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# Below-ground nitrogen transfer from oak seedlings facilitates *Molinia* growth: $^{15}\text{N}$ pulse-chase labelling

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## Abstract

**Aims** Belowground carbon transfer from plant to plant has been extensively described, but such transfer for nitrogen has been less thoroughly investigated when the donor is a non- $\text{N}_2$ -fixing species. This study, applied to forest regeneration, aimed to determine whether tree seedlings facilitated neighbouring grass growth through

nitrogen transfer at an early stage of development, thus facilitating nitrogen acquisition by understory species.

**Methods** *Quercus petraea* seedlings were planted in pots either sole-grown or mixed-grown with *Molinia caerulea* tufts or another oak seedling.  $^{15}\text{N}$ -urea pulse-chase labelling (cotton wick method) was performed in oak shoots and the fate of  $^{15}\text{N}$  in each soil and plant compartment was tracked for one year. N transfer pathways were investigated using two degrees of physical separation between root systems.

**Results** *Molinia* dry weight was higher when mixed-grown with oak seedlings than when sole-grown. Increase in grass dry weight correlated with N transfer from donor oak to receiver *Molinia*. Interestingly, the presence of *Molinia* increased N rhizodeposition of oak. N allocation in donor oak towards root in winter and shoot in spring was enhanced.

**Conclusions** Oak seedlings facilitated *Molinia* growth through rapid N transfer, underlining the ability of non- $\text{N}_2$ -fixing species to supply N to neighbours.  $^{15}\text{N}$  allocation within donor oak and its rhizodeposition depended on neighbour identity.

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## Abbreviations

|        |                       |
|--------|-----------------------|
| SG     | Sole-grown            |
| Mesh1  | 1 $\mu\text{m}$ Mesh  |
| Mesh30 | 30 $\mu\text{m}$ Mesh |
| MG     | Mixed-grown           |

## Introduction

Clements et al. (1929) describing plant interactions defined resource competition as a “physical process” and “a combined need in excess of the supply”. This implies that plants compete for light, water and nutrients only when they occupy the same zone of air or soil. Competition then occurs when one individual successfully reaches and captures limiting resources to the detriment of another individual. In this way it lowers the performance of its neighbours in biomass production, growth or reproduction. For many years, work on interactions has mainly focused on strategies of competition for resources (Bleasdale 1960; Grime 1974; Tilman 1990; Craine 2005; Craine and Dybzinski 2013), seen as a passive mechanism by which plants draw resources from a common reservoir (*i.e.* soil) with ranging efficiency.

Schreiner and Reed (1907) in an early literature review on the capacity of living roots to excrete matter in the soil pointed out that toxic excretions from roots exhibited deleterious effects on the growth and development of other plants. Allelopathy, or more generally chemical interference, is the process by which plants release allelochemicals into the environment that have significant direct or indirect effects on other plant processes (Molisch 1937; Muller 1969; Rice 1985; Grove et al. 2013). Resource competition and chemical interference can thus occur simultaneously (Mallik 1998; San Emeterio et al. 2007) and are species-specific (Fernandez et al. 2016). For example, the presence of beech (*Fagus sylvatica*) reduced the ability of maple (*Acer pseudoplatanus*) to take up inorganic nitrogen, so slowing its growth (Li et al. 2015), although the precise mechanism was not described.

Chemical compounds can also exert a beneficial influence (Rice 1985; Callaway 1995; Kohli et al. 1998). The molecular entities exuded by living roots include metabolites that include carbon, phosphorus and nitrogen compounds (Virtanen and Laine 1939; Slankis et al. 1964; Rovira 1969). Recent studies have emphasized that nutrient and information exchanges among plants are more generalized than had been thought (Pierik et al. 2013), and play a key role in plant interactions. The process by which living and dead roots exude substances in soil, shed fine roots and sloughed-off cells and tissue, along with cell lysates and decomposed root materials is termed rhizodeposition (Philipps et al. 2006; Scandellari et al. 2010; Mommer

et al. 2016). Legumes offer a well-known case of positive substance release in the soil. Indeed, they can release about 50% of their total N into the soil by rhizodeposition (Boulter et al. 1966; Shamoot et al. 1968; Friedrich and Dawson 1984; Khan et al. 2002; Mahieu et al. 2009; Fustec et al. 2010). Some authors have described the ability of neighbouring grass to take up nitrogen released by legumes (Henzell et al. 1964; Vallis et al. 1967; Whitney et al. 1967). N transfer from legumes to non-leguminous species has been reported in intercrops (Stagnari et al. 2017) such as soybean-weed (Moyer-Henry et al. 2006), pea-corn (van Kessel et al. 1985; Bethlenfalvay et al. 1991), pea-barley (Jensen 1996; Johansen and Jensen 1996) and clover-ryegrass (Haystead et al. 1988). Transfer of nutrients has mainly been studied for N<sub>2</sub>-fixing plants, and only very few examples (“less than a handful” – Teste et al. 2015) have shown exchange of nitrogen between non-fixing plants. These N transfers can be as high as 4% from donor plant N, which is not marginal for a non-N<sub>2</sub>-fixing species (Teste et al. 2015) especially in infertile soils (Eissenstat 1990). Haystead and Marriott (1978, 1979) concluded that transfer was a complex multicomponent process that could involve key actors such as soil microorganisms. N transfer pathways can include root intermingling, potential common mycorrhizal networks or rhizodeposition of diverse molecular entities (Ek et al. 1996; Teste et al. 2015; He et al. 2019). All these interactions may result in facilitation of the targeted species. Studies on forest trees found that birch (*Betula papyrifera*) and Douglas fir (*Pseudotsuga menziesii*) were colonized by the same ectomycorrhizal fungi (Simard and Perry 1997), allowing bidirectional carbon transfer (Simard et al. 1997a, b).

Facilitation is defined as a positive effect of plants on the establishment or growth of other plant species, and that causes harm to neither (Hunter and Aarssen 1988; Bertness and Callaway 1994; Callaway 1995; Bruno et al. 2003). As an example for nitrogen, in a silvopastoral systems in Patagonia, Gargaglione et al. (2014) showed that *Nothofagus* facilitated grass N uptake, probably owing to improved microclimatic conditions or reduced competition for N between soil microorganisms and grasses. However, in some cases, both competition and facilitation can occur between plants. Antagonistic facilitation occurs when a species A has a positive effect on a species B, while B has a negative effect on the species A (Bronstein 2009; Schöb et al. 2014). In the context of forest regeneration, Vernay et al.

(2018) described such a negative effect of the grass *Deschampsia cespitosa* on *Quercus petraea* seedling growth, while *Q. petraea* improved *D. cespitosa* biomass production. The strong ability of grasses to take up nutrients, and particularly nitrogen, has been demonstrated in many cases (e.g. Coll et al. 2004; Balandier et al. 2006), but the mechanisms underlying the facilitative effect of young oak seedlings on the grass are not known. From the literature briefly reviewed above, we hypothesized that nitrogen transfer between interacting plants occurs, even in the absence of  $N_2$  fixation, through facilitation of *Molinia* growth by oak seedlings. We specifically tested this hypothesis on *Q. petraea* seedlings in competition with a common perennial forest grass in temperate forests, *Molinia caerulea*. Plants were grown in pots under controlled conditions.  $^{15}N$  pulse-chase labelling (He et al. 2009) was applied to the oak, and  $^{15}N$  retrieval in soil and *Molinia* was quantified after 3, 5, 8 and 11 months to characterize N fate seasonally. N transfer pathways were also indirectly investigated by inserting a mesh between the two species. Two mesh sizes were used: 1  $\mu m$  to allow only chemical transfer (Mesh1), and 30  $\mu m$  to prevent root contact but allow both hyphae contact and chemical transfer (Mesh30).

## Materials and methods

### Experimental design

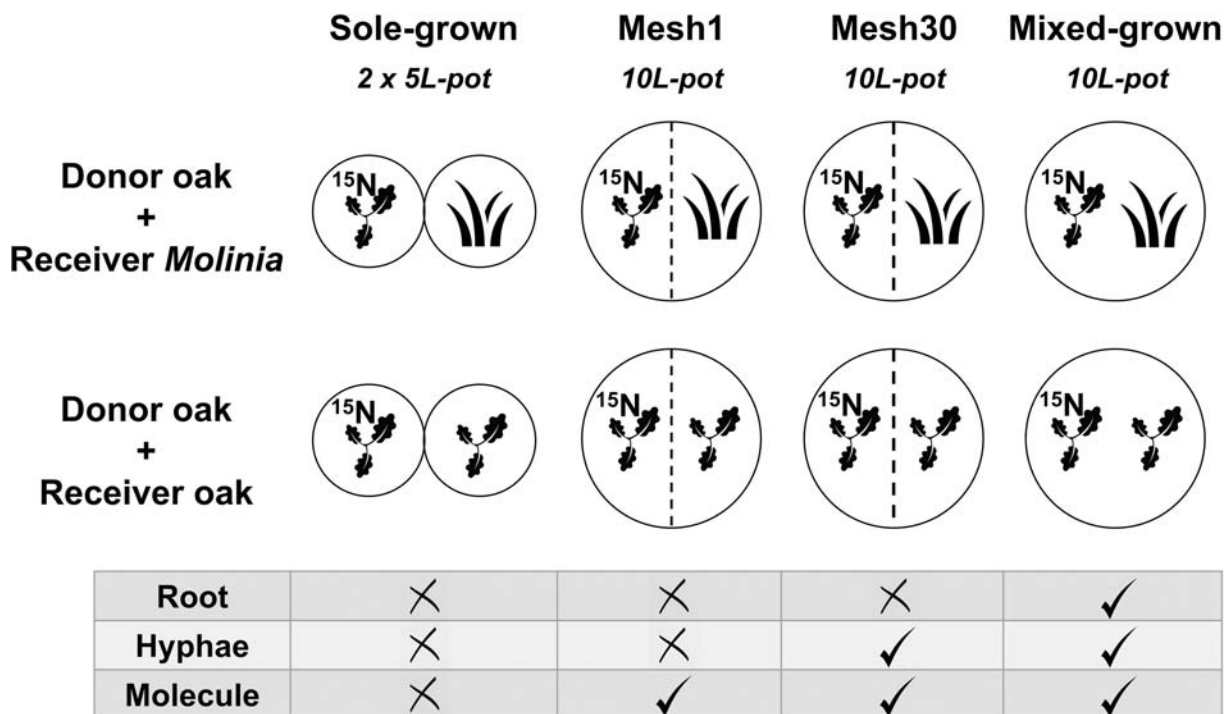
The experiment was conducted in pots under outdoor conditions in Clermont-Ferrand (Auvergne, France, 45°45'N 3°07'E, altitude 394 m a.s.l.) from April 2017 to May 2018 ( $T_{\text{mean}} = 12.9^\circ C$ , Rainfall = 546 mm). 168 one-year-old bare-root oak seedlings (*Quercus petraea* (Matt.) Liebl.) and 56 grass tufts (*Molinia caerulea*, three tufts on average) were planted in plastic pots, separately or together. Oak seedlings were sourced from a local nursery. They were  $21 \pm 6.6$  g (mean  $\pm$  SE) fresh weight,  $41.47 \pm 5.27$  cm in height, and  $4.94 \pm 0.98$  cm in diameter on average. *Molinia caerulea* (three tufts on average) was collected in a local oak forest at Paray-le-Frésil (Auvergne, France; 46°39'N 3°36'E) with  $2.04 \pm 0.97$  g fresh weight per lot. A total of 80 10 L pots and 64 5 L pots were filled with soil (typical luvisol-redoxisol pseudogley, sandy loam) collected in the same forest as the *Molinia*. Forest soil was used to preserve natural colonization by mycorrhizae. Four treatments

based on root system separation or interaction were set up (Fig. 1): (i) two 5 L pots containing either one oak or one *Molinia* tuft were placed side-by-side such that no root interactions were possible (root contact, hyphae contact or chemical transfer) (sole-grown), (ii) oak and *Molinia* tufts were placed in the same 10 L pots but their rooting zones were separated by a nylon (Nitex®) cloth with one of two mesh sizes: either 1  $\mu m$  to allow only chemical transfer (Mesh1) or 30  $\mu m$  to prevent root contact but allow both hyphae contact and chemical transfer (Mesh30), and (iii) oak and *Molinia* tufts were placed in the same 10 L pot to allow full belowground interactions through both root and hyphae contacts and chemical transfer (mixed-grown). Effect of species identity in the interaction, i.e. oak or *Molinia* as a neighbour, was tested, replicating every treatment with receiver oak instead of receiver *Molinia* (Fig. 1). Each treatment was replicated four times with a random spatial pot arrangement. To prevent interaction with water availability, the pots were fitted with probes and irrigated to the field capacity throughout the experiment. No fertilizer of any kind was added to the pots during the experiment.

### Oak shoot $^{15}N$ pulse-chase labelling by the cotton wick technique

To track possible nitrogen transfer from donor oak to receiver *Molinia* or oak,  $^{15}N$  was supplied to donor oaks using the “cotton wick” method. This method supplies  $^{15}N$  through stem injection. Oak stems were pierced using a drill (1 mm hole 2 cm above the ground) to push through a cotton wick, both ends of which were dipped into a  $^{15}N$  solution in a 5 ml Eppendorf tube ( $^{15}N$ -urea, At.  $^{15}N$  98%) through two holes in a cap on the top of the tube (Mahieu et al. 2007; Fustec et al. 2010). Drying and loss of solution from the reservoir and the cotton wick were prevented by sheathing the wick with two silicone tubes sealed to the stem and cap (Terostat®, Henkel Surface Technologies, Gulph Mills, USA).

$^{15}N$ -urea was applied on June 13 and 14, 2017. After oaks seedlings had absorbed more than 3 mL of solution (at 0.5%, weight/volume), the reservoir was replenished with a further 3 mL of  $^{15}N$  urea solution at 0.375% (w/v). When all the solution of  $^{15}N$  was fully absorbed, the reservoir was washed with 1 mL of deionized water, which was in turn absorbed by the plant. This procedure supplied around 18 mg of  $^{15}N$  to oak shoots. 5 L pots without cotton wicks were used as controls to determine



**Fig. 1** Experimental set-up. Oak was mixed-grown with either another oak plant or a *Molinia* tuft in one pot. When mixed-grown, belowground compartments were either fully intermingled (root contact; mixed-grown) or separated with a mesh of size 30  $\mu\text{M}$  (Mesh30) or 1  $\mu\text{M}$  (Mesh1). Mesh limits (cross in the table) the number of pathways of nutrient exchange between two root

compartments: hyphae and molecules could go through Mesh30 (check mark in the table), but only molecules through Mesh1. Oak was sole-grown beside another oak plant or a *Molinia* tuft with no root contact (sole-grown). Superscript  $^{15}\text{N}$  indicates oak was labelled with  $^{15}\text{N}$  urea through cotton wick application (see Materials and methods for details)

the natural  $^{15}\text{N}$  abundance in each compartment (oak shoots and roots, soil, *Molinia* shoots and roots).

#### Plant harvesting

Plants were harvested sequentially after total  $^{15}\text{N}$  urea uptake was completed (August 2017), during leaf senescence (October 2017), during dormancy (February 2018) and after bud break (May 2018). In winter, only sole-grown and mixed-grown conditions were collected, to estimate  $^{15}\text{N}$  allocation, as there is no transfer during dormancy. For this harvest, oak shoots were stem and marcescent leaves, and *Molinia* shoots were senescent leaves.

Plant shoots and roots were collected for both species. For Mesh1 and Mesh30 treatments, soils separated by the mesh were collected individually, and soil in sole-grown and mixed-grown pots was collected as a whole. Soil was then sieved (2 mm) and visible roots were

collected. Roots were washed with 200 mL of a 0.5 mM  $\text{CaCl}_2$  solution to remove  $^{15}\text{N}$  from their surface and to maintain cell membrane integrity (Epstein 1961). This solution was then sieved and mixed with the soil. About 200 mL of this mixture was used to determine N content and isotope abundance. At each harvest and for each treatment, four replicates of each compartment were collected.

#### N content and isotopic analysis

Root, shoot and soil samples were dried at 60 °C for at least 48 h, weighed and ground to a fine powder. Total N content and  $^{15}\text{N}$  abundance were then determined with an elemental analyser (vario ISO-TOPE cube, Elementar, Hanau, Germany) in line with a gas isotope ratio mass spectrometer (IsoPrime 100, Isoprime Ltd, Cheadle, UK) at the Silvatech platform, INRA Nancy-Lorraine.



## Calculations

Total N amount (mg) was calculated as follows:

$$N_{\text{tot}} = \frac{\%N_{\text{total}} \times \text{DW}}{100}, \quad (1)$$

where  $\%N_{\text{total}}$  is N content (% DW) and DW is dry weight (mg).

$^{15}\text{N}$  amount (mg) in each tissue sample was calculated as follows:

$$^{15}\text{N}_{\text{amount}} = \frac{(\text{At. } \%^{15}\text{N} - \text{At. } \%^{15}\text{N}_{\text{unlabelled sample}}) \times N_{\text{tot}}}{100}, \quad (2)$$

where At.  $\%^{15}\text{N}$  is isotopic abundance in samples of donor oak and receiver neighbour, defined as follows:

$$\text{At. } \%^{15}\text{N} = \frac{^{15}\text{N}}{(^{14}\text{N} + ^{15}\text{N})} \times 100. \quad (3)$$

Relative  $^{15}\text{N}$  allocation (supp. data, Figure S1 and S2) was calculated as follows:

$$\%^{15}\text{N} = \frac{^{15}\text{N}_{\text{amount in compartment } i} \times 100}{\sum ^{15}\text{N}_{\text{amount}}}, \quad (4)$$

where  $\sum ^{15}\text{N}$  amount is the sum of  $^{15}\text{N}$  amounts in each compartment (shoots and roots in donor, receiver *Molinia*, receiver oak and in the soil).

## Statistics

Statistical analysis was performed using R software (R studio, Version 1.0.153). Data are means of  $n = 4$  biological replicates ( $\pm$  SE). All variables were tested for normality and homoscedasticity using the Shapiro-Wilk and Levene tests. Statistical analyses were conducted using one-way ANOVA or  $t$  test. For factors with more

than two levels (seasons, root separation treatment and compartment), ANOVA was followed by Tukey's honestly significant difference *post hoc* test for mean comparison at a 90% level.

## Results

*Molinia* dry weight increased when mixed-grown with oak (Fig. 2 and Tables S1–S5)

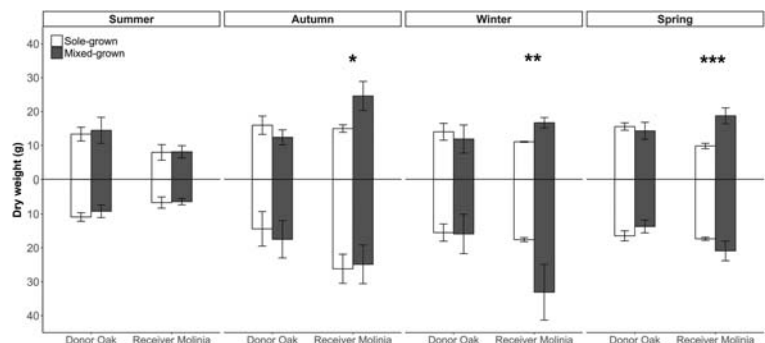
Donor oak dry weight was constant across seasons either sole-grown or mixed-grown with *Molinia* (Fig. 2).

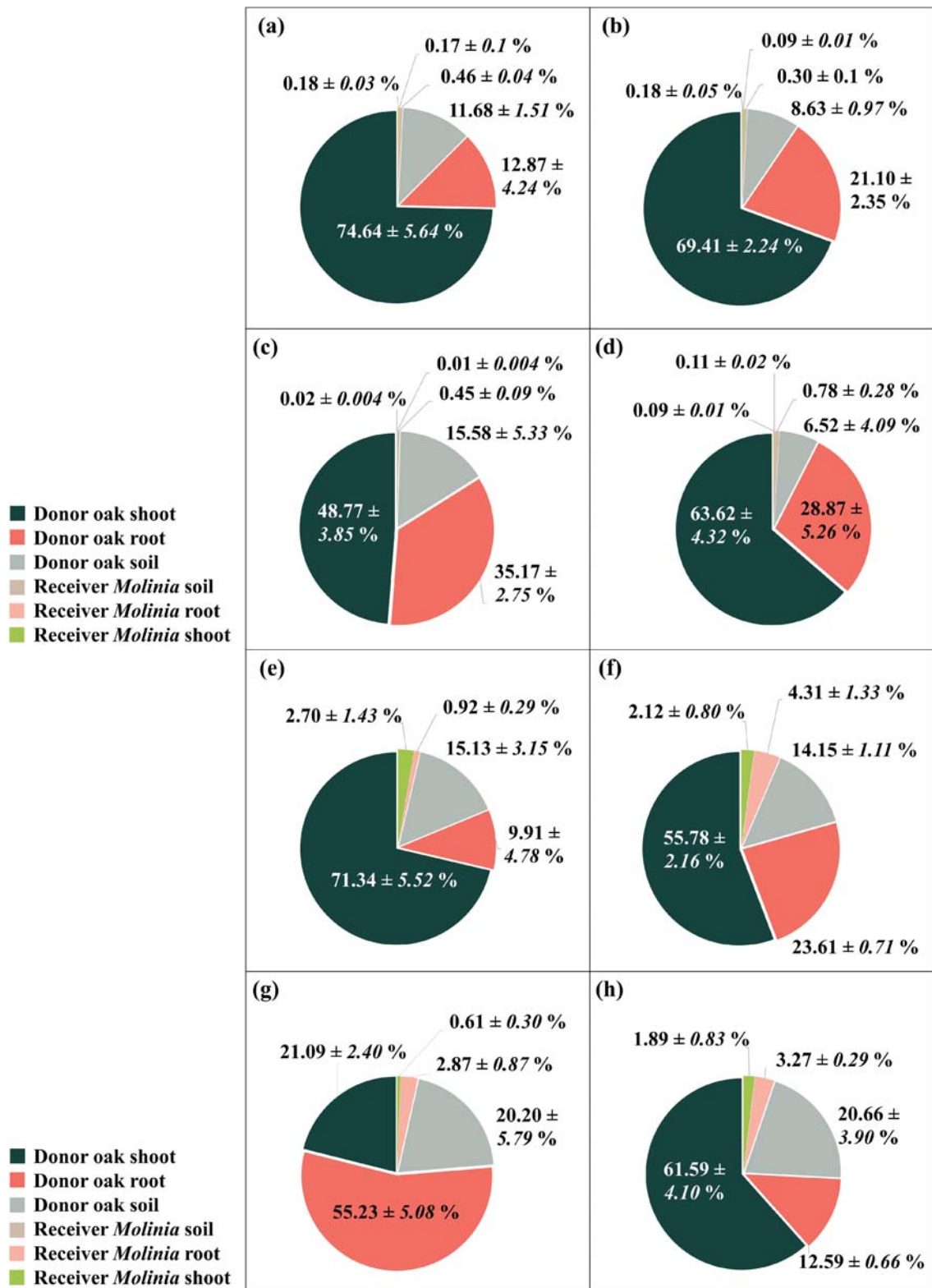
By contrast, growth in *Molinia* tufts increased appreciably from summer ( $7.95 \pm 2.31$  g for shoots and  $6.62 \pm 1.65$  g for roots) to autumn ( $14.99 \pm 1.11$  g for shoot and  $26.18 \pm 4.3$  g for root) in sole-grown ( $p = 0.01$  for shoot and  $p = 0.0007$  for roots) and then remained constant until spring (Tables S1, S2, S3 and S4). In mixed-grown conditions, *Molinia* shoot growth increased from summer ( $8.11 \pm 1.81$  g) to autumn ( $24.61 \pm 4.29$  g,  $p = 0.005$ ) (Table S1), but larger root biomass was only observed in winter. Remarkably, *Molinia* shoot biomass tended to be higher when grown with oak rather than sole-grown in autumn ( $p = 0.07$ ), winter ( $11.06 \pm 0.12$  g in sole-grown and  $16.7 \pm 1.55$  g in mixed-grown,  $p = 0.04$ ) and spring ( $9.83 \pm 0.8$  g and  $18.74 \pm 2.37$  g in mixed-grown,  $p = 0.027$ ) (Fig. 2 and Tables S1–S5).

Fate of rhizodeposited N from donor oak seedlings (Figs. 3, 4 and Tables S6–S14)

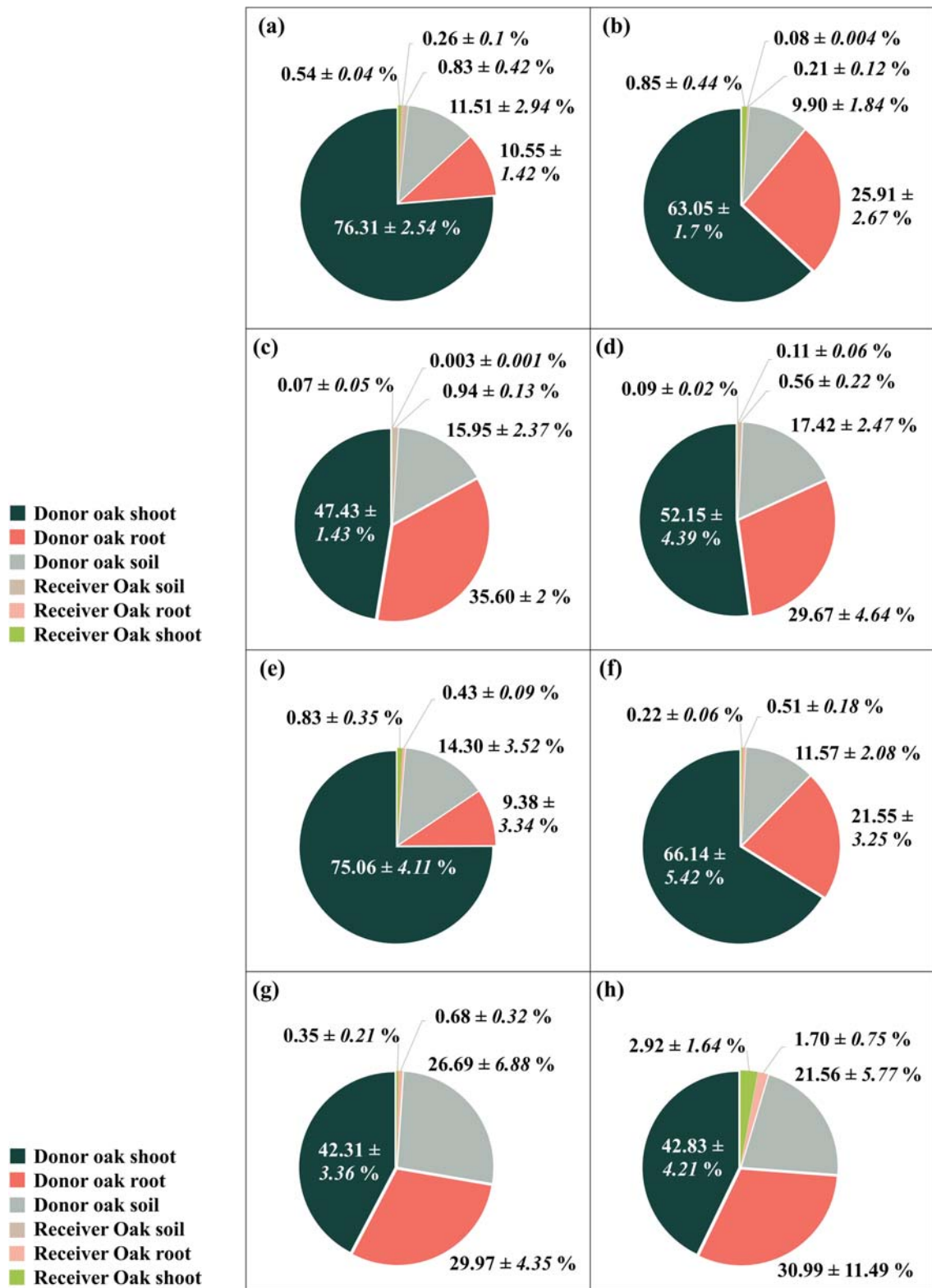
Overall, about 73% of the measured  $^{15}\text{N}$  amount in all compartments was found in donor oak, either sole-grown or mixed-grown with *Molinia* in summer (Fig.

**Fig. 2** Above- and belowground dry weight for donor oak and receiver *Molinia*, for each season (summer, autumn, winter and spring). Plants were grown with either no root contact (sole-grown, white bars) or root contact (mixed-grown, grey bars). Values are reported as means  $\pm$  SE ( $n = 4$ ). \*, \*\* and \*\*\* correspond to  $p < 0.1$ , 0.05 and 0.01, respectively





**Fig. 3**  $^{15}\text{N}$  allocation (expressed as % of total  $^{15}\text{N}$  measured in all compartments) among shoots, roots and soil in donor oak and receiver *Molinia*. Species were sole-grown (a, b, c and d) or mixed-grown (e, f, g and h). Values are reported as means  $\pm$  SE ( $n = 4$ )



**Fig. 4**  $^{15}\text{N}$  allocation (expressed as % of total  $^{15}\text{N}$  measured in all compartments) among shoots, roots and soil in donor oak and receiver oak. Species were sole-grown (a, b, c and d) or mixed-grown (e, f, g and h). Values are reported as means  $\pm$  SE ( $n = 4$ )



3a and e).  $^{15}\text{N}$  percentage in soil was statistically similar among seasons, treatments and neighbours.

### *Sole-grown plants*

Surprisingly,  $^{15}\text{N}$  was detected in receiver neighbour (significantly different from zero) but at a very low level (Figs. 3 and 4 – sole-grown and Table S15). In receiver oak,  $^{15}\text{N}$  amount was significantly different from zero in summer, autumn and winter in shoots, roots and soil respectively. In spring, both donor oak shoots and soil contained significant amounts of  $^{15}\text{N}$ . In *Molinia*,  $^{15}\text{N}$  amount was significantly different from zero in all cases except in roots in summer ( $p = 0.17$ ). In donor oak,  $^{15}\text{N}$  proportion in oak donor shoots decreased from summer to winter (from about 75% to 50%,  $p = 0.004$ ) while that in the roots and soil rose (Figs. 3c, 4c, S1 – sole-grown and Table S15 – Receiver *Molinia*) near either receiver oak or *Molinia*.

### *Mixed-grown plants*

When mixed-grown,  $^{15}\text{N}$  transfer from donor oak to receiver neighbour, either *Molinia* or oak, was statistically significant.  $^{15}\text{N}$  allocation patterns in shoots and roots were similar to that of sole-grown, but  $^{15}\text{N}$  amount in receiver was higher (Fig. 3, 4 and S2). However, decrease in  $^{15}\text{N}$  allocation to shoots and a concomitant increase in roots in winter were much larger when mixed-grown with receiver *Molinia* (Fig. 3e and 3g; from about 70% to 20%,  $p < 0.0001$ ) than with receiver oak (Fig. 4e and g; from about 75% to 40%,  $p < 0.001$ ) (Figure S1j and k). In spring,  $^{15}\text{N}$  in donor oak was reallocated to shoots more when mixed-grown with *Molinia* ( $p < 0.001$ ) than with another oak ( $p \approx 1$ ). Allocation was much greater in receiver *Molinia* roots from autumn onwards than in receiver oak (Figure S2k). In autumn,  $^{15}\text{N}$  relative amount in shoots was higher in *Molinia* than in receiver oak (Figure S2j).

### *Mesh (supp. data)*

Overall, mesh had a very small effect on  $^{15}\text{N}$  allocation in either donor oak, receiver oak or receiver *Molinia* (Figure S1 and S2). However, in Mesh30,  $^{15}\text{N}$  allocation was greater in shoots in autumn in donor oak mixed-grown with another oak than in donor oak grown with *Molinia* (Figure S1g).  $^{15}\text{N}$  allocation to shoots was much higher in receiver *Molinia* than in receiver oak

in summer and autumn (Figure S2g).  $^{15}\text{N}$  allocation patterns in root and soil were not statistically different according to receiver oak or *Molinia* (Figure S2h, i).

## Discussion

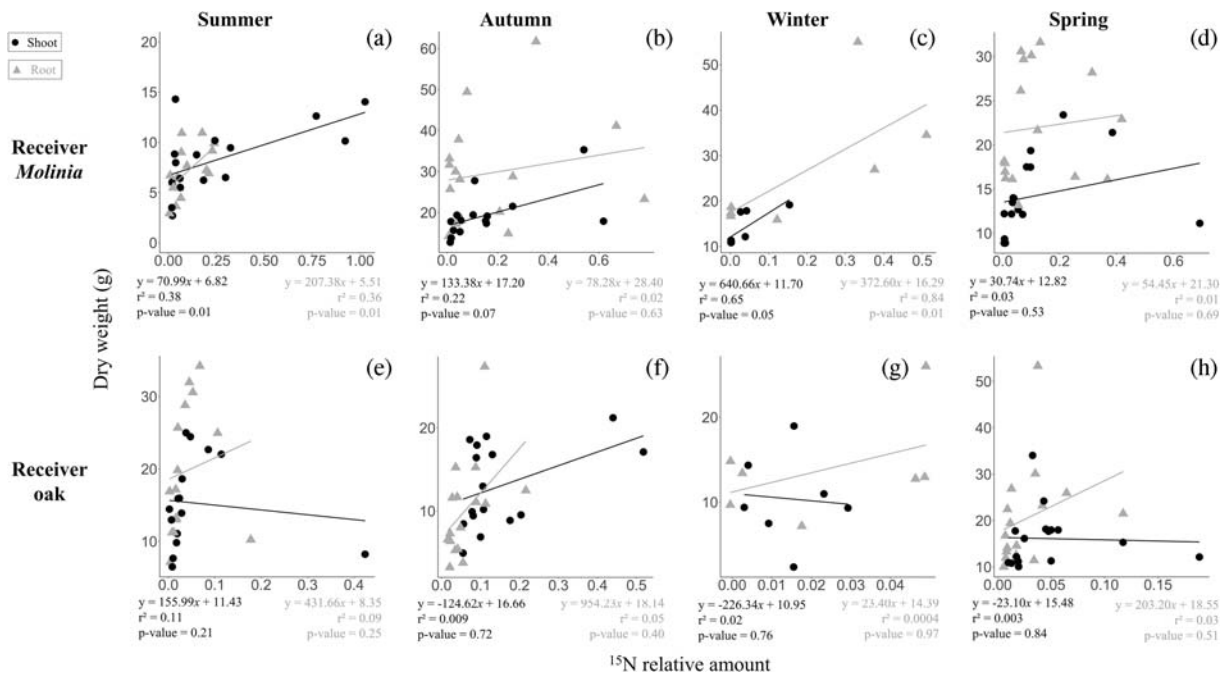
Our results show that *Molinia* biomass was rapidly favoured (within a few months) when mixed-grown with oak. Tracking  $^{15}\text{N}$  fate validated our hypothesis that the presence of *Molinia* in the same pot with oak drove higher oak root N release for the benefit of *Molinia*.

### Oak facilitated *Molinia* growth

The positive effect of oak seedlings on *Molinia* appeared in the early stage of cohabitation. When mixed-grown with oak, *Molinia* above- and belowground biomass values were greater from autumn and winter onwards, respectively, than those measured in *Molinia* sole-grown. These results are in line with previous findings that highlighted a facilitative effect of oak saplings on grass (Vernay et al. 2018). Unexpectedly, we did not find a detrimental effect of *M. caerulea* on oak seedling biomass. These results are thus not consistent with antagonistic facilitation, which had been shown in a previous experiment when *Q. petraea* and *D. cespitosa* were grown together, possibly owing to higher density of tufts in pots (Vernay et al. 2018). No significant biomass difference was observed in oak among seasons, not even when mixed-grown. Low growth may have failed to reveal an effect of *Molinia* on oak seedling growth.

### N allocation pattern in oak was affected by the presence of *Molinia*

After oak shoot labelling by the cotton wick method,  $^{15}\text{N}$  was rapidly found in oak stems and roots in summer. As expected, a higher  $^{15}\text{N}$  allocation from shoots towards roots was observed in autumn and winter irrespective of the treatment (*i.e.* mixed-grown or sole-grown), in accordance with the conservative strategy of oaks.  $^{15}\text{N}$  in roots was then re-allocated towards shoots to sustain bud break and early growth of emerging leaflets (Millard 1994). Interestingly, *Molinia* presence accentuated both allocations of  $^{15}\text{N}$  to roots during winter and remobilization of  $^{15}\text{N}$  towards shoots in oak during spring. Larger N mobilization to leaves might improve C capture, and ultimately shoot and root growth in oak mixed-grown with



**Fig. 5** Correlation between dry weight and  $^{15}\text{N}$  relative amount in receiver *Molinia* in summer (a), autumn (b), winter (c) and spring (d) and in receiver oak in summer (e), autumn (f), winter (g) and

spring (h). Dark point and regression line represent shoots and grey triangle and regression line represent roots. Equation line (y),  $r^2$  and  $p$ -value are reported above each graph

*Molinia* (Bhatt et al. 2011). However, N distribution to roots was uncorrelated with concomitant biomass increment, suggesting greater N storage. Such a conservative strategy has already been reported, namely higher N allocation in oak roots than in oak shoots mixed-grown with *D. cespitosa* (Vernay et al. 2018). In spring, harvest coincided with bud bursting and early foliar development, a period generally associated with N remobilization (see Millard and Gwen.-Aelle 2010 for a review), which was particularly accentuated from roots to shoots in oak grown with *Molinia*. One explanation for this observation might be a greater soil N depletion by *Molinia*, leading to a higher demand for internal N remobilization in oak to sustain budburst and leaf development (Nielsen et al. 2000; Bausenwein et al. 2001). It has been shown that the level of N availability during bud break in oak is a critical factor affecting leaf C capture (Bazot et al. 2016; Vernay et al. 2016, 2018). Thus the magnitude of internal N recycling from roots might not depend only on limited soil inorganic N amounts but also on neighbour presence and neighbour identity (Welker et al. 1991; Kaitaniemi et al. 2018). Moreover, some internal N can be released in soil according to neighbour presence and identity (Dhamala et al. 2016).

A non-negligible part of  $^{15}\text{N}$  is found in the soil

Nitrogen rhizodeposition processes have been described in legume and grass species and are known to occur a few days after labelling (Ribeiro Paula et al. 2015). Conversely, in fruit trees, N rhizodeposition is mainly attributed to root mortality, so  $^{15}\text{N}$  is found in soil to a large extent in winter (Scandellari et al. 2010). However, in our study, oak seedlings exuded  $^{15}\text{N}$  during the first weeks after labelling, and the presence of a neighbour modified N flux from roots to soil. We hypothesized that neighbours (both oak and *Molinia*) might exude specific molecular entities to increase active exudation by oak roots. Abiotic factors influencing root rhizodeposition such as soil nitrogen availability or  $\text{CO}_2$  level (Hale et al. 1971; Philipps et al. 2006; de Graaff et al. 2007; Tückmantel et al. 2017; Bowsher et al. 2018) and interaction through root exudates (Suriyagoda et al. 2012; Semchenko et al. 2014; Mommer et al. 2016) are well-described. Recent studies on belowground signalling interactions have demonstrated that neighbour detection, response strategies and communication are mediated by root-secreted signalling chemicals (Chen et al. 2012; Rasmann and Turlings 2016; Kong et al. 2018;

Canarini et al. 2019; Huang et al. 2019). Intraspecific neighbours can improve or impair nutrient uptake of nitrogen, involving both a mutually beneficial cooperative relationship and a competitive relationship among neighbours (Hong et al. 2017). However, to our knowledge, the effect of neighbour presence and identity on nutrient rhizodeposition has not been described. This is therefore probably the first time that such a flux from roots to soil in oak-*Molinia* interference has been characterized and given an ecologically sound basis.

In winter, the increase of  $^{15}\text{N}$  in soil was probably due to the shedding of fine roots and sloughing of living cells (Scandellari et al. 2010).

N transfer between plants may contribute to the growth facilitation process

In *Molinia*, biomass increase was positively correlated with  $^{15}\text{N}$  relative amounts in shoots and roots (Fig. 5 and Table S16) especially in summer, autumn and winter (Fig. 5a, b and c, respectively). Conversely, the relationship was never significant for receiver oak, whatever the season. Overall, these results suggest that *Molinia* was able to obtain  $^{15}\text{N}$  from oak for its own growth (Fig. 5e, f, g and h). These results support our hypothesis of *Molinia* growth facilitation by oak seedlings through N transfer. It emphasizes the importance of the strategy and identity of neighbours in influencing plant-plant N transfer.

Our experiment clearly shows that a significant albeit relatively modest amount of  $^{15}\text{N}$  was transferred from oak to *Molinia* (maximum 6.5% of the total  $^{15}\text{N}$  found in all compartments). However, we used a pulse labelling technique (at a given limited time) so that we do not know what the contribution of oak N was to the nutrition of *Molinia* over the whole season with a permanent N flux. Previous reports on the contribution of N released by a donor to total N amount in a receiver species showed that N amount varied widely according to combination of species, species age and the method used to estimate N transfer (Teste et al. 2015; Montesinos-Navarro et al. 2017). This contribution ranged from 5% (Frey and Schüepp 1993) to 80% in soybean-weed (Moyer-Henry et al. 2006). The cotton wick method was most often used on non-woody plants, but the technique can succeed on tree seedlings as demonstrated. Such transfer from plant to plant has been extensively described for carbon (Simard et al. 1997b; Simard and Perry 1997; Klein 2016) but this is the first time to our

knowledge that N transfer from a non-fixing nitrogen species to a graminoid has been evidenced at the very early stage of development in oak. This underlines the importance of nutrient exchanges, such as that of nitrogen, occurring below ground.

What are the different possible pathways of N transfer between plants?

When plants were grown with a 1  $\mu\text{m}$  mesh as a barrier to roots and mycorrhizal hyphae,  $^{15}\text{N}$  was found in receiver neighbour and soil, suggesting that some N transfer occurred as free molecules moving through the mesh (Figure S2 and Table S6). Mesh30 was designed to let through mycorrhizal hyphae, and a significant  $^{15}\text{N}$  amount was found on the other side, at a level higher than in Mesh1, at least in autumn. This suggests that mycorrhizae might contribute to N transfers, in line with other studies (He et al. 2003, 2006, 2009; Govindarajulu et al. 2005; Jalonen et al. 2009), but our results do not enable us to conclude on the role of mycorrhizae in N transfer between oak and *Molinia*. Further experiments are now required to identify and quantify mycorrhizal networks in both oak and *Molinia* roots. We hypothesized that only vesicular arbuscular mycorrhizae (VAM) were involved in N transfer between young oak and *Molinia*, as *Molinia* is only colonized by VAM (Taylor et al. 2001). However, to our knowledge, it is not known whether *Q. petraea* is colonized by VAM, although it has been observed in other oak species (Dickie 2001).

Unexpectedly,  $^{15}\text{N}$  was also found in the neighbouring pot in the separate-pot treatment (sole-grown).  $^{15}\text{N}$  amounts were significantly different from natural abundance in many cases (Table S15), although the difference was small (1% at most). In receiver oak,  $^{15}\text{N}$  was first different from zero in shoots (summer), in roots (autumn) and then in soil (winter). In spring,  $^{15}\text{N}$  was significantly different from zero in all compartments. This N flux suggests emission of volatile nitrogen compounds from donor oak and uptake by its neighbour, first in the aboveground part. The ability of plants to take up nitrogen by leaves has been demonstrated in several studies (Wittwer and Teubner 1959; Eilers et al. 1992; Feng et al. 2015; Guo et al. 2017). After above-ground uptake, N may have been transferred to roots and translocated from roots to soil during dormancy. This spatial and temporal pathway from shoot to root, and then to soil, suggests that volatile

$^{15}\text{N}$  was first absorbed by shoots, then transferred to roots in autumn, and rhizodeposited in winter.

In *Molinia* sole-grown,  $^{15}\text{N}$  amount was different from zero in each condition except in roots in summer ( $p < 0.15$ ) and in soil in winter ( $p < 0.095$ ), supporting the hypothesis of volatile nitrogen compounds. Emissions of volatile organic compounds by plants is known (Fehsenfeld et al. 1992; Guenther et al. 1995; Holzinger et al. 2000), but only a few studies have described emissions of volatile nitrogen compounds by above-ground organs (Tukey 1966). To our knowledge it is not known whether volatile nitrogen transfer between plants occurs, or to what extent  $^{15}\text{N}$  in receiver neighbours could derive from soil N volatilization. The belowground pathway is still the main contributor to N transfer from oak to either oak or *Molinia* (Table S17 – Ratio MG/SG). Additionally, total  $^{15}\text{N}$  amount in all pots decreased across seasons especially between August and October (June–August: –9%; August–October: –31%; October–February: –0.4%; February–May: –18%) supporting the hypothesis of soil  $^{15}\text{N}$  loss. Involvement of microbial communities in nitrogen transformation (Robertson and Groffman 2007; Hayatsu et al. 2008) can explain  $^{15}\text{N}$  diminution across seasons. Although the impact of rhizodeposits on soil microorganisms has been well-studied in the past 20 years (Paterson et al. 2006; Wichern et al. 2008; Schenck Zu Schweinsberg-Mickan et al. 2012), it would be of interest to characterize more precisely (quantitatively and qualitatively) their role in rhizodeposition processes, according to species, and how it can impact on nutrient transfer (Gorka et al. 2019).

## Conclusions

Our experiment clearly demonstrates N transfer from oak to *Molinia* qualitatively. However, pulse-chase labelling only enabled us to track the fate of  $^{15}\text{N}$  supplied in summer. A continuous supply of  $^{15}\text{N}$  throughout the whole experiment would have allowed quantification of the actual N amount effectively transferred from oak to *Molinia*. Compartmental modelling based on testing different pathways of N transfer may help identify and rank these fluxes. Soil properties and microorganisms such as mycorrhizae need to be considered in further studies to gain a better understanding of transfer processes between plants. Some substances might be emitted by roots and/or shoots: such allelopathic substances have not been well-identified and their role in such interference is under-

researched. Applying exudates from *Molinia* roots around oak seedlings to monitor any changes in growth and functioning (such as mycorrhizal symbiosis) and then analysing these exudates chemically may prove helpful in identifying allelopathic substances.

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