Mycorrhizae differentially influence the transfer of nitrogen among associated plants and their competitive relationships

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Abstract

The formation of a common mycorrhizal network among roots of different plant species growing close to each other can influence plant community dynamics, regulating plant relationships through the differential transfer of nutrients from one plant to another. However, knowledge of the mechanisms that regulate this process is poor. Here we quantify the contribution of arbuscular mycorrhizae to the transfer of N among heterospecific plants growing adjacent to each other and examine whether the differential transfer of N within the plant community via mycorrhizae can alter competitive relationships among plant species. Plants of four species (wheat, pea, flax, and chicory) were grown in four-compartment pots (one species per compartment) under three conditions: no belowground interaction permitted among the compartments (Clo-sys); belowground interaction limited to soil microorganisms (including arbuscular mycorrhizal [AM] fungi) and soil solution (Res-sys); and belowground interaction permitted, so the crossing of roots, soil microorganisms, and soil solution was allowed (Ope-sys). Each condition was tested in both the absence (–myc) and presence (+myc) of AM symbiosis. The transfer of N from pea to the three non-legume companion species (assessed via direct ^{15}N labelling) was greater in Ope-sys than Res-sys for wheat and chicory but not for flax. In general, N transfer was greater in +myc than –myc in all species. In wheat the positive effects of AM symbiosis on N transfer were pronounced in Ope-sys but not in Res-sys, whereas in flax and chicory mycorrhization had similar effects in both Ope-sys and Res-sys. In Res-sys and in the absence of AM symbiosis, wheat intercepted about 50% of the total N transferred from pea, chicory about 40%, and flax about 10%. Mycorrhization altered these ratios, reducing the proportion of N transferred to wheat while increasing the proportion transferred to the other two species (especially to chicory), thus favouring the weakest components of the mixture. Similar effects of AM symbiosis were observed in Ope-sys. Our study shows that AM symbiosis affects

the distribution of N and as a consequence the competitive relationships among adjacent plants of different species.

Keywords

Arbuscular Mycorrhizal Symbiosis; Common Mycorrhizal Networks; Nitrogen Transfer; Plant-plant interactions; ^{15}N labelling.

1. Introduction

Most plants, including crop species, can establish symbiotic relationships with arbuscular mycorrhizal (AM) fungi. This symbiosis can significantly affect the growth and productivity of the plants (Lin et al., 2015); the uptake of nutrients (Saia et al., 2014b; Thirkell et al., 2016), in particular less mobile elements; and resistance to biotic (Pozo et al., 2010) and abiotic (Grümberg et al., 2015; Fileccia et al., 2017) stresses. The presence of AM fungi can also improve the soil aggregation (Rillig et al., 2015), promote C sequestration (Wang et al., 2016), and stimulate the growth and development of other microorganisms of the rhizosphere involved in the many biological processes essential for the plant (e.g., symbiotic N fixation; Püschel et al., 2017; Ingraffia et al., 2019) and for soil fertility (decomposition of organic matter, humification, nitrification, etc.; Veresoglou et al., 2012; Paterson et al., 2016; Bukovská et al., 2018).

High variability in plant growth response to the presence of different AM fungi has been observed: Some plants are highly dependent on mycorrhizae, whereas others perceive mycorrhizal fungi as antagonists (Francis and Read, 1995; Klironomos, 2003). Moreover, within a single plant species, interactions with AM fungi and their contribution to plant growth vary widely according to inter- and intraspecific variability in fungi (De Novais et al., 2014). In very fertile soils, the plant often does not gain any benefit, and its growth can even be compromised by the substantial demand for C by the fungi (Smith et al., 2009). Similarly, in poor soils, AM fungi may compete for nutrients with plants, often with negative effects on plant growth and yield (Püschel et al., 2016; Ingraffia et al., 2020). Therefore, the effects of mycorrhization vary by plant species as well as the environmental conditions (climate, soil type, etc.) in

which the plant grows and agronomic practices adopted (fertilisation strategy, tillage technique, rotation, etc.; Johnson et al., 2006; van der Heijden and Horton, 2009).

Most AM fungi are not host specific, and a single fungus can simultaneously colonise a large number of plants of the same species as well as of different species. Thus, AM fungi are capable of creating hyphal networks to connect the root systems of neighbouring plants in the field, and this can favour the exchange of nutrients, carbohydrates, and lipids among plants (He et al., 2003; Walder and van der Heijden, 2015). Such a network is called a *common mycorrhizal network* (CMN). CMNs can also influence the distribution of resources among associated species and consequently influence their competitive relationships, differentially affecting the different components of the plant community. Indeed, the cost–benefit ratio of being connected to a mycorrhizal network is not the same for all plant species, and different plant species benefit to different extents from the network; in some cases CMNs can mitigate the aggressiveness of dominant components, whereas in others they can exacerbate it or have no influence (Grime et al., 1987; van der Heijden et al., 1998; Hartnett and Wilson, 1999).

The role of mycorrhizal networks in regulating plant interaction and plant community dynamics is poorly understood (van der Heijden and Horton, 2009; Wipf et al., 2019). Some authors have stated that CMNs can have a profound effect on plant community (Selosse et al., 2006), whereas others believe that their role in plant-plant interaction is not qualitatively different from that of other microbes shared by coexisting plant species in nature (Bever et al., 2010). Among the mechanisms that can underlie the different responses, an important role can be attributed to differentiated nutrient transfer between the associated species. In fact, not all plants receive equal benefits from the mycorrhizal network (Scheublin et al., 2007). Some research has shown that the more a plant invests in the fungal network, the more benefits it receives (Bücking and Shachar-Hill, 2005; van der Heijden and Horton, 2009; Teste et al., 2010), whereas other authors have shown that small seedlings receive more advantages than larger plants (van der Heijden, 2004), even though the latter provide more carbohydrates for the fungus. Other research has shown that the benefits plants in a single community derive from a mycorrhizal network are not correlated with the amount of carbon they invest (Walder et al., 2012).

The presence of legumes increases N in the rhizosphere through different pathways: decomposition of thin roots and root nodules, the release of root exudates rich in soluble nitrogenous compounds, and an

increase in the activity of microbial communities involved in biochemical N cycling (De Deyn et al., 2008; Thilakarathna et al., 2016). According to Fustec et al. (2010), N rhizodeposited by various legume species as a percentage of the plant N vary from 7% to 57%. In a mixture, non-legume plants can intercept available N, as N released by legumes into the rhizosphere can reach other plants' roots in the growth medium (Paynel et al., 2001; Høgh-Jensen and Schjoerring, 2001; Høgh-Jensen, 2006). N transfer can be increased by AM extraradical mycelium absorption and translocation of soil N coming from the legumes. Furthermore, the AM fungi can promote direct transfer from legume plants to non-legume plants through the hyphal network, which connects the roots of nearby plants. Some researchers have shown that N transfer via a CMN from N_2-fixing mycorrhizal to non-N_2-fixing mycorrhizal plants is consistent (He et al., 2009; He et al., 2019), whereas others have shown that nutrient transfer via a CMN does not occur in significant quantities (Jakobsen and Hammer, 2015). Moreover, other studies have reported that N transfer via AM networks can be bidirectional and that the flow from non-N_2-fixing species to N_2-fixing species is generally low (Johansen and Jensen, 1996; He et al., 2003; Li et al., 2009). The amount of N transferred through mycorrhizal hyphae and the relative importance of the different pathways by which this process occurs vary greatly depending on soil characteristics, climatic conditions, plant and fungi taxa, the size and structure of the microbial community, and so on (He et al., 2003; He et al., 2019). These factors can affect the amount of N of the substrate that is potentially transferable (influencing plant growth; organic matter decomposition rates; and the size, diversity, and structure of the soil microbial community) as well as the extent of mycorrhization and activation of an efficient CMN. Therefore, it should not be surprising that the data in the literature are highly variable; further variability also results from the different methods used to quantify N transfer.

The present study, conducted on four species (pea, durum wheat, flax, and chicory) grown in a mixture, aimed to answer the following questions: 1) Does N transfer mediated by AM fungi from a legume to companion plants vary by species? 2) If so, which species are favoured by AM fungi? and 3) Can possible differences in N transfer alter competitive relationships among associated species? The plant species included in the experiment are all typically grown in the Mediterranean area and have similar growing seasons. Our intention was to create a plant community of species with different nutritional needs, root systems, and abilities to establish associative relationships with soil microorganisms

(Whiteley and Dexeter, 1982; Dalpé and Monreal, 2004; Frenda, personal observations). Although the four species we chose as model plants are never grown in mixture in practice, we believe that the diversity imposed by our selection can provide insight into the different responses of CMNs in mitigating (or exacerbating) the aggressiveness of some components of a plant community.

2. Materials and methods

2.1. Experimental design and plant management

The experiment was conducted outdoors in a wire house under a transparent plastic roof (pots were protected from the rain) with open sides at the Pietranera farm (S. Stefano Quisquina, AG, Italy; 37°53′ N, 13°51′ E; 162 m a.s.l.). Four-compartment plastic pots were used. In each compartment (which represents the experimental subunit) was grown one of the following four species: durum wheat (*Triticum durum* cv. Antalis), pea (*Pisum sativum* cv. Baccarà), flax (*Linum usitatissimum* cv. Sideral), or chicory (*Cichorium intybus* cv. Spadona). The experiment was arranged in a completely randomised design with six replications. The treatments were i) AM fungal inoculation, with plants grown in the presence (+myc) or absence (–myc) of AM fungi; and ii) belowground interaction, with plants grown under the following three different conditions:

- **Closed system (Clo-sys)**: no belowground interaction; plastic partition walls prevented any interaction among belowground environments (crossing of roots, passage of AM fungal mycelia or other microorganisms or soil solution).
- **Restricted system (Res-sys)**: interaction limited; a septum consisting of nylon fabric with 16 µm mesh prevented the crossing of roots but permitted the passage of AM extraradical mycelia, soil microorganisms, and soil solution.
- **Open system (Ope-sys)**: maximum interaction; no partition walls were present between compartments, so crossing of roots, soil solution, and soil microorganisms (including AM fungi) was allowed between compartments.

Hence, a total of 36 pots (144 subunits) were used (2 levels of AM fungal inoculation × 3 levels of belowground interaction × 6 replications). In fact, a total of 60 pots (240 subunits) were prepared, as 24

more pots (96 subunits) were included in the trial to assess N transfer from the legume (pea) to the companion species and the relative contribution of AM fungi (see subsection 2.2). The four-compartment pots were constructed by joining together foursquare pots (8 cm side, 25 cm height, 1.6 L capacity). In the Clo-sys condition, the adjacent walls of each pot were left intact. In the other two conditions, either an 18 × 5 cm slit was drilled in the adjacent walls and then a septum of nylon fabric with 16 µm mesh (Intertessile srl, Appiano Gentile, Como, Italy) was applied (Res-sys) or an 18 × 5 cm slit was drilled without any septum (Ope-sys; Fig. S1).

Each pot was filled with 2.0 kg of a mixture composed of silica sand (Gras Calce srl, Trezzo sull'Adda, Italy), whose N concentration (Kjeldahl) and P (Olsen) were 0.11 g kg^{-1} and 7.44 mg kg^{-1}, respectively, and agricultural soil (sand:soil ratio of 70:30 by weight). The soil was collected from the first 30 cm of a well-structured clay soil classified as Vertic Haploxerept and having the following characteristics: 267 g kg^{-1} clay, 247 g kg^{-1} silt, and 486 g kg^{-1} sand; pH 8.0; 10.8 g kg^{-1} total C (Walkley-Black); 0.86 g kg^{-1} total N (Kjeldahl); 40.1 mg kg^{-1} available P (Olsen P); 598 mg kg^{-1} total P; 26 cmol kg^{-1} cation exchange capacity; 1.70 dS m^{-1} electrical conductivity (saturated paste at 25°C). Given the poor nutrient content of the substrate, fertilisation was performed soon after the emergence of seedlings. We added to each compartment of each pot 200 mL of an N-free nutrient solution with the following composition: 15 mg L^{-1} P, 48 mg L^{-1} K, 350 mg L^{-1} Ca, 10 mg L^{-1} Mg, 134 mg L^{-1} S, 0.3 mg L^{-1} B, 2.5 mg L^{-1} Fe, 1.0 mg L^{-1} Mn, 0.5 mg L^{-1} Zn, 0.2 mg L^{-1} Cu, and 0.097 mg L^{-1} Mo. The pH of the solution was 6–6.5, and the electrical conductivity was 2–2.2 dS m^{-1}.

The substrate was sterilised as follows: humidification, 24 h at room temperature and 24 h at 130°C, for a total of three cycles. Furthermore, to avoid contamination, all pots and seeds were sterilised by immersion in a solution of sodium hypochlorite 2.5% for 4 min. Before sterilisation, we extracted the natural soil microflora by suspending the soil in distilled water in a 1:4 w:w ratio, stirring for 20 min (140 oscillations per minute), and then filtering with filter paper (16 µm mesh) to remove the spores of AM fungi present in the native microbial community of the soil.

Sowing was performed on January 29, 2019. Three seeds of each species were distributed in the centre of each single compartment (subunit). This design meant that each species had two species on either side and one opposite; this could have had possible repercussions for root system interaction, when

allowed, between the two opposite species. To limit this effect, we alternated the arrangement of the species in the pots in the six replicates of each treatment to replicate twice all three possible combinations (wheat opposite pea, chicory opposite pea, flax opposite pea). Ten days after emergence, thinning was performed, which left one plant of each species per experimental subunit.

In the +myc treatments, inoculation of the AM fungi was performed with the addition of 6 g per experimental unit (1.5 g for each experimental subunit) of a commercial inoculum (AEGIS IRRIGA, Italpollina SpA, Rivoli Veronese, Italy) consisting of a mix of two AM fungi species, *Rhizophagus irregularis* and *Funneliformis mosseae*, equally present at a density of 700 spores g^{-1}. This commercial inoculum also had 1×10^7 rhizosphere bacteria. To isolate the effects of the AM fungi, we extracted the bacterial community of the inoculum using the same protocol used for the natural soil microbial community reported above; the bacterial community of the inoculum was added to the –myc treatments at sowing time. The inoculation was performed in two stages: two thirds (1 g for each subunit) was added during pot filling by mixing with the substrate, and the remaining one third (0.5 g for each experimental subunit) was added at sowing close to the seeds. Furthermore, immediately after sowing, the native microbial soil community, extracted as described previously, was reintroduced into all pots (both +myc and –myc) with the addition of 80 mL per pot of soil filtrate (20 mL for each subunit).

The temperature regime during the experimental period is shown in Fig. S2. The plants were kept in optimal conditions in terms of water supply throughout the trial; watering was pot specific. When the soil water content reached approximately 60% of the available substrate water capacity, a volume of tap water (0.58 dS m^{-1} electrical conductivity at 25°C) was added sufficient to bring the substrate back to field capacity. We determined the available substrate water capacity (moisture content between –33 and –1500 kPa) at the beginning of the experiment using the pressure-plate extractor method (Richards, 1941). To determine when to irrigate, we weighed all pots every 2 days. Variation in weight was attributed to evapotranspiration.

2.2. ^{15}N labelling of the legume plant

The two treatments in which belowground interaction was allowed (Res-sys and Ope-sys) were duplicated, that is, 24 more pots (96 subunits), resulting from 2 levels of AM fungal inoculation × 2

levels of belowground interaction × 6 replications, were included in the trial. In these pots, the legume (pea) was labelled with the isotope ^{15}N so we could quantify possible N transfer from the legume to the companion species and the contribution of AM fungi to this process. We labelled the legume using a modified leaf immersion method (McNeill et al., 1997; de Graaff et al., 2007) using the pea tendril instead of the leaf (Lam et al., 2013). ^{15}N from both tendrils and leaves reaches the roots (McNeill et al., 1997), and therefore the method is suitable for estimating rhizodeposition of N and its cycling through various pools, as shown by Merbach et al. (2000) and Hertenberger and Wanek (2004). Two tendrils were cut at the tip (1–2 mm), and each of them was immersed in a single 2 mL Eppendorf tube containing 1 mL ammonium nitrate solution labelled with ^{15}N (0.7124 g labelled NH_4NO_3 at 98% in ^{15}N in 100 mL distilled water; Sigma-Aldrich) for 72 h, during which the tube was covered with a layer of Parafilm to minimise evaporation. In addition, to monitor evaporation loss, we arranged four control Eppendorf tubes (identical to those used for the ^{15}N labelling but containing only 1 mL distilled water) among the experimental units. It can be assumed that the evaporation loss observed in the control Eppendorf tubes was equal to that of the tubes containing the ^{15}N-labelled solution. The control tubes were weighed before and after the ^{15}N labelling treatment, and no appreciable differences were found between the two weighings. The labelling was done in two stages: at 76 days after sowing (DAS) using the tendrils of the fourth or fifth nodes and at 92 DAS using the tendrils of the 11th or 12th nodes.

2.3. Biomass harvesting and analyses

The experiment ended when the wheat plants reached the heading stage (105 DAS), at which point we collected above- and belowground biomass from each subunit (species). We harvested aboveground biomass by cutting the plants at the ground level and recorded the fresh and dry weight. To extract the roots, we carefully cut the pots vertically and removed the substrate with abundant washing in water; in Ope-sys, the roots of the different species were carefully separated. Then they were oven-dried at 40°C until a constant weight and the dry weight was recorded. We extracted a root subsample of each plant species, cleared it with 10% KOH, stained it with 0.05% trypan blue using the Phillips and Hayman method (Phillips and Hayman, 1970), and used it to quantify the percentage of AM fungi infection using the method developed by McGonigle et al. (1990).

Both above- and belowground biomass was ground to a fine powder, gathered in a single sample (mixing 30% of the total shoot weight and 30% of the total root weight), and analysed for the concentration of total N with the Dumas method (DuMaster D-480; Büchi Labortechnik, Flawil, Switzerland). Samples of treatments in which ^{15}N was applied were analysed for the concentration of the ^{15}N isotope with an elemental analyser (NA1500; Carlo Erba, Milan, Italy) paired with a mass spectrophotometer (Isoprime, GV, Cheadle, UK). Data obtained from the ^{15}N-enriched pots were used only to calculate N transfer and all related parameters.

2.4. Calculations and statistical analyses

We obtained N uptake by multiplying the total biomass by its N concentration. Data on above- and belowground biomass were used to calculate the competitive ability of each plant species in the two belowground interaction treatments (Res-sys and Ope-sys) as follows:

$$PS_{ca}\% = \frac{PS_{BI} - PS_{Clo-sys}}{PS_{Clo-sys}} \times 100,$$

where PS_{ca} (%) is the competitive ability of the plant species, PS_{BI} is the above- or belowground biomass of the plant when grown in one of the two belowground interaction treatments (Res-sys and Ope-sys), and $PS_{Clo-sys}$ is the above- or belowground biomass of the plant when grown in the absence of belowground interaction (Clo-sys).

We obtained N transfer using the yield-independent method proposed by Chalk and Smith (1997):

$$P_{non-leg(\leftarrow leg)} = \frac{E_{non-leg}}{E_{leg}},$$

where $P_{non\text{-}leg(\leftarrow leg)}$ represents the proportion of N of each non-legume species deriving from the legume, $E_{non\text{-}leg}$ represents the isotopic enrichment of the biomass of each non-legume species, and E_{leg} represents the isotopic enrichment of the biomass of the legume species. The amount of N derived from pea found in each of the non-legume species ($N_{non\text{-}leg(\leftarrow leg)}$) was obtained as follows:

$$N_{non-leg(\leftarrow leg)} = P_{non-leg(\leftarrow leg)} \times N_{upt-non-leg},$$

where $N_{upt\text{-}non\text{-}leg}$ is the N uptake of each non-legume species. The total amount of N transferred from pea to the other non-legume plants ($N_{leg(\rightarrow non\text{-}leg)}$) was obtained as the sum of each $N_{non\text{-}leg(\leftarrow leg)}$. Finally, we

obtained the proportion distribution of N transferred from the legume to the three non-legume species ($P_{N\text{-}received}$) as follows:

$$P_{N-received} = \frac{N_{non-leg(\leftarrow leg)}}{N_{leg(\rightarrow non-leg)}} \times 100.$$

We created four subsets of data (one for each plant species) to investigate the effects of the applied treatments within each plant species. Each subset of data was subjected to a two-way factorial analysis of variance (ANOVA) with belowground interaction treatment, AM fungal inoculation, and their interaction as explanatory variables. The analyses were performed with R 3.6.0 (R Core Team, 2019). Also, to eliminate variability attributable to the opposite species in the arrangement of the four plant species within each pot, we included this parameter as a covariate in the model. We assessed the normality of the distribution of the model residues using the Shapiro test, and we assessed their homoscedasticity using the Bartlett test. When the ANOVA assumptions were not respected, the data were transformed accordingly. Following the ANOVA, we used pairwise comparisons using the emmeans package (Lenth, 2019) to investigate the effects of mycorrhization within each belowground interaction treatment. All p values derived from pairwise comparisons are reported in figures to avoid the problem of p value dichotomous cutoffs (Wasserstein and Lazar, 2016; Betensky, 2019; Wasserstein et al., 2019). Using the same approach, we investigated the effects of the two factors (belowground interaction, AM fungal inoculation, and belowground interaction × AM fungal inoculation) on the overall performance of the four species mixture using data obtained from the entire experimental unit in each belowground interaction treatment.

AM fungi root colonisation was assayed in all pots; however, we performed ANOVA on this trait using only the subset of inoculated treatments (+myc) and using belowground interaction and plant species and their interaction as explanatory variables. Moreover, for this trait only the value observed in the +myc treatments is reported graphically. We decided to use this approach because we observed negligible root colonisation in the non-inoculated treatments (–myc) and we wanted to achieve more accurate comparisons of belowground interaction treatments and of plant species. Untransformed data are reported in figures. The tidyverse package (Wickham, 2017) was used to represent the data graphically.

3. Results

3.1. AM root colonisation and plant growth

In the +myc treatments, root colonisation varied among the four species (Fig. S3). The highest values were observed in pea and chicory (42.7% and 40.8%, respectively), with no appreciable differences between the treatments. In flax and wheat, the percentage of roots colonised by AM fungi was 32% on average, but lower values were observed in the treatment in which belowground interaction was precluded (Clo-sys).

Aboveground growth was influenced markedly by treatment (Table 1 and Fig. 1). In particular, wheat showed increased growth as the belowground interaction between species increased, ranging from 3.1 g pot^{-1} in Clo-sys, to 5.1 g pot^{-1} in Res-sys, to 6.9 g pot^{-1} in Ope-sys. In contrast, for the other three species (pea, flax, and chicory), an increase in belowground interaction was met by a decrease in aboveground biomass produced (Clo-sys < Res-sys < Ope-sys). On average, the presence of AM fungi resulted in a reduction in the aboveground growth of wheat; however, the differences between the –myc and +myc treatments increased with the increase in interaction, as the p values of the individual contrasts within each treatment (Fig. 1). The opposite behaviour was observed in the pea plants, as mycorrhizal symbiosis resulted in a marked increase in aboveground biomass ($P < 0.001$); in this case, moreover, the differences decreased as interaction increased. The presence of AM fungi resulted in less consistent effects in flax and chicory, with marginal advantages only in Res-sys for flax and in Ope-sys for chicory. Very similar trends were observed for belowground biomass for each of the four species as influenced by the root interaction treatment and AM fungi inoculation (Fig. 2 and Table 1).

On average, wheat plants showed a much greater competitive ability than the associated species in terms of both above- and belowground biomass production (Fig. 3); this ability was even greater when the plants were grown with maximum belowground interaction (Ope-sys). The other three species were dominated by wheat, as shown by the almost always negative values for this index. However, competitive ability was on average higher in pea than in flax and chicory and usually decreased with greater belowground interaction (Res-sys < Ope-sys). The AM fungi inoculum had null or negative effects on wheat in general. The presence of AM fungi led to consistent improvement in the ability to compete for resources among pea plants, but only for belowground biomass production. The AM fungi

inoculum increased the competitive ability of chicory for both above- and belowground biomass production (but not for the latter in Ope-sys), whereas positive effects were observed in flax for aboveground biomass only.

Above- and belowground total biomass production of the four-species mixture increased with the increase in belowground interaction among the plants (Ope-sys > Res-sys > Clo-sys; Fig. 4 and Table 1). Overall, AM fungi did not have any appreciable effects on the total production of either above- or belowground biomass. AM symbiosis resulted in an increase in overall N uptake in Res-sys only.

3.2. N uptake and N transfer

The N concentration of the biomass in wheat and flax increased progressively with an increase in belowground interaction among species (in particular flax), whereas no differences were observed in pea and chicory (Fig. S4 and Table 1). The presence of AM fungi led to marked increases in the biomass N concentration in flax in all belowground interaction systems. A positive effect of mycorrhizal symbiosis was observed in pea only in Res-sys. Finally, in wheat and chicory, no difference was found in N concentration due to mycorrhizal symbiosis in any belowground interaction system.

The increase in interaction between associated plants resulted in a large increase in total N uptake in wheat (from 31.6 to 87.9 mg pot^{-1}), a decrease in chicory (from 47.9 to 20.2 mg pot^{-1}), and small or no differences in pea and flax (Fig. 5). Mycorrhizal symbiosis led to large increases in N uptake in the legume in all treatments, whereas in chicory and flax increases were observed only in Res-sys; finally, in wheat, N uptake decreased in the presence of AM fungi in Ope-sys.

In the absence of mycorrhization, N transfer from pea to the three non-legume species (as a proportion of the total N of each species) was greater when maximum belowground interaction between the species was allowed (Ope-sys) compared to no interconnections between roots (Res-sys) for wheat and chicory but not for flax (Fig. 6). On average, the presence of AM fungi resulted in an increase in N transfer, but the three species reacted differently when belowground interaction varied. In wheat AM fungi inoculation produced much greater positive effects in Ope-sys than in Res-sys, whereas in flax and chicory the effects were similar for both treatments.

On average, the amount of N transferred from pea to the associated species (calculated from the isotope enrichment values detected in the phytomass of the legume and associated species; Table S1) increased as belowground interaction increased (Ope-sys > Res-sys). Overall, the presence of mycorrhizae greatly increased N transfer and markedly influenced the distribution of the N transferred among each of the companion plants (Fig. 7). In Res-sys in the absence of inoculation, wheat received about 50% of the total N transferred from the legume, chicory received about 40%, and flax received less than 10%. The presence of mycorrhizae altered these ratios, reducing in percentage terms the contribution to wheat and increasing that of the other two species. In the treatment in which maximum belowground interaction among the four species was allowed (Ope-sys) and in the absence of inoculation, wheat was the greatest beneficiary of N transferred from pea (more than 81%), whereas about 16% and 2% of the total N transferred went to chicory and flax, respectively. Also, in this case, the presence of AM fungi favoured the weaker components (chicory and flax), even if, unlike what was observed in Res-sys, the differences between +myc and –myc treatments were rather small.

4. Discussion

Mycorrhization had diverse effects on N uptake and plant growth both above- and belowground in each species. On average, mycorrhizal symbiosis favoured growth and N accumulation in pea and (less so) in flax and chicory, adversely affected growth in wheat. It appears that the cost-to-benefit ratio of AM symbiosis varied depending on the plant species (Walder et al., 2012, 2015). It is interesting to note that pea, the species in which mycorrhizal symbiosis conferred the most robust advantages, was also the species in which colonisation was the greatest; in contrast, wheat, a species disadvantaged by mycorrhizal symbiosis, was the least colonised by AM fungi. To understand these results, remember that in this experiment the substrate was extremely poor in N but had adequate availability of other nutrients. It is possible that under such conditions, given the high N demand of both wheat and the AM fungi, there was marked competition for the element. This confirms the results obtained by Ingraffia et al. (2020), who found that in the absence of N fertilisation, mycorrhization in wheat resulted in reduced plant growth, biomass N concentration, and N uptake (which shows how AM fungi strongly compete for scarce available N), whereas small benefits were sometimes observed when N availability increased.

In pea, N was not a limiting factor thanks to its ability to activate symbiotic relationships with specific rhizobia capable of fixing N_2; for this species, the benefits of mycorrhizal symbiosis are probably due to its positive effects on the process of symbiotic fixation, as evidenced by Goss and de Varennes (2002), Antunes et al. (2006), and Saia et al. (2014a). In most cases, the increase in N fixation due to symbiosis with AM fungi seems to be the result of greater absorption of P and other essential micronutrients, with positive effects on growth and yield and with a consequent increase in N demand (Barea et al., 1989; Azcón-Aguilar and Barea, 1992). Other studies have highlighted how differences in N fixation between mycorrhizal and non-mycorrhizal plants disappear if the plants are grown in a substrate adequately supplied with readily available P (Morgan et al., 2005; Püschel et al., 2017). However, our research was conducted in a substrate deficient in N but sufficiently supplied with other nutrients, and thus we believe that other factors may have caused the increase in N fixation in pea as highlighted previously in other species (Saia et al., 2014a); this certainly deserves further study. As far as flax, mycorrhization conferred a slight advantage on the species. In this case, N was certainly not a limiting factor as the plant, having had a reduced growth, had extremely low N requirements that were certainly satisfied by the availability of N in the substrate (N removals of flax were less than 1/10 compared to those of wheat grown under the same conditions). The results for chicory are more difficult to interpret. This species, in the absence of belowground interaction among crops, was able to exploit a remarkable amount of N from the substrate (even greater than that taken up by wheat). Under these conditions, given the scarce availability of the element in the substrate, there should have been competition between the plant and AM fungi for N, as we found for wheat; however, this was not observed. Thus, we stress the need to conduct new research to understand the modalities and response mechanisms of this species to mycorrhizal symbiosis. Wheat was the most competitive species, as evidenced by the increases in yield and N uptake observed as interaction among belowground systems increased. The strong effort exerted by wheat resulted in progressive reductions in yield and N uptake in the other crops, especially flax and chicory. In particular, in the absence of mycorrhization, an increase in biomass of 125% in wheat and a decrease in biomass of more than 65% in chicory were observed in the treatment in which interaction between belowground systems of associated species was possible (open system) compared to the treatment in which belowground interaction was precluded (closed system). In the absence of mycorrhization, a

decrease in biomass, both above- and belowground, was observed in the restricted compared to closed system in flax and particularly in chicory; in contrast, an increase in biomass was observed in the restricted compared to closed system in wheat. This suggests that in the restricted system the high nutrient and water uptake from wheat activated the diffusion of water and solutes from neighbouring compartments toward the cereal, favouring its growth and reducing the availability of resources for nearby plants and penalizing their growth.

The use of the ^{15}N isotope permitted us to ascertain the existence of N transfer from the legume to the companion species, and this was also possible thanks to the high isotope enrichment achieved in the leguminous species through direct ^{15}N labelling via tendrils. However, the amounts of N transfer observed were low overall compared to those found in other studies (Xiao et al., 2004; Meng et al., 2015). Our results may have been affected to some extent by the relatively short period of growth and the short interval between ^{15}N supply (labelling) and the end of the experiment (cutting); both of these factors certainly limited the possibility of transfer. In the restricted system, in which direct interaction between the root systems of the associated species was prohibited, the amount of N transferred from the legume in the absence of mycorrhizal symbiosis was just 0.18 mg. This confirms the existence of a diffusive flux of N from the legume pot (in which there was a higher concentration of N due in particular to root exudate) to the non-legume pots, in particular wheat, which was able to use more water and nutrients than the other species, thus determining the conditions for a more highly diffusive flux toward its compartment. In the treatment in which the maximum belowground interaction between species was allowed, the amount of N transferred from pea to the other species was 0.59 mg. The increases observed in the restricted system were evident in wheat but were almost nil in flax and chicory; clearly, the advantage to the latter two species of exploring the soil compartment of pea with their root systems was counterbalanced by the negative effects of the high competitiveness of the wheat, which had the same possibility. In the presence of AM fungi, the amount of N transfer increased to 0.31 mg in the restricted system (compared to 0.18 observed in –myc treatment). This indicates the important role of AM by extraradical mycelium absorption and translocation of soil N coming from the legume. In the open system, the presence of mycorrhizae resulted in a substantial increase in transferred N (0.92 mg). Therefore, the results of this experiment show how the amount of N transferred to non-fixing species is

420 determined predominantly by the N released from the legume into the substrate (through root exudates
421 and the mineralisation of senescent roots, even if the latter probably contributes little considering the
422 short interval between leaf labelling and measurement). Furthermore, mycorrhizal symbiosis positively
423 influences this process, which confirms the findings of Li et al. (2009) and Ingraffia et al. (2019), who
424 observed an increase in N transfer from bean to intercropped cereal due to the presence of the hyphal
425 linkage. AM fungi may have favoured N transfer to the non-fixing species by 1) creating the conditions
426 necessary for direct transfer (i.e., interconnecting the root system of the legume to those of the associated
427 species), 2) intercepting efficiently the N released by legume root exudates, and 3) increasing the amount
428 of N released by the root exudates of the legume (He et al., 2003) as well as the mineralisation of its
429 senescent roots through their direct interaction with soil microorganisms (Veresoglou et al., 2012;
430 Hodge and Storer, 2015; Bukovská et al., 2018). Unfortunately, our data do not allow us to establish the
431 contributions of the different modes of N transfer from pea to associated species but only allow us to
432 quantify the total.
433 The mycorrhizae appeared capable of altering the competitive relationships between associated species,
434 reducing both the above- and belowground biomass of the more aggressive species (wheat) and
435 favouring, even if a little, the weaker species (flax and chicory). Mycorrhizal symbiosis can help reduce
436 the overlaps in ecological niches between associated species, thus reducing interspecific competition
437 and increasing complementarity between associated plants, with positive effects on the productivity of
438 plant communities. AM fungi are also able to reduce disparities in competitive capacity between
439 dominant and subordinate species (Mariotte et al., 2013; Urcelay and Diaz, 2003; Wagg et al., 2011).
440 Asymmetry in terms of trade in a CMN involving flax was observed by Walder et al. (2012), who found
441 that although flax invested little C in the symbiosis, it gained more nutrients than a companion species
442 (sorghum) that transferred a massive amount of C in the CMN. However, AM fungi can also favour
443 dominant components, as the largest plants can provide the most C to their associated AM fungi (Merrild
444 et al., 2013) and obtain the most nutrients from CMNs (Kiers et al., 2011; Weremijewicz and Janos,
445 2013). Hartnett and Wilson (1999) found that in a plant association the dominant C_4 plants obtained the
446 maximum benefit from AM fungi, thereby reducing the diversity of the plant community; similar results
447 were obtained by Connell and Lowman (1989) in research conducted on tropical rainforests. Ingraffia

448 et al. (2019), investigating a wheat–faba bean association, also found an advantage of mycorrhizal
449 symbiosis for the stronger competitor in the mixture (wheat).
450 The mechanisms by which AM fungi alter the competitive relationships between associated plants are
451 not yet clear. According to Watkinson and Freckleton (1997), the effects of mycorrhizal symbiosis on
452 competitive relationships between associated plants vary by plants' dependence on mycorrhizal
453 symbiosis; according to this hypothesis, the largest benefits are obtained by plants whose growth
454 depends to a greater extent (or in full) on the establishment of an efficient symbiotic relationship with
455 AM fungi. In this study, the species in which an increase in competitive ability was observed due to
456 mycorrhizal symbiosis were those in which a greater percentage of mycorrhized roots was observed
457 (pea and in particular chicory). It is likely that many other factors drive the nutrient dynamic in CMNs
458 and the effects of mycorrhizal symbiosis on the growth of associated components. Among these we must
459 surely include characteristics of the substrate itself, the availability of nutrients, differences in AM
460 fungal species, the diversity of the plant community, partners' sink strength, and density ratios; all of
461 these factors generate different effects of mycorrhizal symbiosis on interspecific interaction and
462 consequently on the plant community (van der Heijden and Horton, 2009; Walder and van der Heijden,
463 2015).
464
465 **5. Conclusions**
466 In this research the advantage conferred by mycorrhizal symbiosis on the weaker components (chicory
467 and flax) seems in some way attributable to the transfer of N (an element that certainly limited the
468 growth of the non-fixing species) to the associated components; in fact, using the ^{15}N isotope as a tracer
469 allowed us to ascertain how the recipients of the N transferred from the legume to the associated
470 components changed because of the presence of the AM fungi. Our data show that N transfer increased
471 to a greater extent in plants in which the percentage of mycorrhizal colonisation was higher (chicory).
472 This is particularly evident for the treatment in which belowground interaction was allowed only through
473 fungal hyphae but not for the treatment in which the roots of the plants were left free to fully colonise
474 the substrate. This suggests that the effects of mycorrhizae on N transfer occur predominantly through
475 the interception by AM fungi of the element released by legume root exudates and the mineralisation of

its senescent roots and root nodules; when it was possible for the wheat roots to interact intimately with the legume roots, the effects of mycorrhization on the differentiated transfer of N were drastically diminished. Overall, our research shows that AM fungi can affect the distribution of N and consequently the competitive relationship between neighbouring plant species. Given that a wide range of crops establish symbiotic relationships with AM fungi, mechanistic understanding of the nutrient dynamics in CMNs is essential for maximising both nutrient acquisition and biomass yield and developing sustainable strategies for intercropping systems.

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Figure captions

Fig. 1. Aboveground biomass of the four species at varying levels of belowground interaction (Clo-sys, no belowground interaction; Res-sys, interaction precluded to roots but not to arbuscular mycorrhizal fungi and soil microorganisms; Ope-sys, maximum belowground interaction) in the absence (–myc; grey plots) or presence (+myc; coloured plots) of AM fungal inoculum. Circles inside plots represent means, with whiskers representing ± SE (n = 6). The width of the plot shows the density distribution of values. P values for pairwise comparisons between +myc and –myc within the same belowground interaction treatment are reported above the plots.

Fig. 2. Belowground biomass of the four species at varying levels of belowground interaction (Clo-sys, no belowground interaction; Res-sys, interaction precluded to roots but not to arbuscular mycorrhizal fungi and soil microorganisms; Ope-sys, maximum belowground interaction) in the absence (–myc; grey plots) or presence (+myc; coloured plots) of AM fungal inoculum. Circles inside plots represent means, with whiskers representing ± SE (n = 6). The width of the plot shows the density distribution of values. P values for pairwise comparisons between +myc and –myc within the same belowground interaction treatment are reported above the plots.

Fig. 3. Competitive ability at varying levels of belowground interaction in the absence (–myc; grey plots) or presence (+myc; coloured plots) of arbuscular mycorrhizal (AM) fungal inoculum. Upper graphs: competitive ability measured on aboveground biomass. Lower graphs: competitive ability measured on belowground biomass. Res-sys, interaction precluded to roots but not to arbuscular mycorrhizal fungi and soil microorganisms; Ope-sys, maximum belowground interaction. Circles inside plots represent means, with whiskers representing ± SE (n = 6). The width of the plot shows the density distribution of values. P values for pairwise comparisons between +myc and –myc within the same belowground interaction treatment are reported above the plots.

Fig. 4. Total above- and belowground biomass (g per pot) and total N uptake (mg per pot) of the four-species mixture at varying levels of belowground interaction in the absence (–myc; grey plots) or presence (+myc; coloured plots) of arbuscular mycorrhizal (AM) fungal inoculum. Clo-sys, no belowground interaction; Res-sys, interaction precluded to roots but not to arbuscular mycorrhizal fungi and soil microorganisms; Ope-sys, maximum belowground interaction. Circles inside plots represent means, with whiskers representing ± SE (n = 6). The width of the plot shows the density distribution of values. P values for pairwise comparisons between +myc and –myc within the same belowground interaction treatment are reported above the plots.

Fig. 5. Total N uptake (mg per pot) of the four species at varying levels of belowground interaction (Clo-sys, no belowground interaction; Res-sys, interaction precluded to roots but not to arbuscular mycorrhizal fungi and soil microorganisms; Ope-sys, maximum belowground interaction) in the absence (–myc; grey plots) or presence (+myc; coloured plots) of AM fungal inoculum. Circles inside plots represent means, with whiskers representing ± SE (n = 6). The width of the plot shows the density distribution of values. P values for pairwise comparisons between +myc and –myc within the same belowground interaction treatment are reported above the plots.

Fig. 6. N transfer from the legume (pea) to the non-legume species (wheat, flax, and chicory) at varying levels of belowground interaction in the absence (–myc; grey plots) or presence (+myc; coloured plots) of arbuscular mycorrhizal (AM) fungal inoculum. Upper graphs: proportion (%) of the total N uptake of each companion species. Lower graphs: amount of N (mg pot^{-1}) transferred to each companion species. Res-sys, interaction precluded to roots but not to arbuscular mycorrhizal fungi and soil microorganisms; Ope-sys, maximum belowground interaction. Circles inside plots represent means, with whiskers representing ± SE (n = 6). The width of the plot shows the density distribution of values. P values for pairwise comparisons between +myc and –myc within the same belowground interaction treatment are reported above the plots.

Fig. 7. N transfer (mg per pot) from pea to the non-legume species (wheat, flax, and chicory) and related percent distribution (mean values ± SE; n = 6) at varying levels of belowground interaction (Res-sys, interaction precluded to roots but not to arbuscular mycorrhizal fungi and soil microorganisms; Ope-sys, maximum belowground interaction) in the absence (–myc) or presence (+myc) of AM fungal inoculum. P values for pairwise comparisons between +myc and –myc within the same belowground interaction treatment are reported.

Supplementary material

Fig. S1. The design of the four-compartment pots. Clo-sys, no belowground interaction was allowed, as each pot was separated from the neighbouring pots by a plastic wall; Res-sys, arbuscular mycorrhizal fungal mycelium and soil solution were allowed to pass between pots through slits (18 × 5 cm) filled with a septum of nylon fabric with 16 µm mesh; Ope-sys, each pot was connected by slits (18 × 5 cm) but no septum was applied.

Fig. S2. Ten-day mean air temperature (blue) and 10-day maximum temperature (red) during the experiment. The times of sowing (S) and plant harvest (H) are indicated.

Fig. S3. Root colonisation (%) of the four species at varying levels of belowground interaction (Clo-sys, no belowground interaction; Res-sys, interaction precluded to roots but not to arbuscular mycorrhizal fungi and soil microorganisms; Ope-sys, maximum belowground interaction).

Fig. S4. N concentration (%) of the biomass of the four species at varying levels of belowground interaction (Clo-sys, no belowground interaction; Res-sys, interaction precluded to roots but not to arbuscular mycorrhizal fungi and soil microorganisms; Ope-sys, maximum belowground interaction) in the absence (–myc; grey plots) or presence (+myc; coloured plots) of AM fungal inoculum. Circles inside plots represent means, with whiskers representing ± SE (n = 6). The width of the plot shows the density distribution of values. P values for pairwise comparisons between +myc and –myc within the same belowground interaction treatment are reported above the plots.

Figure 1

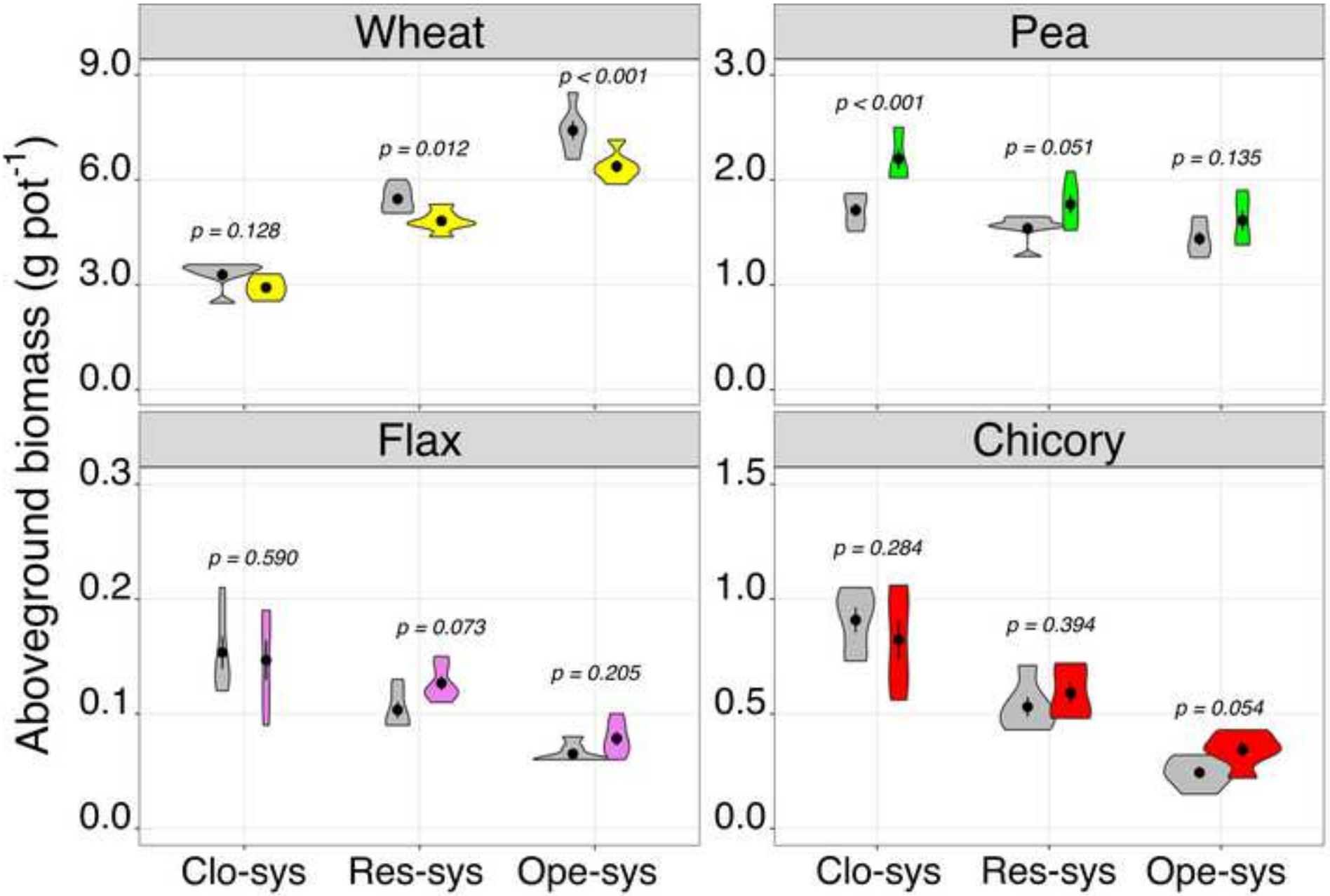
Wheat
Pea
Flax
Chicory
Aboveground biomass (g pot-1)
9.0
6.0
3.0
0.0
3.0
2.0
1.0
0.0
0.3
0.2
0.1
0.0
1.5
1.0
0.5
0.0
p = 0.128
p = 0.012
p < 0.001
p < 0.001
p = 0.051
p = 0.135
p = 0.590
p = 0.073
p = 0.205
p = 0.284
p = 0.394
p = 0.054
Clo-sys
Res-sys
Ope-sys
Clo-sys
Res-sys
Ope-sys

Figure 2

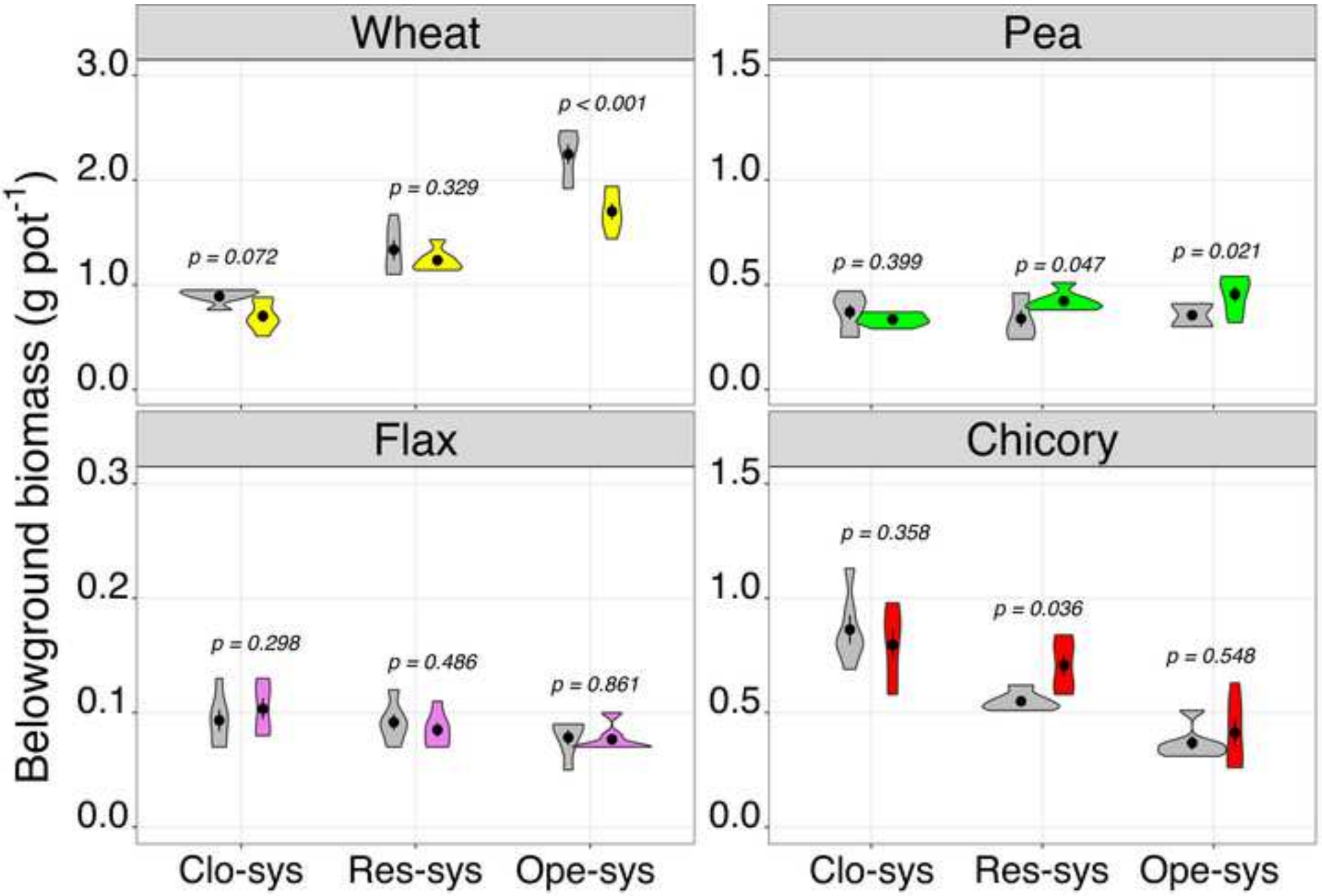
Wheat
Pea
Flax
Chicory
Belowground biomass (g pot-1)
p = 0.072
p = 0.329
p < 0.001
p = 0.399
p = 0.047
p = 0.021
p = 0.298
p = 0.486
p = 0.861
p = 0.358
p = 0.036
p = 0.548
Clo-sys
Res-sys
Ope-sys

Figure 3

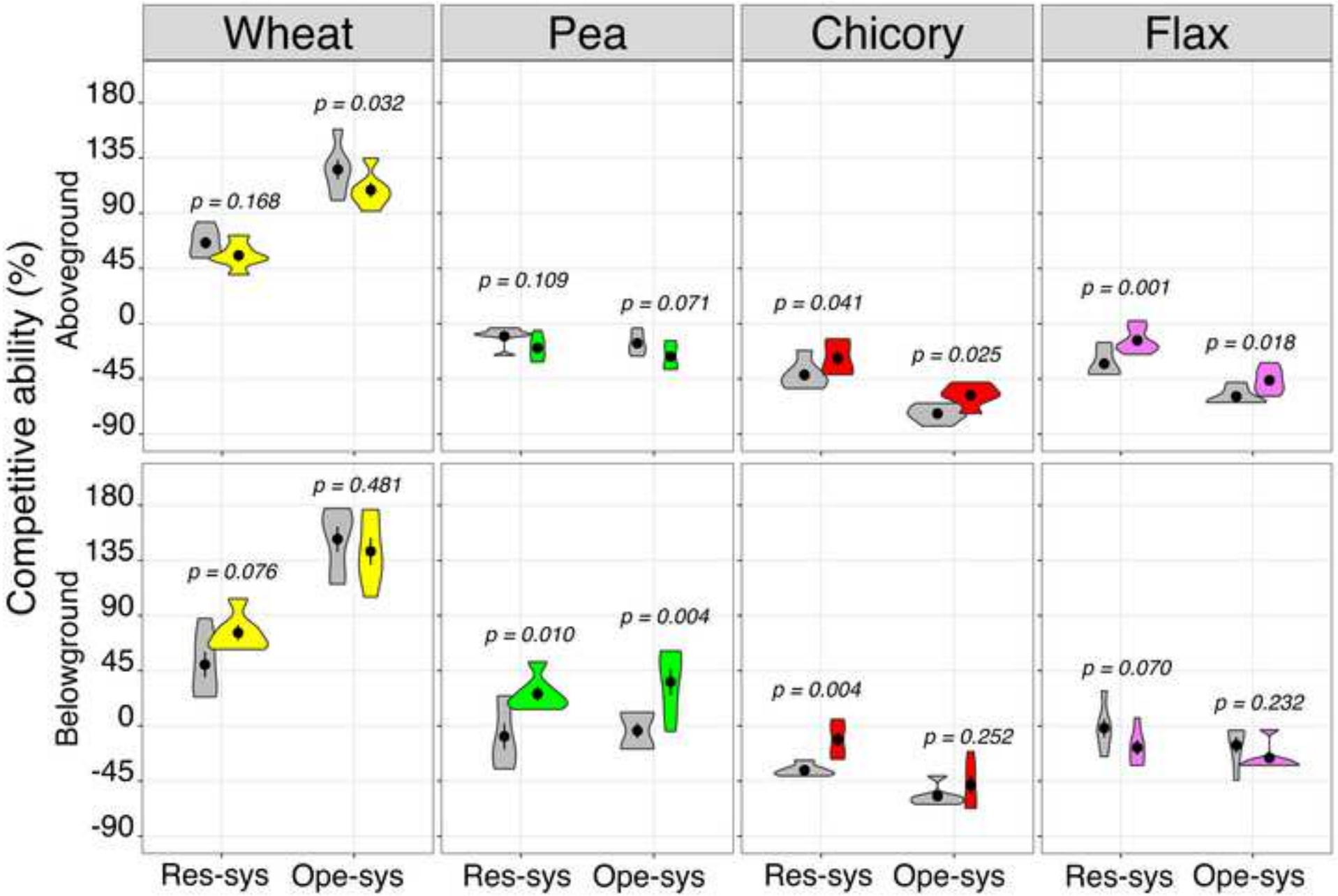
Wheat
Pea
Chicory
Flax
Competitive ability (%)
Aboveground
Belowground
180
135
90
45
0
-45
-90
p = 0.168
p = 0.032
p = 0.109
p = 0.071
p = 0.041
p = 0.025
p = 0.001
p = 0.018
p = 0.076
p = 0.481
p = 0.010
p = 0.004
p = 0.004
p = 0.252
p = 0.070
p = 0.232
Res-sys
Ope-sys

Figure 4

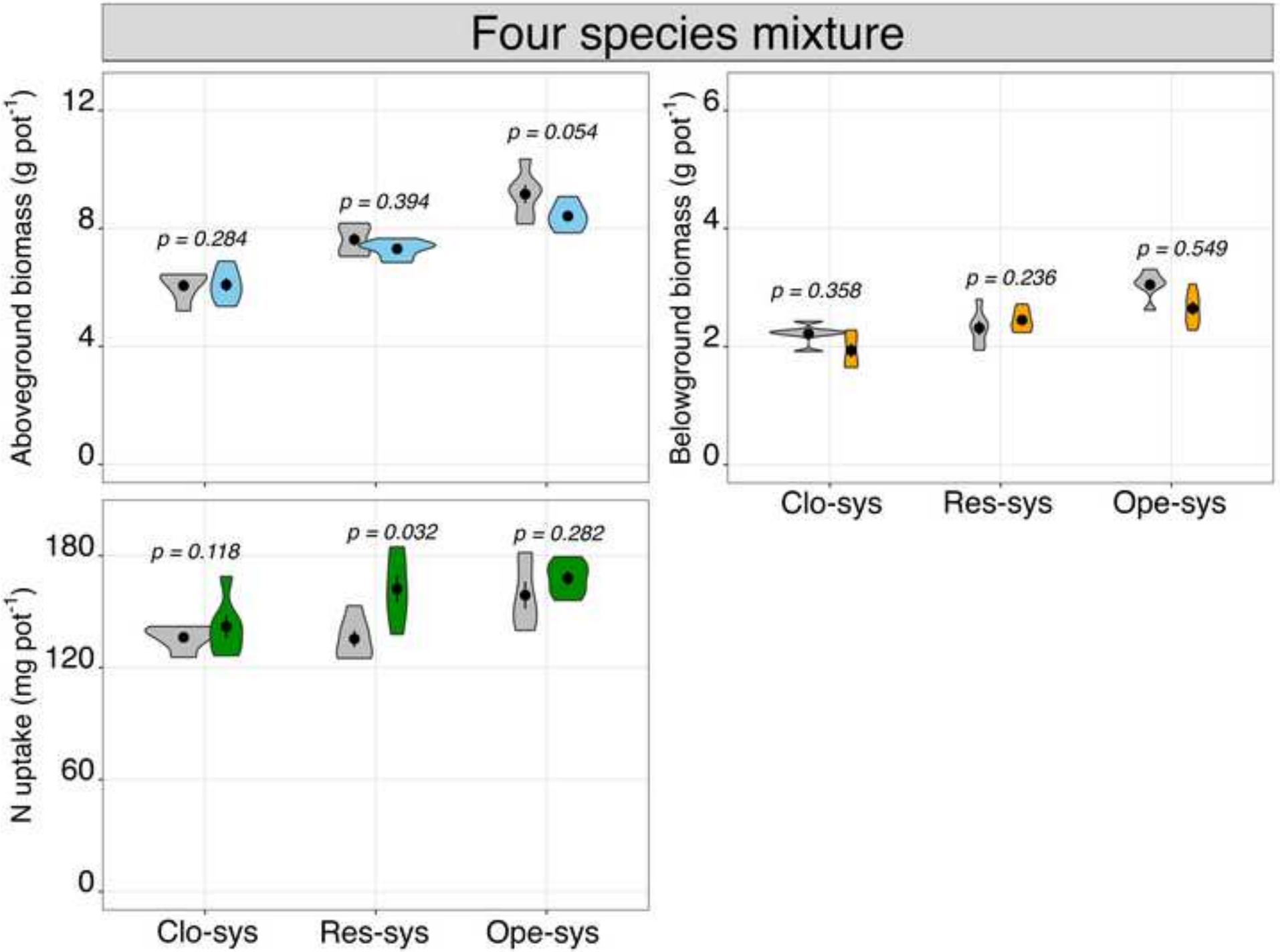
Four species mixture
Aboveground biomass (g pot-1)
12
8
4
0
p = 0.284
p = 0.394
p = 0.054
Belowground biomass (g pot-1)
6
4
2
0
p = 0.358
p = 0.236
p = 0.549
N uptake (mg pot-1)
180
120
60
0
p = 0.118
p = 0.032
p = 0.282
Clo-sys
Res-sys
Ope-sys
Clo-sys
Res-sys
Ope-sys

Figure 5

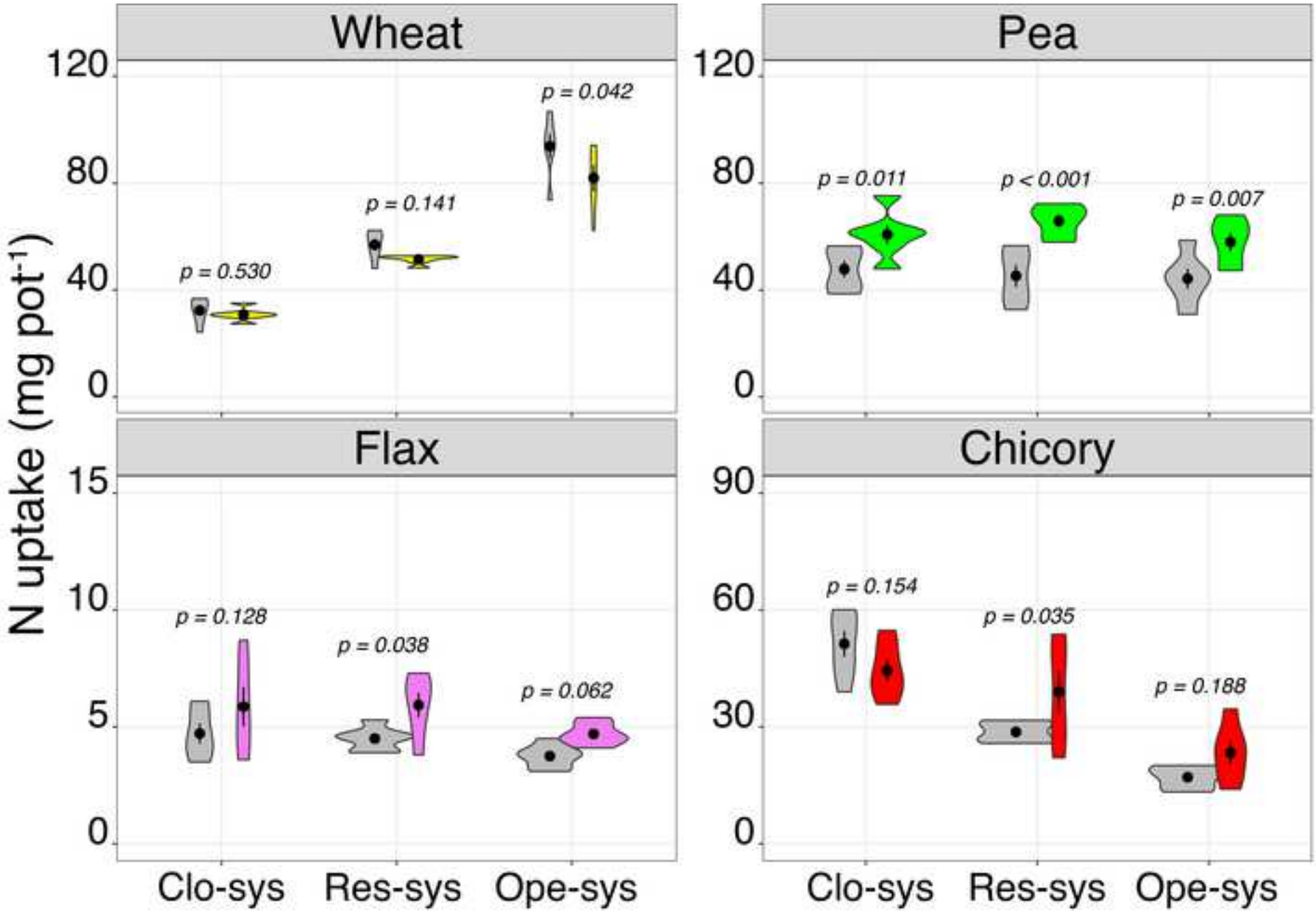
Wheat
Pea
Flax
Chicory
N uptake (mg pot-1)
120
80
40
0
15
10
5
90
60
30
p = 0.530
p = 0.141
p = 0.042
p = 0.011
p < 0.001
p = 0.007
p = 0.128
p = 0.038
p = 0.062
p = 0.154
p = 0.035
p = 0.188
Clo-sys
Res-sys
Ope-sys

Figure 6

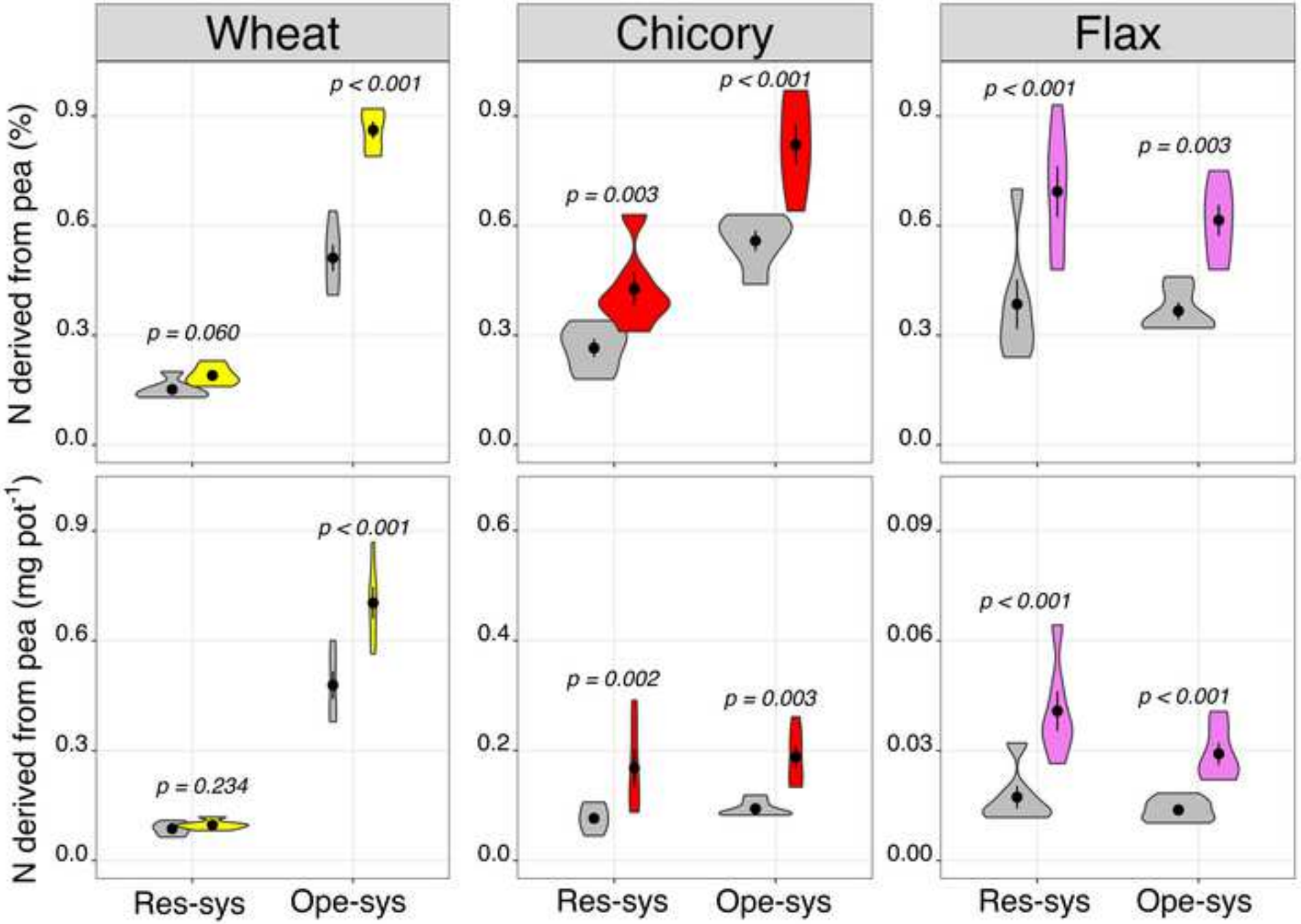
Wheat
Chicory
Flax
N derived from pea (%)
N derived from pea (mg pot-1)
p = 0.060
p < 0.001
p = 0.003
p < 0.001
p < 0.001
p = 0.003
p = 0.234
p < 0.001
p = 0.002
p = 0.003
p < 0.001
p < 0.001
Res-sys
Ope-sys
Res-sys
Ope-sys
Res-sys
Ope-sys

Figure 7

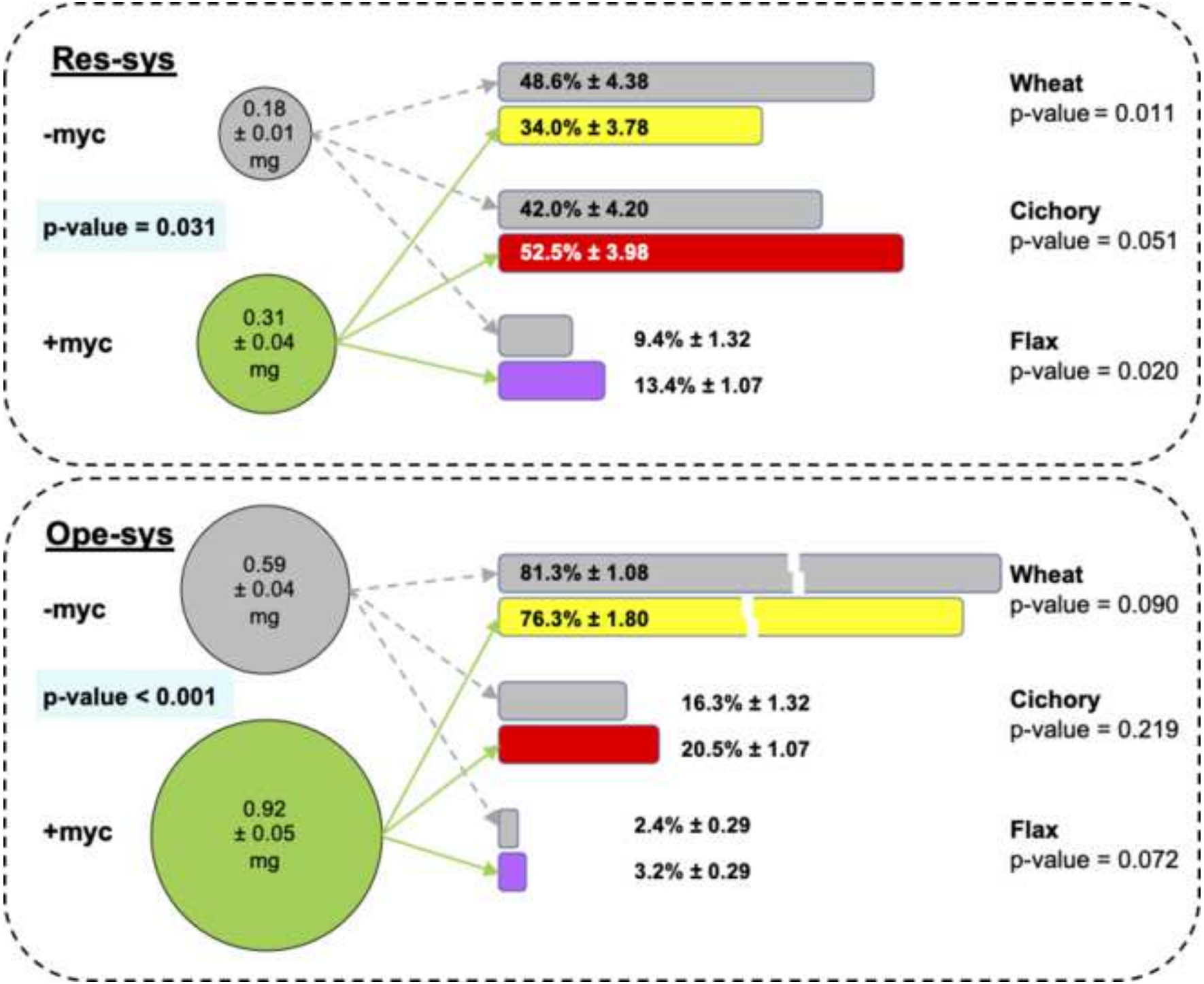
Res-sys
-myc
0.18 ± 0.01 mg
p-value = 0.031
+myc
0.31 ± 0.04 mg
48.6% ± 4.38
34.0% ± 3.78
Wheat
p-value = 0.011
42.0% ± 4.20
52.5% ± 3.98
Cichory
p-value = 0.051
9.4% ± 1.32
13.4% ± 1.07
Flax
p-value = 0.020
Ope-sys
-myc
0.59 ± 0.04 mg
p-value < 0.001
+myc
0.92 ± 0.05 mg
81.3% ± 1.08
76.3% ± 1.80
Wheat
p-value = 0.090
16.3% ± 1.32
20.5% ± 1.07
Cichory
p-value = 0.219
2.4% ± 0.29
3.2% ± 0.29
Flax
p-value = 0.072

Table 1. P values for the analysis of variance. BI, belowground interaction (Clo-sys, no belowground interaction; Res-sys, interaction precluded to roots but not to arbuscular mycorrhizal fungi and soil microorganisms; Ope-sys, maximum belowground interaction); I, inoculum (+myc, presence of inoculum; –myc, absence of inoculum).

Species or mixture	Factor	Above-ground biomass	Below-ground biomass	N concentration	N uptake	Proportion of N derived from pea†	Amount of N derived from pea‡	N transfer distribution	Aboveground competitive ability	Belowground competitive ability
Wheat	**BI**	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001
	I	< 0.001	< 0.001	0.180	0.020	< 0.001	0.002	0.004	0.016	0.420
	BI x I	0.064	0.010	0.669	0.574	< 0.001	0.066	0.476	0.536	0.082
Pea	**BI**	< 0.001	0.210	0.087	0.396	< 0.001	< 0.001	-	0.127	0.412
	I	< 0.001	0.043	0.038	< 0.001	0.149	< 0.001	-	0.020	< 0.001
	BI x I	0.1280	0.053	0.409	0.465	0.483	0.131	-	0.807	0.775
Cichory	**BI**	< 0.001	< 0.001	0.747	< 0.001	< 0.001	0.1530	< 0.001	< 0.001	< 0.001
	I	0.3120	0.290	0.403	0.2362	< 0.001	< 0.001	0.029	0.004	0.005
	BI x I	0.1040	0.105	0.541	0.0377	0.7150	0.8300	0.569	0.863	0.156
Flax	**BI**	< 0.001	0.015	< 0.001	0.037	0.3540	0.0283	< 0.001	< 0.001	0.075
	I	0.1423	0.920	0.002	0.003	< 0.001	< 0.001	0.005	< 0.001	0.038
	BI x I	0.2231	0.450	0.965	0.906	0.562	0.332	0.648	0.423	0.063
Four-species mixture	**BI**	< 0.001	< 0.001	-	< 0.001	-	-	-	-	-
	I	0.312	0.290	-	0.322	-	-	-	-	-
	BI x I	0.104	0.105	-	0.039	-	-	-	-	-

† For pea: proportion of N from pea to others

‡ For pea: amount of N from pea to others

Declaration of interests

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.

RI, ASF, DG, and GA: conceptualized and elaborate the data. ASF, DG, and GA: acquired the funds to conduct the experiment. RI and ER: carried out the formal analysis. RI wrote the first draft of the paper. ASF, DG, GA, ER and PR: collaborated on the ideas, and contributed critically to the drafts. All authors gave the final approval for publication.

Mycorrhizae differentially influence the transfer of nitrogen among associated plants and their competitive relationships

Rosolino Ingraffia, Dario Giambalvo, Alfonso S. Frenda*, Eliseo Roma, Paolo Ruisi, Gaetano Amato

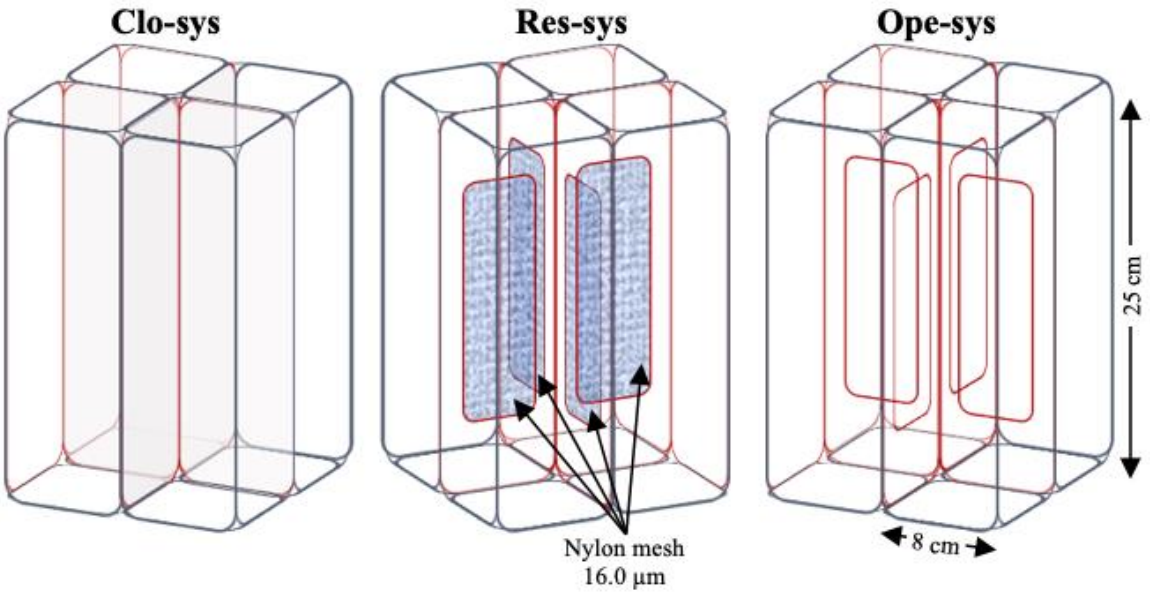

Fig. S1. The design of the four-compartment pots. Clo-sys, no belowground interaction was allowed, as each pot was separated from the neighbouring pots by a plastic wall; Res-sys, arbuscular mycorrhizal fungal mycelium and soil solution were allowed to pass between pots through slits (18 × 5 cm) filled with a septum of nylon fabric with 16 µm mesh; Ope-sys, each pot was connected by slits (18 × 5 cm) but no septum was applied.

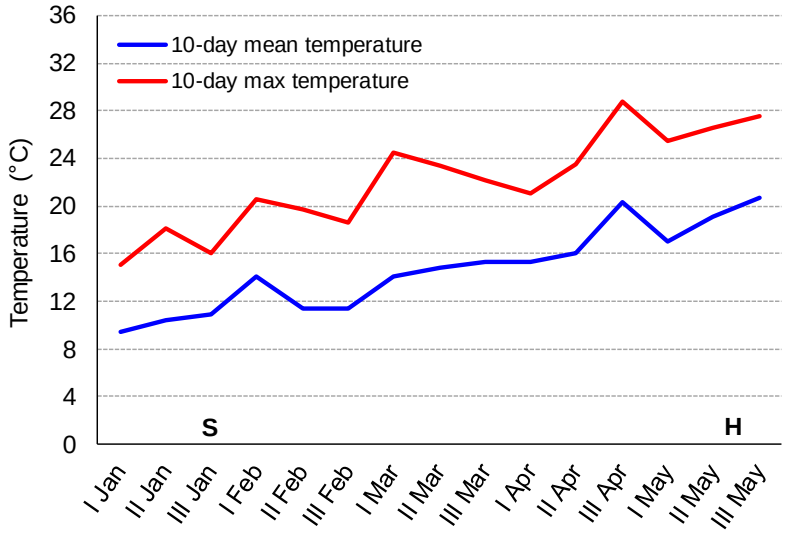

Fig. S2. Ten-day mean air temperature (blue) and 10-day maximum temperature (red) during the experiment. The times of sowing (S) and plant harvest (H) are indicated.

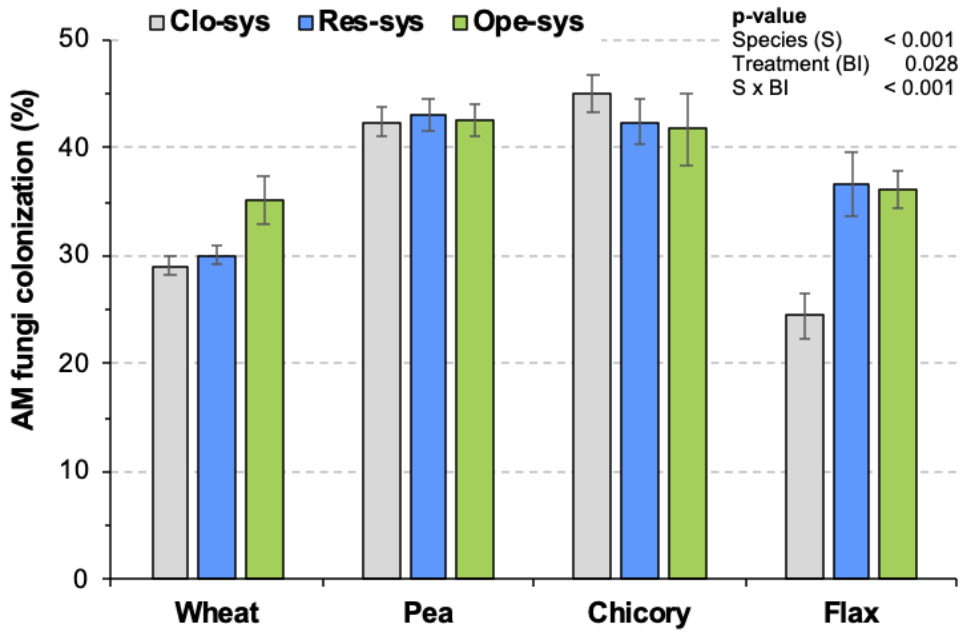

Fig. S3. Root colonization (%) of the four species at varying levels of belowground interaction (Clo-sys, no belowground interaction; Res-sys, interaction precluded to roots but not to arbuscular mycorrhizal fungi and soil microorganisms; Ope-sys, maximum belowground interaction).

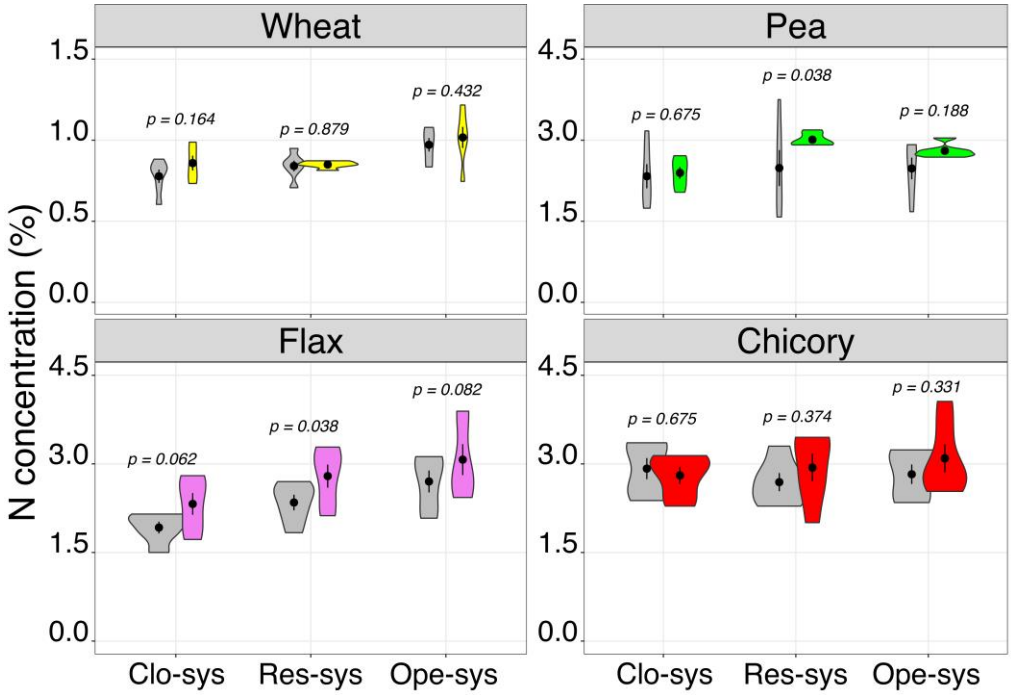

Fig. S4. N concentration (%) of the biomass of the four species at varying levels of belowground interaction (Clo-sys, no belowground interaction; Res-sys, interaction precluded to roots but not to arbuscular mycorrhizal fungi and soil microorganisms; Ope-sys, maximum belowground interaction) in the absence (–myc; grey plots) or presence (+myc; coloured plots) of AM fungal inoculum. Circles inside plots represent means, with whiskers representing ± SE (n = 6). The width of the plot shows the density distribution of values. P values for pairwise comparisons between +myc and –myc within the same belowground interaction treatment are reported above the plots.

Table S1. Atom ^{15}N values (%) detected in the phytomass of the legume and associated species. Mean values ± SE (n = 6).

Species	**Restricted system**		**Open system**		**Closed system**	
	+myc	-myc	+myc	-myc	+myc	+myc
Cicory	0.389 (±0.002)	0.383 (±0.006)	0.430 (±0.004)	0.403 (±0.002)	0.370 (±0.004)	0.370 (±0.002)
Wheat	0.377 (±0.001)	0.378 (±0.002)	0.432 (±0.002)	0.400 (±0.002)	0.372 (±0.003)	0.371 (±0.003)
Flax	0.404 (±0.004)	0.391 (±0.018)	0.414 (±0.003)	0.391 (±0.002)	0.370 (±0.001)	0.370 (±0.002)
Pea*	5.782 (±0.743)	7.130 (±3.469)	8.057 (±0.749)	6.981 (±0.841)	0.369 (±0.002)	0.368 (±0.001)

* Crop labelled with the isotope ^{15}N (in Restricted system and in Open system) using a modified leaf immersion method (tendril instead of the leaf).