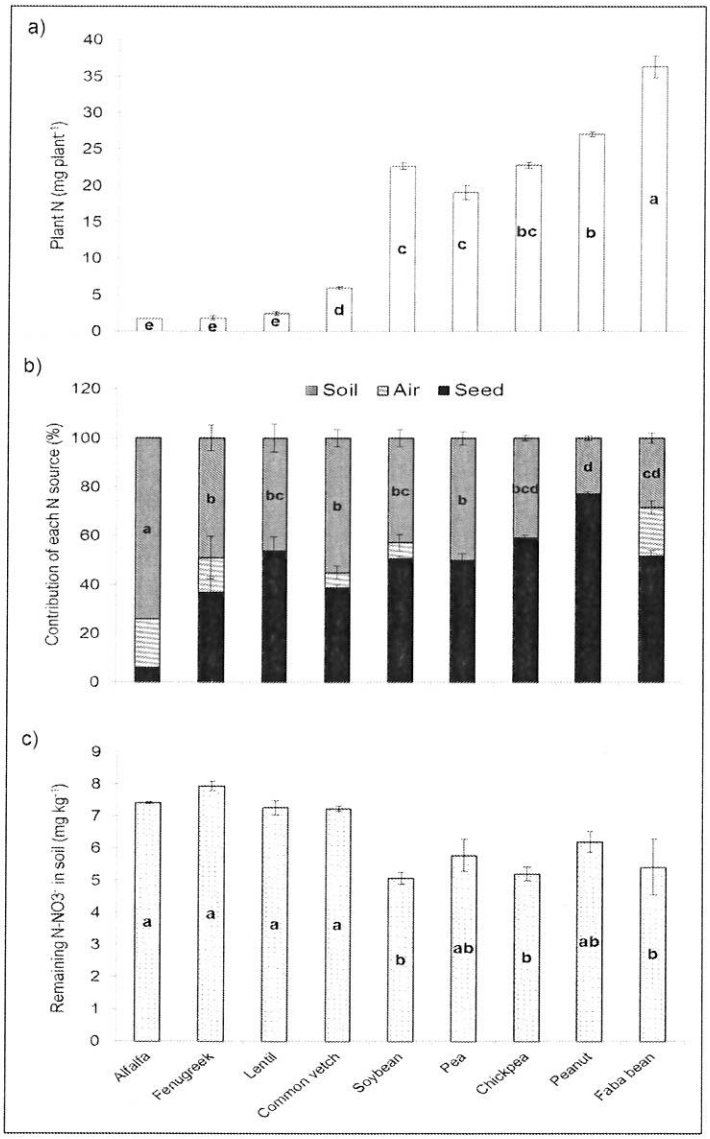

Fig. 1. Plant N (a), the percentage of N derived from seed, air and soil (b) in the total plant N and remaining N-NO3⁻ in soil (c) for nine legume species grown without N supply. Values are means (n = 4, except for fenugreek, where n = 3) ± SE (standard error). Means with different letters are significantly different ($P < 0.05$).

uptake in relation to the different shoot and root traits, while seventeen percent of the variation, accounted for by PC2, was mainly due to differences in N_2 fixation. The biomass of the plant, shoot, root, and initial seed were also correlated, and the remaining mineral soil N was negatively correlated with the plant biomass. The soil N uptake was also correlated to the lateral and vertical expansion, but not with the ability of a species to initiate biological N_2 fixation. The nodule growth and activity were higher for species having a low seed reserve depletion.

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Identification of groups of species – The PCA analysis separated plant species with a low or high soil N uptake ability (Fig. 2b). With the highest ability to deplete soil N combined with the highest biological N_2 fixation, faba bean shows a particular profile. Three relatively homogeneous groups of species were determined by hierarchical clustering analysis (Fig. 3a). The first cluster includes fenugreek, lentil, alfalfa and vetch, the four species characterized by the lowest soil N uptake, seed mass and shoot and root biomass and lateral root expansion. The second cluster included peanut, pea, chickpea, and soybean, four species characterized by a higher soil N uptake, shoot biomass and lateral root expansion. Faba bean was again separated from the others (Cluster 3), having a higher nodule biomass, a higher N_2 fixation and a lower seed reserve depletion than the other species.

3.2. Growth and N acquisition at a high mineral N level

In the rhizotrons supplied with mineral N (N treatment), a large variation among species was also observed in the growth of shoots and roots (Fig. 4). The total biomass did not differ between the two levels of mineral N ($p > 0.05$), except in lentil. However, the root:shoot ratio was significantly lower in the N treatment than in the 0N for faba bean (-32%), chickpea (-27%) and soybean (-23%). The N supply did not significantly change the vertical root penetration rate ($p > 0.05$). A higher N availability modified the lateral root expansion only in soybean by reducing it by 34%. With a mineral N supply, the N accumulation in plants increased on average by 37% compared to the 0N treatment, with a large contribution of soil N to total plant N. Nodule number and biomass were largely reduced by the N supply for all species. Nodules were less affected by the mineral N supply in soybean and faba bean than in alfalfa, chickpea and pea, which did not initiate any nodules. The remaining soil N at 35 DAG, in the N treatment, varied across legume species from approximately 25 to 31 mg kg^{-1} of dry soil, in the soybean and vetch treatments, respectively. The remaining soil N for soybean, peanut, chickpea, pea and faba bean were similar to those measured for wheat (24.4), and all legumes had a lower soil N depletion than that of oilseed rape (18.5).

A second principal component analysis (Fig. 5a) was performed to investigate whether the correlations between variables are modified with a higher soil N availability. The first two PCs comprised 79% of the total variability. The first component (PC1, 65% variation) consisted mostly of the level of soil N uptake in relation to the shoot and root biomass, initial seed mass and lateral root expansion, and PC2 (14% variation) accounted primarily for vertical root penetration, root: shoot ratio and number of nodules. Similar to the 0N treatment, in the N treatment, soil N uptake was mainly correlated with the total DM, shoot DM, root DM, initial seed mass and the lateral root expansion. The soil N uptake was less correlated to the lateral root expansion than to the depth root penetration. The lateral root

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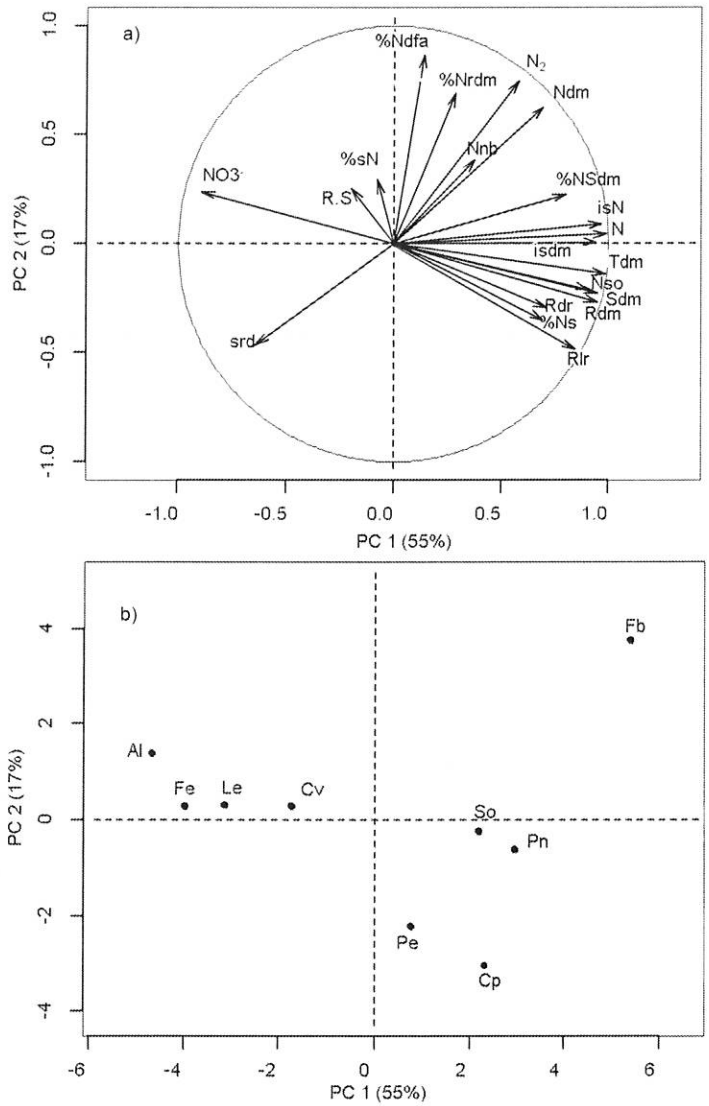

Fig. 2. (a) Factor loadings for the variables measured on nine legume species grown under low soil N availability, for the first two axes of the principal component (PC) analysis. The percentage of the total variance explained by the first two principal components is shown in parentheses. (b) Projections of the different species on the first two axes of the principal component analysis. Variables: Shoot dry matter (Sdm), root dry matter (Rdm), total dry matter (Tdm), root:shoot ratio (R.S), initial seed dry matter (isdm), initial seed N (isN), N content in seeds (%sN), seed reserve depletion (srd), root lateral expansion rate (Rlr), root depth penetration rate (Rdr), nodule number (Nnb), nodule dry matter (Ndm), plant N (N), percentage of plant N derived from seeds (%Ns), N_2 fixed (N_2), percentage of exogenous N derived from air (%Ndfa), soil N uptake (Nso), N content in shoot dry matter (%NSdm), N content in root dry matter (%Nrdm), remaining N-NO_3^- in soil (NO_3^-). Species: Alfalfa (Al), Fenugreek (Fe), Lentil (Le), Common vetch (Cv), Soybean (So), Pea (Pe), Chickpea (Cp), Peanut (Pn) and Faba bean (Fb).

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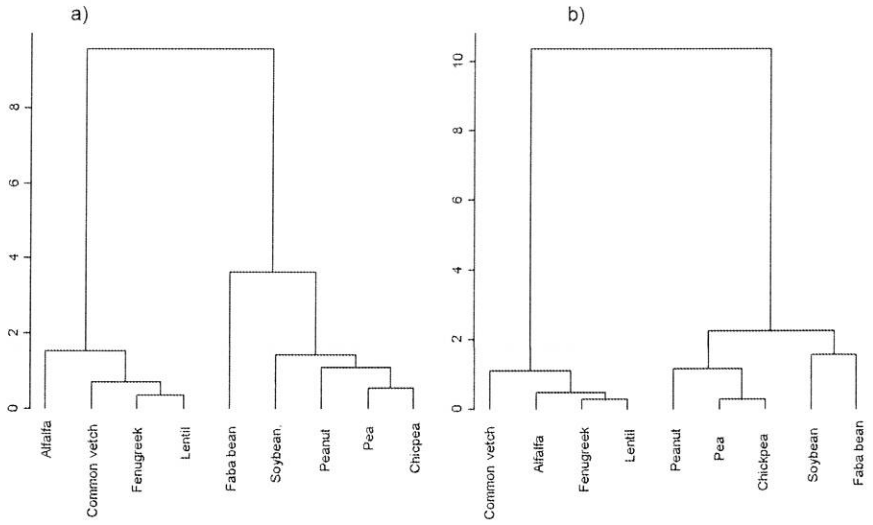

Fig. 3. Dendrograms of relationships among species, calculated using Ward's hierarchical clustering of species, which accounts for the distance matrix comparing all variables (used in PCA analysis) of each species grown under low N availability (a) or high soil N availability (b).

expansion and vertical root penetration were less correlated in the N treatment than in the 0N (Figs. 2a and 5a).

Under high soil N availability, the dispersion (Fig. 5b) and the classification of the species within the groups identified in 0N were slightly modified (Fig. 3a, b). The first cluster included the same species as in 0N (fenugreek, lentil, alfalfa, vetch) and corresponded to the legume species having low soil N uptake ability. The second cluster included three species, peanut, pea, and chickpea, was mainly characterized by a high lateral root expansion. In the N treatments, faba bean and soybean are grouped into a cluster, with nodule number and biomass higher than those in the other species combined with a small seed reserve depletion. Soybean was more similar to faba bean than to peanut, pea and chickpea in the N treatment, because of reduced lateral root expansion and a lower effect of N on nodule establishment than in other species.

4. Discussion

During the early growth observed in this experiment, the soil N uptake was low for all legume species compared to non-legumes due to the high dependency of the plant on the seed N and to low N demand at this stage. A large proportion of the nitrogen accumulated in the shoots and roots came from the seed (up to 77%). Large and N-rich legume seeds contain a substantial amount of N and are potentially able to sustain seedling growth for a significant part of the plant cycle (Herdina and Silsbury, 1990). This particular trait of legumes probably contributes to the late utilization of exogenous N (mineral soil N and N_2 from biological N_2

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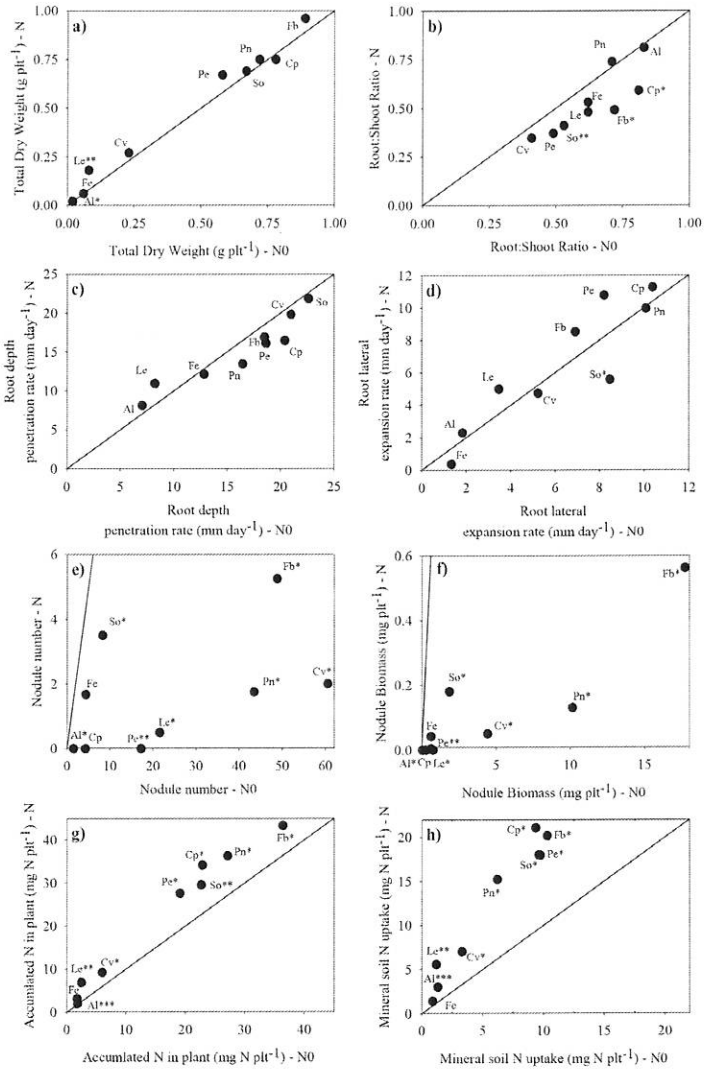

Fig. 4. Dry matter (a) and root:shoot ratio (b), root depth penetration (c) and lateral root expansion (d), nodule number (e) nodule dry matter (f), plant N (g), soil N uptake (h) with (N) and without N supply (0N) for nine legume species: Alfalfa (Al), Fenugreek (Fe), Lentil (Le), Common vetch (Cv), Soybean (So), Pea (Pe), Chickpea (Cp), Peanut (Pn) and Faba bean (Fb). ***, **, * indicate significant differences between the two levels of soil N availability at $p < 0.001$, $p < 0.005$, $p < 0.01$, respectively.

fixation) for growth compared to that of non-legume species. In our experiment, because of the high contribution of the seed N to the total plant N, the plants could satisfy their N demand during early growth even in the 0N treatment, where the mineral N availability was low. Consistently, we did not find any significant difference in the total plant N between the N and 0N treatments, except for lentil.

Although legume species showed a low soil N uptake ability, a large variability among them was observed for all investigated traits, with consequences on the remaining soil N, regardless of the level of soil N availability. In the early growth

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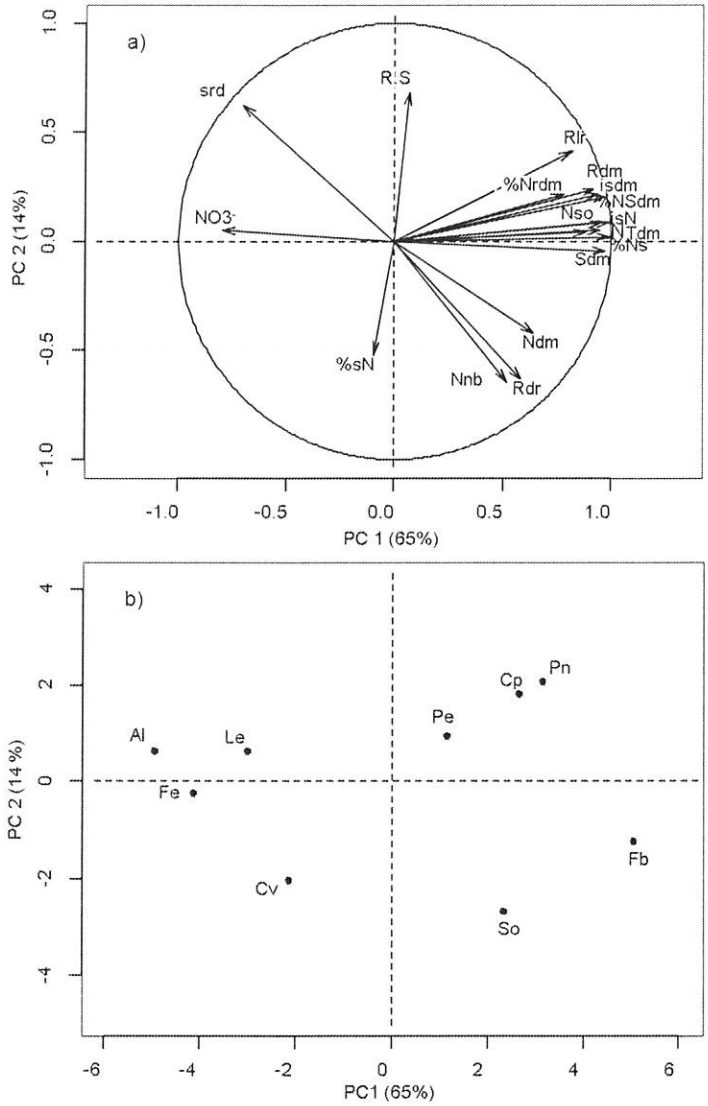

Fig. 5. (a) Factor loadings for the variables measured on nine legume species grown under high soil N availability for the first two axes of the principal component (PC) analysis. The percentage of the total variance explained by the first two principal components is shown in parentheses. (b) Projection of the different species on the first two axes of the principal component analysis. Variables: Shoot dry matter (Sdm), root dry matter (Rdm), total dry matter (Tdm), root:shoot ratio (R.S), initial seed dry matter (isdm), initial seed N (isN), N content in seeds (%sN), seed reserve depletion (srd), root lateral expansion rate (Rlr), root depth penetration rate (Rdr), nodule number (Nnb), nodule dry matter (Ndm), plant N (N), percentage of plant N derived from seeds (%Ns), Soil N uptake (Nso), N content in shoot dry matter (%NSdm), N content in root dry matter (%Nrdm), remaining N-NO_3^- in soil (NO_3^-). Species: Alfalfa (Al), Fenugreek (Fe), Lentil (Le), Common vetch (Cv), Soybean (So), Pea (Pe), Chickpea (Cp), Peanut (Pn) and Faba bean (Fb).

phase, consistent with Fayaud et al. (2014), large differences among species were correlated with the seed mass. The growth and N acquisition were better correlated with the initial seed mass than with the initial seed N. However, legumes with a seed mass greater than 0.17 g such as soybean, pea, chickpea, peanut and faba bean

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had similar shoot biomass but displayed contrasting values for the root traits. Legumes often have a slow root depth penetration compared to of non-legumes (Bellostas et al., 2003; Corre-Hellou et al., 2007; Thorup-Kristensen, 2001). We found large differences both in the depth and lateral root development among legume species. Furthermore soil N uptake was correlated with the root lateral expansion rate. In the early growth phase, the root lateral expansion appeared to be a more important trait than the root depth penetration for the soil N uptake. Branch roots may allow exploring the soil better than taproot during early stages. Moreover, in our experiment, the soil N availability did not modify the root depth penetration as observed in previous studies (Corre-Hellou and Crozat, 2005) but could modify the root lateral expansion. The root:shoot ratio tended to be reduced with a high soil N availability, as observed in other studies (Corre-Hellou and Crozat, 2005; Hamblin et al., 1990). This ratio was high and differed among species; however, in our study, it was not a key trait involved in the soil N uptake.

The nodule biomass per plant differed across species and was not correlated with the seed mass. Within each species, the relationships between the seed size and nodule biomass per plant have been observed in different lima bean (*Phaseolus lunatus* L.) cultivars (Dobert and Blevins, 1993), in some clover species (Evers, 1982), and in three varieties of soybean (*Glycine max* L.) (Smith and Ellis, 1980). Other authors have also demonstrated differences in the onset of nodule initiation among species due to the different distributions between above- and below-ground organs with hypogeal or epigeal emergence (Sprent and Thomas, 1984). The type of emergence did not explain the differences in nodulation and N_2 fixation during the early growth stage in our study. All species had reduced nodule establishment and N_2 fixation at high levels of soil N. However, faba bean and soybean were able to establish nodules even under high soil N availability. Other studies have demonstrated that faba bean maintains high levels of nitrogen fixation at high levels of soil N availability compared to other legumes which are more sensitive to available soil N (Harper and Gibson, 1984; Turpin et al., 2002).

We demonstrated that the ability to initiate a larger number of nodules early and to start N_2 fixation was not correlated with soil N uptake under the early growth stages. The root exploration (mainly lateral expansion) and early biomass in relation to the seed mass were the major traits relative to N uptake, whatever the level of soil N availability.

The cluster analysis classified the species into three groups. Peanut, pea, chickpea and soybean, with a rapid early shoot growth and a high lateral root expansion, had a better soil N uptake. These characteristics may result in a better competitive ability with neighbouring plants (crops and weeds) for soil N at the beginning of the crop's life cycle. Conversely, fenugreek, lentil, alfalfa, and vetch could be considered weakly competitive legumes for soil N due to their small seed mass,

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small plant biomass and low lateral root expansion. At the beginning of the crop cycle, they may leave a larger part of the available N for weeds or another associated crop. Among the species studied, faba bean had several unique traits. Faba bean used only 66% of the seed reserves in this early phase. Furthermore, it exhibited an ability to fix atmospheric nitrogen and simultaneously to take up soil nitrate at a much greater rate than the other species studied. Indeed, faba bean tended to benefit from different sources of nitrogen for its root and shoot growth. Hence, a strong competition against other associated species for both light and N may be predicted in faba bean in this early growth phase. The classification of species may differ according to the soil N availability depending on the specific response of each trait, but we observed only slight differences. Larger differences between species in response to the N availability might occur during the later stages.

Fenugreek and vetch are mainly used for forage or cover crops in the time period between two main crops or as living mulch within a main crop. Their slow establishment and low ability for soil N uptake at the beginning of the crop cycle may affect their yield when harvested and their ability to play expected roles such as the reduction of N leaching and increased cover allowing weed control. Whatever the type of use (forage or grain), legumes species can be intercropped with non-legume species to increase soil N uptake, the competitive ability of the cover against weeds and to reduce N losses during the beginning of the crop cycle and after (Corre-Hellou et al., 2011; Hiltbrunner et al., 2007; Hauggaard-Nielsen et al., 2009). Mixing legumes with different levels of competitive abilities can also be a relevant strategy (Mikić et al., 2015).

Cool-season legumes and warm-season legumes have rarely been compared under same growth conditions. Nevertheless, warm-season legumes are also relevant species for temperate cropping systems used as frost-sensitive cover crops in a mixture with other main crops such as wheat or oilseed rape.

Further experiments are required in field conditions to assess whether differences in the soil N uptake ability are also observed and maintained at later stages and to see how the shoot and root traits are influenced by various soil and climate conditions and interspecific competition. Such an analysis can be extended to identify differences across genotypes within a species. Some root traits may differ significantly across cultivars as shown, for example, for chickpea (Kashiwagi et al., 2006), lentil (Sarker et al., 2005) and lupin (Chen et al., 2012). The interspecific variability during the early stages documented here has identified traits involved in early N acquisition allowing for the classification of species among legumes with different strategies and competitiveness for N. These findings will help us to better understand and manage interspecific competition for soil N between legumes and other species (crops and weeds) at the beginning of the crop's life cycle.

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Declarations

Author contribution statement

Elana Dayoub: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Analyzed and interpreted the data; Wrote the paper.

Guenaelle Corre-Hellou, Christophe Naudin, Guillaume Piva: Conceived and designed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Steve Shirtliffe, Joelle Fustec: Conceived and designed the experiments; Contributed reagents, materials, analysis tools or data; Wrote the paper.

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Competing interest statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

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