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RESEARCH ARTICLE

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Tripartite symbioses regulate plant–soil feedback in alder

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Abstract

1. Plant–soil feedbacks regulate plant productivity and diversity, but potential mechanisms underpinning such feedbacks, such as the allocation of recent plant assimilate, remain largely untested especially for plants forming tripartite symbioses.
2. We tested how soils from under alder *Alnus glutinosa* and beneath other species of the same and different families affected alder growth and nutrition, and colonization of roots by nitrogen-fixing *Frankia* bacteria and ectomycorrhizal fungi. We also measured how the soil environment affected carbon capture and allocation by pulse labelling seedlings with $^{13}CO_2$. We then tested for linkages between foliar nutrient stoichiometry and carbon capture and allocation and soil origin using statistical modelling approaches.
3. Performance of alder and nitrogen nutrition were best on home and birch *Betula pendula* soils (both Betulaceae), whereas performance on Douglas fir *Pseudotsuga menziesii* (Pinaceae) soil was poor. Plants growing in *P. menziesii* soil were virtually devoid of *Frankia* and ectomycorrhizas, and the natural abundance ^{15}N signatures of leaves were more enriched indicating distinct nitrogen acquisition pathways. Seedlings in these soils also had smaller ^{13}C fixation and root allocation rates, leading to smaller ^{13}C respiration rates by microbes.
4. Statistical models showed that the best predictors of foliar N concentration were ^{13}C allocation rates to fine roots and net CO_2 exchange from the mesocosms. The best predictors for foliar phosphorus concentration were net CO_2 exchange from the mesocosms and soil origin; seedlings in home soils tended to have greater foliar phosphorus compared to birch soils while seedlings from Douglas fir soils were no different from the other treatments. Foliar phosphorus concentration was not correlated with plant available or total soil phosphorus for any of the soils. Home soils also resulted in distinct ectomycorrhizal communities on seedlings roots, which could be responsible for greater foliar phosphorus concentration.
5. Our findings show how the association of alder with nitrogen-fixing *Frankia* relieved nitrogen limitation in the seedling triggering a performance feedback loop. We propose that relief of nitrogen limitation likely increases plant phosphorus demand, which may promote the formation of ectomycorrhizas in nutrient-deficient soils. The formation of tripartite symbioses therefore generates positive plant–soil

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feedbacks, which enables plants to acquire mineral nutrients otherwise inaccessible in trade for carbon.

KEYWORDS

Alnus glutinosa, carbon allocation, carbon-13, ectomycorrhiza, *Frankia*, nitrogen, phosphorus

1 | INTRODUCTION

Plant–soil feedback occurs when reciprocal interactions with abiotic and biotic components of the soil environment lead to positive or negative effects on plant performance (Bever et al., 2010). Feedbacks can shape the composition of vegetation communities (Bever, 2003; Bever et al., 1997; Klironomos, 2002; Kulmatiski et al., 2012) and are often influenced by interactions with soil micro-organisms (Bennett & Klironomos, 2019; Van der Putten et al., 2013). For example, interactions with soil biota have direct effects on plant nutrition or health, such as mycorrhizal and pathogenic fungi, and nitrogen (N_2-)-fixing bacteria strongly influence plant–soil feedbacks (Bennett et al., 2017; Kardol et al., 2013; Revillini et al., 2016). Classic plant–soil feedback experimental designs rely on the comparison in plant performance between 'home' (conspecific) soil and sterilized soil, or 'home' and 'away' (heterospecific) soil (Brinkman et al., 2010). However, a key open question is as follows: what regulates the relative effects of competitors, mutualists and pathogens from home and away soils? The exchange of goods and services provided by soil micro-organisms is strongly influenced by resource availability and the allocation of photosynthate below-ground, but this idea remains largely untested in the context of plant–soil feedback experiments.

Moreover, most fundamental work on plant–soil feedback has been conducted in grassland, and much less is known about mechanisms regulating feedbacks for trees (Kulmatiski et al., 2012; Lekberg et al., 2018). In one of the few studies addressing the importance on mycorrhizal type on plant–soil feedbacks, the productivity of ectomycorrhizal (ECM) trees was greater in their own soil compared to away soils, whereas arbuscular mycorrhizal (AM) trees generally performed worse in home soil (Bennett et al., 2017). Indeed, recent findings suggest a key role of extraradical fungal networks produced by different mycorrhizal types in regulating the direction of plant–soil feedbacks (Liang et al., 2020, 2021). In line with these findings, Teste et al. (2017) reported that plant responses to soil biota differed according to their nutrient acquisition strategy, including positive feedbacks for ECM plants and negative feedback for N-fixing plants. The mechanisms underlying such effects are unclear but the ability to resist pathogens, while maintaining associations with beneficial nutritional symbionts, is likely to be a key factor.

An intriguing adaptation that may exaggerate positive effects of mutualists is the formation of tripartite symbioses. In trees, genera such as *Alnus* forms associations with both ECM fungi and N_2-fixing bacteria in the genus *Frankia* (Kennedy & Hill, 2010; Molina et al., 1993; Tedersoo et al., 2009). Such tripartite associations may have striking effects on plant–soil feedback through the contrasting impacts of the symbionts on host tree nutrition, and thus species forming tripartite symbioses may be ideal models to test fundamental ecophysiological drivers of plant–soil feedback. For example, *Frankia* may have affected coevolution between ECM fungal communities and *Alnus* by shifting host nutritional needs such that they favour fungi adept at acquiring growth-limiting nutrients other than from N, notably phosphorus (P). *Alnus* species may therefore selectively associate with ECM fungi that have enhanced abilities towards P acquisition (Giardina, 1995; Houlton et al., 2008; Johnson et al., 2010; Nasto et al., 2014). Indeed, some studies suggest that ECM fungal communities associated with *Alnus* trees are especially proficient at P acquisition (Chatarpaul et al., 1989; Horton et al., 2013; Walker et al., 2014). The requirement to form symbioses with both *Frankia* and ECM fungi to acquire different nutrients may therefore be important determinants of plant–soil feedback in plants forming tripartite symbioses and provide a putative mechanism underpinning plant–soil feedback in these species.

If the requirement for mineral nutrients regulates plant–soil feedback, then inevitably, provision of carbon (C) or other plant-derived resources to symbiotic microbial communities becomes an important consideration (Kiers & Denison, 2008; Smith & Read, 2008). While we still lack detailed quantification of the amount of C plants allocate to mycorrhizal fungi across species, growth stages and environmental contexts, the current evidence suggests plants allocate in the region of 5%–20% of photosynthate to mycorrhizal fungi (Smith & Read, 2008). Biological market models have been developed to describe how the inter-play between mineral nutrient uptake and allocation of plant-derived C to symbionts can shape plant fitness (or indices of fitness) (Hoeksema & Schwartz, 2003; Johnson, 2010; Schwartz & Hoeksema, 1998; Werner et al., 2014) and even provide a mechanism for the stability of the symbiosis (Kiers et al., 2011). Biological market models suggest that in order for a symbiosis to act as a mutualism, the amount of C allocated to symbiotic microbes should be smaller than the amount of C needed to directly uptake those nutrients by roots. These models therefore suggest that the outcome of nutritionally based symbiotic relationships is dependent on the availabilities of photosynthate and mineral nutrients to each partner.

A further feature of soil microbial symbionts relevant to understanding plant–soil feedbacks is the relationship between their specificity and functionality (Revillini et al., 2016). If such a relationship exists, then the degree of specificity shown by fungal and bacterial symbionts to their host plants is also likely to be an important factor determining net positive or negative plant–soil feedback.

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We therefore aimed to link plant–soil feedback effects in trees to key ecophysiological processes and the availability of compatible soil-borne mutualists. We tested how conspecific or heterospecific soil influences fitness proxies (growth and nutrition) of *Alnus* seedlings, and related these effects to the availability of soil symbionts (ECM fungi and *Frankia*) and below-ground allocation of recent photosynthate using a ^{13}C tracer experiment. We grew alder seedlings in independent soil mesocosms from monospecific stands of alder (*Alnus glutinosa* [L.] Gaertn.) (home soil), birch (*Betula pendula* Roth) (family soil) and Douglas fir (*Pseudotsuga menziesii* [Mirb.] Franco) (away soil). First, we tested how soil origin and its associated symbiotic partners shaped alder seedling performance. We predicted that (1) the overall performance of seedlings grown in home soils would be superior to other soils given the presence of specialist symbionts, leading to positive plant–soil feedback. Second, we tested (2) the relationship between seedling nutritional status and allocation of ^{13}C to fine roots and symbiotic partners in home, family and away soils. We predicted that (2a) leaf N content in seedlings would be tightly linked to *Frankia* nodulation and to C allocation below-ground; and (2b) P content in leaves would be greater in seedlings grown in home soils because specialist ECM fungi enhance P acquisition. These hypotheses were tested using mesocosms comprising intact soil cores collected from the field and maintained in a controlled environment facility. This approach enabled us to conduct a suite of analyses related to carbon and mineral nutrient cycling and interactions with key biodiversity groups to gain mechanistic insight into plant–soil feedbacks.

2 | MATERIALS AND METHODS

2.1 | Soil core collection and mesocosm setup

Mesocosms were created by collecting intact soil cores from under pure alder, birch and Douglas fir (10 cores per species, $N = 30$ in total) stands from the Crathes Estate (Aberdeenshire, Scotland, UK; 57.0588°N, 2.4139°W). We selected stands that covered a minimum area of 16 m × 16 m and were surrounded by a minimum 3 m buffer zone to nearest neighbouring tree of different species. Within each stand, we established a 10 m × 10 m grid, and selected sampling locations based on randomly generated sets of coordinates. We hammered 80 mm diameter × 200 mm length PVC pipe sections into the soil, removed the intact cores and fixed a mesh over the base with duct tape to allow drainage. The mesocosms were transported back to the laboratory and placed in a climate chamber at 20°C, with 14-hr daylight (PFD: 200 μmol m^{-2} s^{-1}).

From each mesocosm, we removed two 20-mm diameter soil cores and re-filled each hole by inserting a 20 cm × 15 mm plastic tube with open windows covered by 40 μm root-excluding nylon mesh and filled by twice-autoclaved field-collected soil with a high sand content (Culbin Forest, Morayshire, Scotland). These cores exclude roots but allow ingrowth of extra-radical fungal mycelium (Johnson et al., 2001, 2002a). The root-excluding cores were used to capture respiration of recent photosynthate as part of the pulse labelling experiment (see below).

Alder seeds (Forestart Ltd) were surface sterilized (30% H_2O_2 for 20 min) and germinated on sterile, 1% water agar. One non-mycorrhizal alder seedling was transplanted into each of 10 mesocosms from each of the three stands once they had developed 4–6 leaves. Seedling height and number of leaves were recorded at planting, and monthly thereafter until harvest. Mesocosms were harvested after 6 months, and plant height data indicated most plants were actively growing.

2.2 | Mesocosm $^{13}CO_2$ isotope pulse labelling, harvest and tracing of ^{13}C-photosynthate

Two days before harvest, the mesocosms were pulse labelled with synthetic air in which the CO_2 was replaced with 99 atom% ^{13}C-CO_2 at 380 ppm (BOC Group plc). Mesocosms were transferred to a bespoke transparent chamber (2 m^3) within the controlled environment facility, whereupon we placed an Eppendorf tube containing 0.5 ml of 1 M NaOH placed in the headspace of the root-excluding mesh cores (Johnson et al., 2002b). Leaf samples were clipped from each seedling twice before the labelling (to determine the natural abundance of ^{13}C). The plants were then provided with a continuous flow of ^{13}C-enriched air for 6 hr that maintained ambient CO_2 concentrations. Further leaf samples were taken immediately after labelling (to estimate ^{13}C captured), and then every 12 hr until harvest (48-hr post-label) to estimate the rate of ^{13}C loss from leaves due to respiration and reallocation of photosynthate. This sampling generated five enriched and two natural abundance leaf samples per plant in total. During the experiment, we harvested on average 215 ± 25 mg, 194 ± 22 mg and 141 ± 15 mg leaf material from seedlings grown in home family and away soils, respectively. We expect the small amount of tissue removed for these analyses would have a negligible impact on fluxes of ^{13}C or other aspects of plant performance. At each leaf sampling point, we also captured in the NaOH traps the release of ^{13}C-CO_2 from the root-exclusion mesh cores to give a relative measure of the dynamics of ^{13}C released from microbial (including fungal) activity.

Whole-system CO_2 flux was measured on each mesocosm prior to harvest (Licor 8100 portable IRGA) under light and dark conditions in the growth chamber. Net CO_2 exchange was later calculated as a measure of C accumulation in the mesocosms (negative values reflecting C accumulation). Shoots, roots, soil and in-growth cores were all harvested separately. Specific leaf area (SLA) for each seedling was measured following Pérez-Harguindeguy et al. (2013). All soils were gently rinsed from the fresh root systems, and roots were separated into primary root, lateral roots and fine roots (≤2 mm), and all *Frankia* nodules were removed. Ectomycorrhizal colonization (%) was estimated by randomly sampling 10 cm sections of the fine root fraction, and observing (typically) 50–100 root tips on each section under a dissecting microscope. Mycorrhizal tips were morphotyped (Agerer, 2001), and representative subsamples removed for molecular analysis (see below for details). All shoot and root components were dried at 80°C for 48 hr and weighed. The leaf samples were

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milled in a ball mill and analysed for total foliar C, N and P in the same manner as soil samples.

Ground leaf and fine root samples were analysed for ^{13}C content by cavity ring-down laser spectrometry using a Picarro G2201-i coupled with a combustion module and Small Sample Isotope Module, and calibrated against certified reference gases (Air Liquide UK Ltd). The amount of ^{13}C initially taken-up by plants during the labelling period was estimated from the ^{13}C concentration of leaf tissue removed just after labelling, and scaled up to absolute amounts based on the total C content of all green leaves, harvested at the end of the pulse-chase period. Allocation of ^{13}C below-ground was partitioned into fine roots and to *Frankia* nodules (5 nodules analysed per seedling). To release CO_2 from the NaOH traps, we transferred a 0.1-ml aliquot of NaOH to hydrogen-flushed exetainers (Labco, UK) and added 0.5 ml of 1.3 M H_3PO_4 to acidify the solution. The samples were analysed for $^{13}C/^{12}C$ ratio by either continuous-flow isotope ratio mass spectrometry (CF-IRMS) at the James Hutton Institute, Scotland UK, or by cavity ring-down spectrometry, using suitably cross-calibrated instruments. In addition, a subset of samples was analysed for foliar ^{15}N natural abundance using CF-IRMS.

2.3 | Soil nutrient pools

Total and exchangeable nutrient pools were measured. To assess exchangeable K, Ca, Mg, P, NO_3^- and NH_4^+, nutrient supply rates were measured using in situ ion exchange membranes (PRS™ Probes; Western AG). At harvest, soil from each mesocosm was homogenized and analysed for total concentrations of C, N and P. Carbon and N were analysed with an elemental analyser (Vario EL Cube, Elementar) and P using a colorimetric segmented flow analyser AA3 (Seal Analytical, UK). The nutrient pools for each soil origin are summarized in Table 1.

2.4 | Root tip fungal DNA extraction sequencing

DNA was extracted from multiple representative tips of each ECM morphotype using 10 μl of Extract-N-Amp, and following the manufacturer's protocol (Sigma). The fungal-specific primer combination ITS1f and ITS4 was used to amplify fungal DNA. Briefly, an aliquot of 1 μl of extracted DNA was combined with 10 μl of Extract-N-Amp PCR solution in a 20 μl reaction. The amplifications consisted of an initial denaturation at 94°C for 1 min, followed by 35 cycles 94°C for 30 s, 51°C for 20 s, 72°C for 1 min, with a final extension of 72°C for 8 min. Successful PCR products were purified using ExoSAP-IT (USB). Samples were sequenced by the Genomic Services facility at the University of Manchester. Raw sequence data were processed using BioEdit software and converted into FASTA format prior to comparison with the UNITE database (Kõljalg et al., 2005, 2013). A representative sequence from each ECM taxon was submitted to GenBank under the accession numbers MT499907–MT499914.

2.5 | Data analysis

To test hypothesis 1, seedling performance metrics were analysed with one-way ANOVA using soil origin as a factor, followed by Tukey post-hoc pairwise comparisons to identify differences between treatments. When assumptions of normality and homoscedasticity were not met, the effect of soil origin was tested using log-transformed data. Net CO_2 exchange was calculated as the difference between light and dark CO_2 measurements, with negative values indicating accumulation of C in mesocosms. Principal component analysis was performed on (a) seedling performance and (b) soil chemistry metrics, and biplots were generated with package FACTOEXTRA (Kassambara & Mundt, 2020) to identify trends in the dataset.

One-way ANOVA was also used to test how soil origin affected ^{13}C and ^{15}N pools and fluxes. In the cases where assumptions were not met, data were analysed by a nonparametric Dunn test (dunn.test) followed by Holm–Sidak post-hoc pairwise comparisons. The ^{13}C pool in each compartment of the experimental mesocosms was calculated by multiplying the ^{13}C-atom% excess with the C pool (mg) in each compartment. The ^{13}C-atom% excess values were obtained by converting $\delta^{13}C$ values (‰) to ^{13}C values (^{13}C-atom%) and then by subtracting the ^{13}C-atom% of unlabelled (natural abundance) leaf samples. The percentage ^{13}C allocated to fine roots and *Frankia* from

TABLE 1 Mean concentrations of selected soil nutrients in experimental mesocosms with soil from home (alder; Ag), family (birch; Bp) and away (Douglas fir; Pm). Values represent mean nutrient contents and associated standard errors. $N = 10$. A PCA biplot representing all nutrients across soil origin can be found in Figure S1

	Ag soil		Bp soil		Pm soil	
Variable	Mean	*SE*	Mean	*SE*	Mean	*SE*
Total C (%)	11.13	1.77	6.53	0.53	18.60	3.05
Total N (%)	0.59	0.05	0.45	0.03	0.85	0.12
Total P (%)	0.07	0.01	0.09	0.01	0.08	0.00
NH_4^+ (μg/10 cm^2 per 21 days)	3.37	1.58	8.68	4.02	35.86	9.50
NO_3^- (μg/10 cm^2 per 21 days)	84.63	20.20	39.85	11.58	49.10	16.34
P (μg/10 cm^2 per 21 days)	1.46	0.37	0.96	0.30	25.65	6.14

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the total ^{13}C initially fixed were estimated 2 days after labelling. To dissect the C allocation strategy in each mesocosm treatment, ^{13}C allocation data were analysed in two ways. First, the dynamics of C assimilation and transfer in the system were compared using $\delta^{13}C$ values (i.e. relative abundance of ^{13}C) in leaves, fine roots, nodules and microbial respiration (from analysis of ^{13}C-CO_2 in the headspace of the root-excluding cores). Second, the total mass of ^{13}C in the system was calculated and the percentage of ^{13}C allocation to fine roots and *Frankia* estimated from the total ^{13}C-photosynthate pool 2 days after pulse labelling.

To test hypothesis 2, linear models representing C-for-nutrient trade were fitted to test whether seedling C allocation to the different mesocosm compartments is associated with seedling nutritional status. This approach controls for physiological variation within seedlings that could mask soil origin effects on foliar stoichiometry. The following metrics related to C capture and allocation were added to the models: (a) seedling biomass, (b) foliar C concentration, (c) net CO_2 exchange, which accounts for C accumulation in the mesocosm, (d) foliar $\delta^{13}C$ after labelling, which serves as a proxy for photosynthetic rate, (e) $\delta^{13}C$ in fine roots, which serves as a proxy for allocation rate to fine roots (and symbionts, given the strong correlation between $\delta^{13}C$ values in fine roots and *Frankia* nodules) and (f) $\delta^{13}C$ microbial respiration rate at 12 hr (peak microbial respiration; Figure S8). This set of variables includes those representing both long-term (seedling biomass, foliar C concentration) and short-term (^{13}C assimilation and transfer rates in the mesocosm) C dynamics. Separate 'global' models were used for either foliar N or P concentrations (as response variables), and both sets of models used metrics related to C capture and allocation and soil origin as explanatory variables. Spearman coefficients between C capture and allocation variables were calculated to check for multicollinearity among variables (Figure S4). All continuous explanatory and response variables were scaled to a mean of 0 and a standard deviation of 1, which allowed the comparison of effect sizes between explanatory and response variables.

A model selection and averaging approach, based on Burnham and Anderson (2002), was used to identify the most parsimonious model. First, a full submodel set was generated (including the null model) from the global model with the dredge function implemented in the MuMIn package (Bartón, 2019). In a first set of models (N1 and P1), the model set was restricted to those models containing the factor soil origin (Soil), while in the second set of models (N2 and P2) soil origin was excluded, which allowed to test whether models excluding soil origin could potentially provide a superior fit to the data. The global model structure (in R code) was as follows:

Model N1

$$\text{foliar N} = CO_2\text{flux} + \text{biomass} + \text{foliar C} + \delta^{13}\text{C leaves} + \delta^{13}\text{C fine roots} + \delta^{13}\text{C - } CO_2 + \text{Soil}.$$

Model N2

$$\text{foliar N} = CO_2\text{flux} + \text{biomass} + \text{foliar C} + \delta^{13}\text{C leaves} + \delta^{13}\text{C fine roots} + \delta^{13}\text{C - } CO_2$$

Model P1

$$\text{foliar P} = CO_2\text{flux} + \text{biomass} + \text{foliar C} + \delta^{13}\text{C leaves} + \delta^{13}\text{C fine roots} + \delta^{13}\text{C - } CO_2 + \text{Soil}$$

Model P2

$$\text{foliar P} = CO_2\text{flux} + \text{biomass} + \text{foliar C} + \delta^{13}\text{C leaves} + \delta^{13}\text{C fine roots} + \delta^{13}\text{C - } CO_2$$

The best models were selected using Akaike's information criterion for small sample sizes (AICc). Models within 4 AICc units of that with the lowest AICc were considered as receiving support from the data and were used in model averaging (Burnham et al., 2011). Parameter estimates, their unconditional standard errors, interval of confidence (95%) and relative importance of the predictors were calculated. Variance inflation factors (VIFs) were calculated for the predictors included in the best models with the car package, and no multicollinearity was detected (VIFs < 2). Assumptions of linearity and homogeneity of variances on residuals from the best models were checked graphically. All statistical analyses were performed using R version 3.5.2.

3 | RESULTS

3.1 | Role of soil origin in seedling performance and abundance of symbionts

Soil origin had a significant effect on seedling productivity traits (Figure 1) and explained ~67% of variation in these traits. Seedlings grown in home and family soils were on average twice the height ($p < 0.001$, $F = 10.89$, $df = 2, 27$), had two times foliar biomass ($p < 0.010$, $F = 5.90$, $df = 2, 27$) and greater above-ground:below-ground ratio ($p < 0.001$, $F = 11.34$, $df = 2, 27$) than seedlings grown in away soils. Seedlings from home and family mesocosms had on average three times more total biomass than plants from away soils, but this effect was non-significant ($p = 0.088$, $F = 2.56$, $df = 2, 27$), due to the large amount of variation within treatments (Figure 2). Fine root biomass allocation was (in proportion) greater for seedlings grown on away soils than seedlings grown in home soils ($p = 0.023$, $F = 4.38$ $df = 2, 27$). Overall, seedlings grown in home and family soils allocated more biomass above-ground while seedlings on away soil allocated more biomass below-ground, particularly fine roots (Figures 1 and 2).

All seedlings grown in home and family soils were associated with *Frankia*. By contrast, only one seedling in away soil was associated with a single *Frankia* nodule. Likewise, only three seedlings grown on away soil formed ectomycorrhizas, and <5% of root tips were colonized. All seedlings from home and family soils were colonized by ECM fungi (ranging from 25% to 100% with an average colonization of 60.5% in home soil, and from 1% to 95% with an average colonization of 49.6% in family soil). *Frankia* nodule biomass and root tip colonization were positively correlated ($\rho = 0.67$, $p < 0.001$). Both nodule biomass and the proportion of ECM root tips

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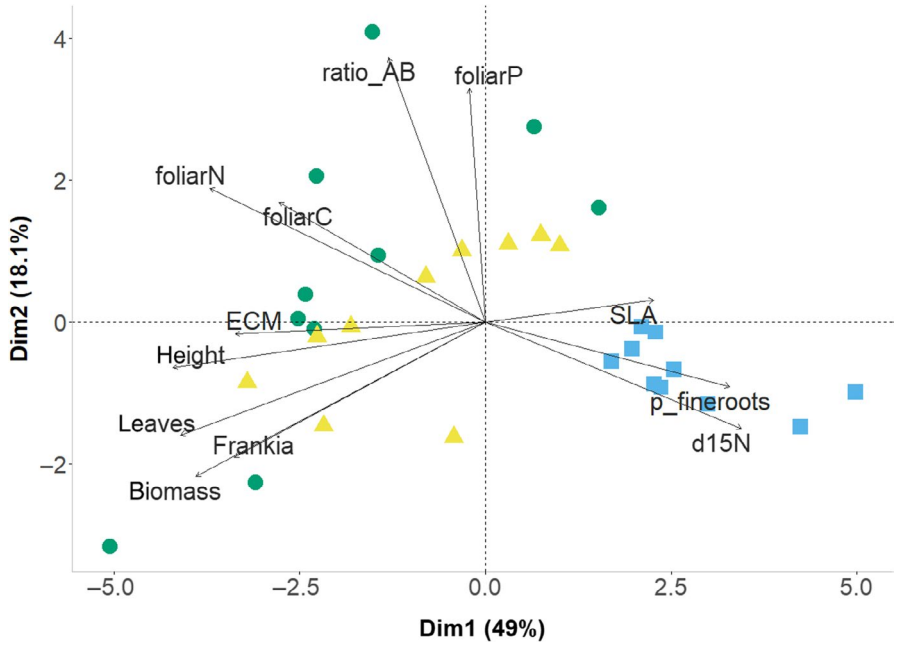


FIGURE 1 PCA biplot for the first two component axes showing alder performance traits when grown on home alder (Ag, circle), family birch (Bp, triangle) and away Douglas fir (Pm, square) soil. $N = 10$. Plant traits: biomass (total dry weight), height, leaf dry weight (leaves), ratio_AB (above-ground:below-ground biomass ratio), SLA (specific leaf area), p_fineroots (fine root:below-ground biomass ratio), *Frankia* (nodule dry weight), ECM (% colonization), foliar C (%C in leaves), foliar N (%N in leaves), foliar P (%P in leaves), d15N (leaf natural abundance $\delta^{15}N$)

were consistently correlated with seedling foliar N (*Frankia* $\rho = 0.73$, $p < 0.001$; ECM $\rho = 0.62$, $p < 0.001$). However, nodule biomass was strongly correlated with seedling productivity (biomass, $\rho = 0.81$, $p < 0.001$, Figure 1) while the strength of the correlation was much weaker for ECM root tips ($\rho = 0.43$, $p < 0.05$; Figure S2).

We successfully identified fungal taxa on 62 root tips from home, 47 root tips from family and 6 root tips from away soils (Table S1). The most common ECM fungus was *Tomentella* sp. 1, which comprised 60% of the identified root tips in home and family soils. We identified two ECM fungal species exclusively in seedlings grown in home soils, namely *Alnicola sphagneti* and *Alnicola alnetorum*.

Natural abundance foliar $\delta^{15}N$ separated plants according to whether or not they were grown in home or away soil ($p < 0.001$, $\chi^2 = 19.38$, $df = 2$; Figure 1) and these data were associated with the presence of symbionts rather than related to biomass. Seedlings grown on home and family were depleted in ^{15}N (average of −1.12 and −0.80, respectively), while seedlings grown in away soils were enriched (average of 7.46; Figure 2). Net CO_2 exchange tended to be more negative in home and family mesocosms than away mesocosms, indicating that these mesocosms were accumulating more C; however, these differences were only marginal ($p = 0.072$, $F = 2.91$, $df = 2, 27$). Seedlings from home and family soil treatments had greater concentrations of foliar *N* ($p < 0.001$, $F = 31.3$, $df = 2, 27$) and C ($p < 0.001$, $F = 15.6$, $df = 2, 27$) than those from away soils (Figure 3). Foliar N was negatively correlated to exchangeable ammonium ($\rho = -0.69$, $p < 0.001$) and soil total N ($\rho = -0.35$, $p < 0.05$, Figure S3).

Foliar P variation was high within treatments (particularly in home soils), and the factor soil origin did not significantly explain P concentrations ($p = 0.24$, $F = 1.50$, $df = 2, 27$, Figure 3). Foliar P was not correlated to either exchangeable P or total P in the soil (Figure S3). Only in the case of away soils there was a positive correlation between foliar P concentration and seedling biomass (Figures S5–S7).

3.2 | Assimilation of ^{13}C by plants and transfer below-ground

We first analysed rates of C assimilation and transfer by focusing on ^{13}C enrichment of key pools and fluxes, and overall, we found significant effects of soil origin on their enrichment. In general, home and away soils had significantly different enrichments, while family soils were intermediate. For example, assimilation of ^{13}C ($\delta^{13}C$ in leaves after labelling) was greater in seedlings from home (226 ± 21.8 ‰) than away soils (158 ± 13.1‰) ($p = 0.025$, $F = 4.24$, $df = 2, 27$), while seedlings on family soil had intermediate enrichment (181 ± 15.0‰; Figure 4). The transfer of ^{13}C was traced into microbial respiration (mesh cores), fine roots and *Frankia* nodules. The highest rate of microbial respiration of recent photosynthate occurred in the period to 12 hr after labelling, after which microbial respiration $\delta^{13}C$ decreased until harvest (Figure S8). Overall, microbial respiration of recent photosynthate in home and family was greater than in away mesocosms for all time points. There was a trend of greater microbial respiration of recent photosynthate in home mesocosms compared to family mesocosms 1 and 2 days after labelling (Figure S8). Fine roots were more enriched in ^{13}C in home mesocosms than away mesocosms ($p < 0.01$, $F = 6.25$, $df = 2, 27$), while seedlings grown on family soils were not different from other soil treatments (Figure 4). *Frankia* nodules were similarly enriched in ^{13}C in both home and family treatments, and the ^{13}C enrichment was not related to nodule dry weight ($p = 0.14$, $\chi^2 = 2.24$, $df = 1$). Overall, there was a significant correlation between $\delta^{13}C$ signatures in all the compartments (Figure S4). In particular, $\delta^{13}C$ signature of *Frankia* nodules was tightly correlated to $\delta^{13}C$ in fine roots ($\rho = 0.62$, $p = 0.004$).

Next, we analysed the mass of ^{13}C allocated to key pools and fluxes. Net ^{13}C assimilation was greatest in seedlings from home

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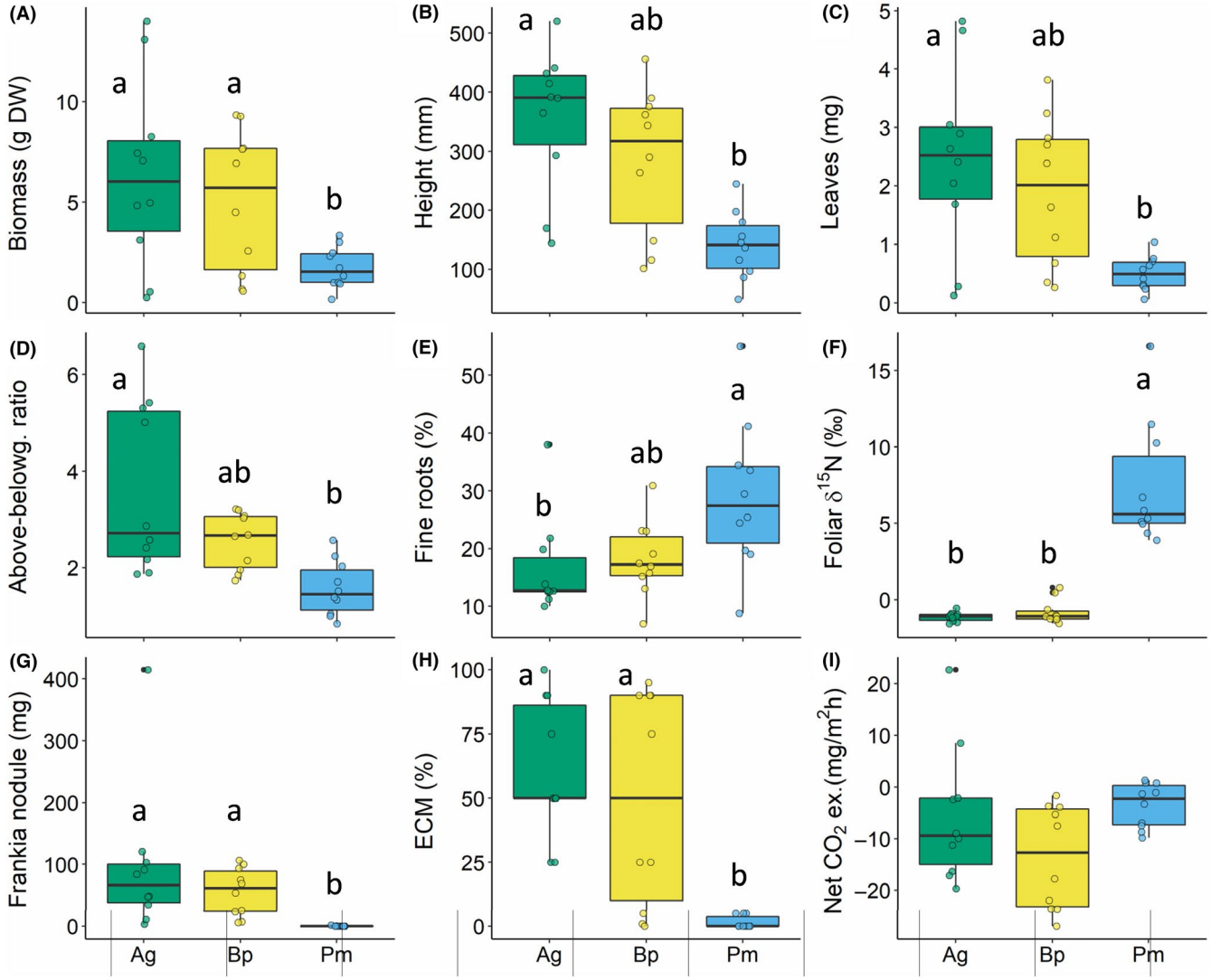


FIGURE 2 Traits of alder seedlings grown on home (alder; Ag, green), family (birch; Bp, yellow) and away (Douglas fir; Pm, blue) soils. (A) total biomass, (B) height, (C) number of leaves, (D) above-ground:below-ground biomass ratio, (E) proportion of fine roots, (F) foliar $\delta^{15}N$, (G) weight *Frankia* nodules, (H) colonization of root tips by ECM fungi and (I) net CO_2 exchange. Differences between soil origins ($p < 0.05$) are indicated by different letters. Boxes surrounding median values (horizontal line) represent the first and third quartiles, and lines show the smaller (and larger) values of 1.5 times the interquartile range. Data points above and below the interquartile range indicate outliers

soils (314 ± 57.7 mg ^{13}C) and family soils (199 ± 41.6 mg ^{13}C) in comparison to away soils (48.3 ± 10.4 mg ^{13}C; $p < 0.0010$, $F = 7.73$, $df = 2, 27$). Net assimilation of ^{13}C in a mesocosm was influenced by both $\delta^{13}C$ in leaves after labelling (e.g. assimilation rate) and leaf biomass (Figure S10). At harvest, seedlings grown in home and family soil retained more recent photosynthate than those from away soils ($p < 0.001$, $F = 7.74$, $df = 2, 27$). The relative amount conserved was of 45.1 ± 4.12%, 53.0 ± 5.2% and 28 ± 4.4% of the net ^{13}C-photosynthate, respectively. There were no significant differences in the proportion of recent ^{13}C-photosynthate transferred to fine roots in soils of different origin and no differences in the proportion of allocation to nodules in treatments where they were present. On average across all treatments, the percentage of plant ^{13}C photosynthate recovered after 2 days below-ground was 2.97 ± 0.38% in fine roots and 1.14 ± 0.13% in *Frankia* nodules.

3.3 | Relationship between recent photosynthate transfer to symbionts and leaf nitrogen and phosphorus concentrations

Foliar N and P concentration at harvest was modelled as a function of metrics related to C capture and allocation of the seedlings and the soil origin in which they were grown. Foliar N across treatments was explained by seedling biomass, the rate of ^{13}C-photosynthate allocation to fine roots and daily net CO_2 exchange (Table 2, model set N2, list of models with AICc < 4 can be found in Table S2). Inclusion of soil origin in the global model (N1) yielded a top ranking model that had a higher AICc than when soil origin was excluded, indicating a less parsimonious model (top ranking model $\Delta AICc_{N1\text{-}N2} = 1.40$, Table S2). Seedling biomass was highly correlated with *Frankia* nodule biomass ($r = 0.81$, $p < 0.001$), and for this reason we tested the

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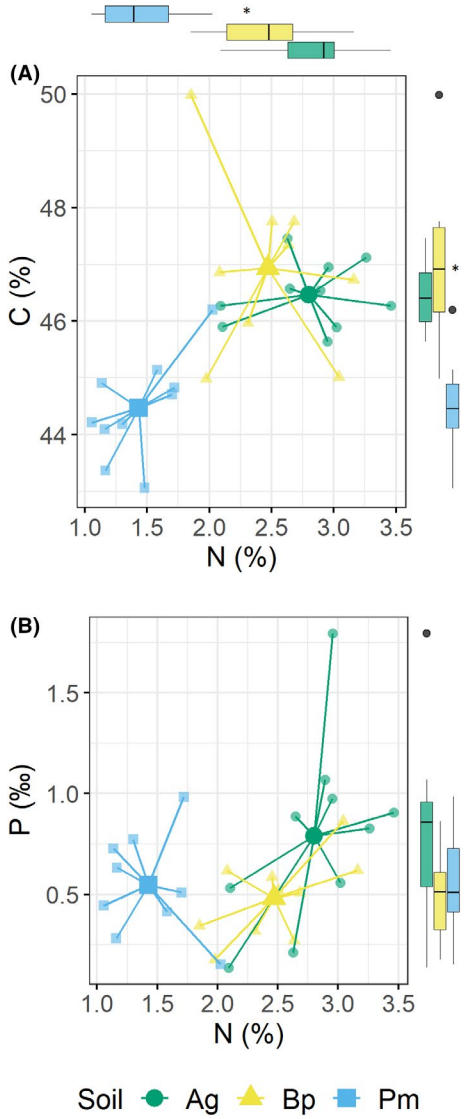


FIGURE 3 Foliar stoichiometry of alder seedlings grown in home (alder; Ag, circle), family (birch; Bp, triangle) and away (Douglas fir; Pm, square) soil. Differences between soil origins are indicated by $^*p < 0.001$. Large symbols represent the average for each soil origin. Boxes surrounding median values (horizontal line) represent the first and third quartiles, and lines show the smaller (and larger) values of 1.5 times the interquartile range. Data points above and below the interquartile range indicate outliers

sensitivity of our model selection by replacing seedling biomass with *Frankia* nodule biomass in the global model, which yielded similar results (Tables S3 and S4).

Foliar P variation across the mesocosms was jointly explained by net CO_2 exchange and soil origin (Table 2, of models with AICc < 4 can be found in Table S2), with seedlings grown on family soils having lower P concentrations than seedlings from home soil. Seedlings grown on away soils did not differ in P from the other soil treatments. There was no correlation between seedling ECM colonization (%) and foliar P between home and family soil treatments (Figures S5–S7).

4 | DISCUSSION

This study aimed at understanding how soil origin (home, family and away) influenced fitness proxies (growth and nutrition) of alder seedlings, and how these effects relate to the availability of symbiotic soil micro-organisms and below-ground allocation of recent photosynthate. The findings supported our first hypothesis, and showed that positive alder-soil feedbacks in terms of seedling growth and stoichiometry are driven by the interactions with compatible symbionts, as well as plant capture and allocation of C. Association of alder with N-fixing *Frankia* relieves N limitation in the seedling triggering a performance feedback loop (e.g. Arnone & Gordon, 1990; Ekblad & Huss-Danell, 1995), independently of the soil origin. The study suggests that this *Frankia*-induced performance loop is not costly for the seedling in terms of C, but likely increases the seedling demand for P. Despite the high variation of foliar P concentration among seedlings in this study, stoichiometric models support the view that home soils (and likely their ECM fungal communities) might have contributed to greater concentrations of foliar P in seedlings than family soils.

Our study suggests fundamental differences in seedling N per C exchange according to the presence of symbionts. In agreement with our initial prediction, seedling foliar N concentration was mainly explained by the transfer of recent photosynthates to fine roots (and *Frankia* nodules where present). Alder can access N *via* three distinct pathways, namely direct uptake by roots, fixation of N_2 by *Frankia* and from mycorrhizal fungi; however, it seems likely that seedlings grown in home and family soils obtained a significant proportion of their N from biological N fixation given the strong correlation of seedling biomass, *Frankia* nodule weight and foliar N. In addition, leaves of seedlings from home and family soils, which supported abundant *Frankia* nodules, had ^{15}N signatures closer to zero compared with those from plants grown on away soil, giving support to the idea that a large proportion of foliar N in those treatments was acquired symbiotically from the atmosphere. Previous studies estimated that *Frankia* supply *Alnus* with 45%–90% of their total foliar N (Domenach et al., 1989; Ekblad & Huss-Danell, 1995; Hurd et al., 2001; Millett et al., 2011). Since ECM fungi are thought to discriminate against ^{15}N during N transfer to host plants which results in host plants being depleted in ^{15}N (Hobbie & Högberg, 2012), N derived from ECM fungi would also contribute to observed $\delta^{15}N$ values of seedlings. While this study does not allow to calculate rates of nutrient obtained per unit C, it shows that overall, nodulated seedlings had higher biomass, exhibited greater ^{13}C assimilation rates and ^{13}C transfer rates to roots, leading to greater ^{13}C enrichment of microbial respiration.

Increased flux of newly fixed C (^{13}C) to roots in nodulated seedlings did not translate to a depletion of foliar C concentration. The C cost related to the nitrogenase activity in intact *Frankia* nodules has been estimated to be in the range of 4.5–5.8 g C respired per g N fixed (Lundquist, 2005). However, both foliar C and N pools were greater in nodulated than in non-nodulated seedlings, suggesting that *Frankia* nodules and N fixation were not limited by availability of C. Also, we show that seedlings in symbiosis with *Frankia* and ECM

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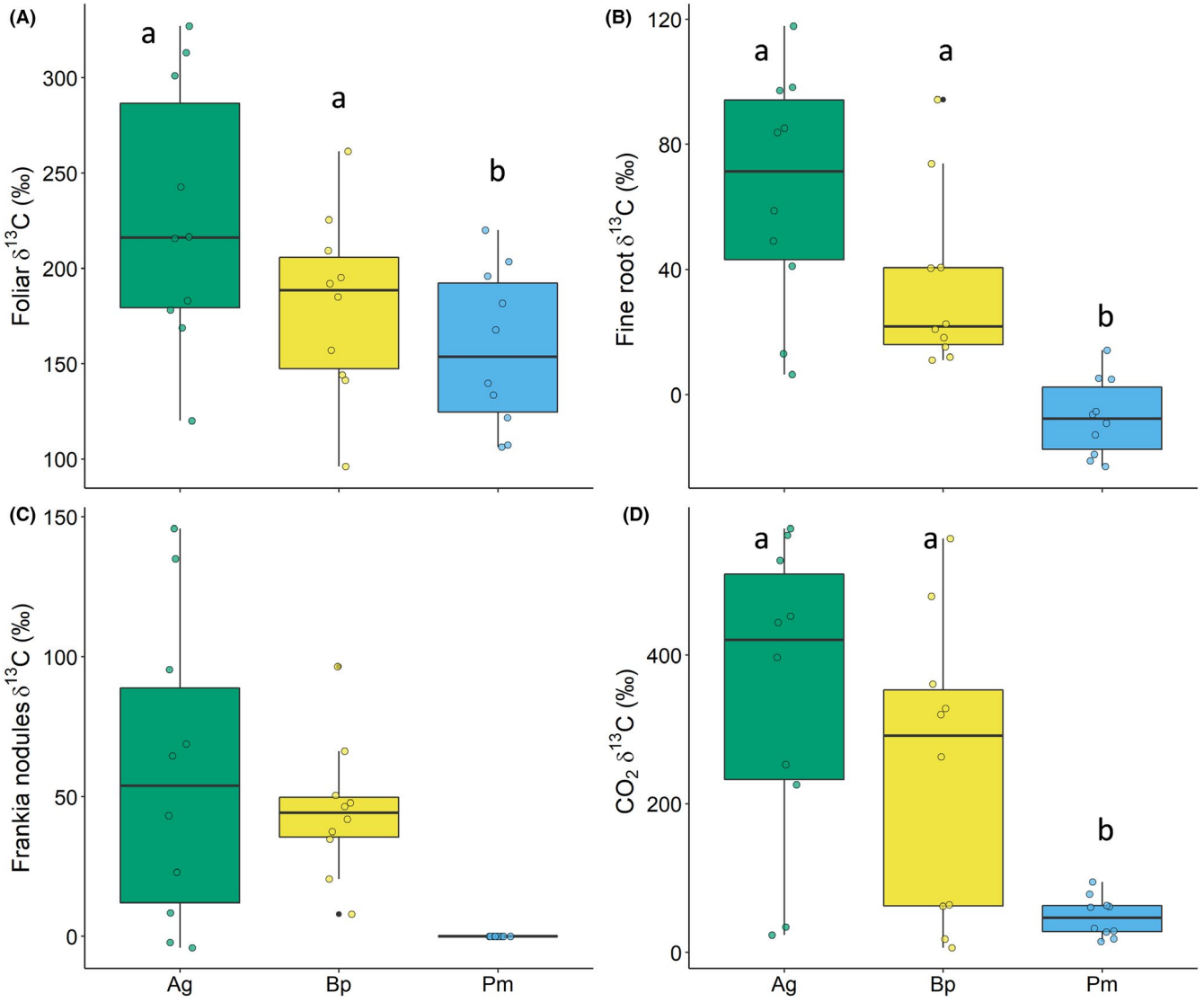


FIGURE 4 Assimilation of ^{13}C by the seedlings grown in home (alder; Ag, green), family (birch; Bp, yellow) and away (Douglas fir; Pm, blue) soils (A), and subsequent transfer to fine roots (48 h) (B) *Frankia* nodules (48 h) (C) and microbial respiration (12 h) after labelling (D). Differences between soil origins ($p < 0.05$) are indicated by different letters. Boxes surrounding median values (horizontal line) represent the first and third quartiles, and lines show the smaller (and larger) values of 1.5 times the interquartile range. Data points above and below the interquartile range indicate outliers

fungi invested proportionally more resources than non-nodulated seedlings into above-ground biomass. By contrast, non-nodulated seedlings had the highest investment in fine root structures despite away soils containing greater concentrations of extractable N, which probably indicates N deficiency for those seedlings. Collectively, these findings imply that for alder seedlings, the cost of direct root nutrient uptake is greater than the cost incurred when in symbiosis with *Frankia*. This supports the notion that symbiotic N-fixing *Frankia* are highly efficient in N provision in terms of C use in nutrient-poor soils (Chatarpaul et al., 1989; Yamanaka et al., 2003), and may therefore be responsible for alder fitness in this experiment.

Alnus spp. consistently associate with a smaller pool of ECM fungal species than other ECM hosts (Massicotte et al., 1994; Molina et al., 1993; Põlme et al., 2013), and many of these fungi are specific to the genus (Bent et al., 2011; Kennedy et al., 2011; Kennedy & Hill, 2010; Roy et al., 2013; Tedersoo et al., 2009). Regardless of any functional differences between host generalist and specialist ECM fungi, such high specificity may affect the ability of plants to colonize new environments where the pool of compatible ECM fungal species is restricted, and thus will be a significant factor in regulating plant–soil feedback. We found plants on home soils tended to have more diverse ECM communities than those on Family and especially away soils (Table S1). Unfortunately, the limited depth of sampling prevents detailed analyses of these effects, but highlights a need to investigate further the functional traits associated with these ECM fungi. A further factor that we are unable to disentangle is that the away soil was from beneath an exotic, non-native species (Douglas fir). This species is known to associate with specialized ECM fungi such as *Suillus amabilis*

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TABLE 2 Model averaging results for foliar N and foliar P starting with the full model including all seedling C economy variables followed by multi-model inference to select the most parsimonious models (the resulting models with AICc < 4 can be found in Table S1). Significant predictors in the models in bold, with confidence intervals not overlapping 0 and $p < 0.05$

	Estimate	Un*SE*	CI (2.5%)	CI (97.5%)	*z* value	*p* value (>|z|)
Model set N1						
(Intercept)	0.3777	0.202	−0.0182	0.7736	1.8699	0.0615
Biomass	**0.2914**	**0.1039**	**0.0877**	**0.4951**	**2.8035**	**0.0051**
Foliar C	0.0381	0.0827	−0.0658	0.3437	0	0.6449
δ^{13}C leaves	−0.0074	0.0385	−0.2558	0.1236	0	0.8478
δ^{13}C fine roots	**0.3861**	**0.143**	**0.1057**	**0.6664**	**0.2699**	**0.007**
δ^{13}C-CO_2	0.0039	0.0361	−0.1781	0.2665	0	0.9131
Net CO_2 ex.	**−0.3457**	**0.0854**	**−0.513**	**−0.1784**	**4.0495**	**<0.0001**
Soil_Bp	−0.3603	0.2345	−0.8199	0.0993	1.5363	0.1245
Soil_Pm	**−0.7728**	**0.3918**	**−1.5408**	**−0.0048**	**1.9723**	**0.0486**
Model set N2						
(Intercept)	0	0.0718	−0.1407	0.1407	0	1
Biomass	**0.4259**	**0.0793**	**0.2704**	**0.5813**	**0.5369**	**<0.0001**
Foliar C	0.1074	0.1006	−0.0054	0.3208	1.0682	0.2854
δ^{13}C leaves	−0.0167	0.0556	−0.2739	0.1132	0	0.7638
δ^{13}C fine roots	**0.6097**	**0.0987**	**0.4162**	**0.8032**	**6.1751**	**<0.0001**
δ^{13}C-CO_2	0.0009	0.0339	−0.2156	0.2372	0	0.9783
Net CO_2 ex.	**−0.2963**	**0.0774**	**−0.448**	**−0.1446**	**3.8275**	**<0.0010**
Model set P1						
(Intercept)	0.5296	0.4066	−0.2673	1	1.3026	0.1927
Biomass	−0.2468	0.2525	−0.803	0.0095	0.9777	0.3282
Foliar C	−0.0115	0.0827	−0.5362	0.3393	0.1394	0.8891
δ^{13}C leaves	0.0016	0.0564	−0.3647	0.4032	0.0281	0.9776
δ^{13}C fine roots	0.2027	0.2898	−0.0852	0.9875	0.6994	0.4843
δ^{13}C-CO_2	0.0764	0.1716	−0.1703	0.7187	0.4454	0.6561
Net CO_2 ex.	**−0.4848**	**0.1741**	**−0.8261**	**−0.1435**	**2.7843**	**0.0054**
Soil_Bp	**−1.1474**	**0.4741**	**−2.0767**	**−0.2181**	**2.4201**	**0.0155**
Soil_Pm	−0.4414	0.7658	−1.9423	1.0596	0.5764	0.5644
Model set P2						
(Intercept)	0	0.1595	−0.3126	0.3126	0	1
Biomass	−0.1421	0.2204	−0.7689	0.1281	0.6449	0.519
Foliar C	−0.1594	0.2014	−0.6624	0.0533	0.7911	0.4289
δ^{13}C leaves	−0.0018	0.0659	−0.4536	0.412	0.0279	0.9777
δ^{13}C fine roots	**0.5116**	**0.2357**	**0.1261**	**0.9501**	**2.1702**	**0.03**
δ^{13}C-CO_2	0.0907	0.2117	−0.28	0.8667	0.4283	0.6685
Net CO_2 ex.	−0.1191	0.172	−0.5869	0.0793	0.6924	0.4887

and *Rhizopogon vinicolor*, as well as generalists, and it is possible that the use of away soil from beneath Douglas fir rather than an unrelated native species may have influenced the outcomes of the experiment.

We also found a strong positive relationship between *Frankia* nodule presence and ECM colonization. Seedlings on home and family soil were all nodulated and with varying levels of ECM colonization while ECM colonization was very poor and *Frankia* were largely absent in seedlings grown on away soils. The pattern in colonization we observed has also been seen for other alder species. Colonization of *A. tenuifolia* seedlings by the ECM fungus *Alpova diplophloeus* was only found when co-inoculated with *Frankia* (Yamanaka et al., 2003); the ECM fungi *Paxillus involutus* was able to colonize *A. incana* seedlings on its own but exhibited greater degree of colonization when co-inoculated with *Frankia* (Chatarpaul et al., 1989). Earlier studies reported low *Frankia*

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nodulation on alder trees grown on conifer sites (Miller et al., 1992); however, we did not expect seedlings grown on *P. menziesii* soils in our experiment to be devoid of *Frankia* and ECM fungi. The lack of ECM colonization on non-nodulated seedlings may be explained by (a) lack of compatible ECM inocula in away soil or (b) lack of receptivity between the host plant and the ECM fungi, as both partners might require minimum levels of N (e.g. colonization by *Frankia* bacteria) to establish the symbiosis (Kennedy et al., 2015). Arnebrant et al. (1993) suggest that ECM fungi can benefit from biological N_2 fixation by *Frankia* in a tripartite symbiosis, by showing that ^{15}N fixed by *Frankia* in symbiosis with alder was transferred to ECM fungal mycelia. Redistributed N fixed by *Frankia* from the atmosphere may therefore contribute to the N demands of ECM fungi, especially those with weaker capacity to acquire and transport N from soil (Wallenda & Read, 1999).

The supply of *Frankia*-derived N likely increases the demand for P by alder to maintain growth. It has also been reported that a minimum level of P is required for *Frankia* to start nodulation and N_2 fixation because these processes are costly in terms of P (Ekblad & Huss-Danell, 1995; Gentili & Huss-Danell, 2003; Jha et al., 1993). It is therefore reasonable to expect a tight linkage between N-fixing symbiotic bacteria and mycorrhizal fungi in nutrient-limited environments. Complementarity between ECM fungi and N-fixing *Frankia* on alder performance has been shown using inoculation experiments (Chatarpaul et al.,1989; Yamanaka et al.,2003). The stoichiometric modelling approach presented here suggests that both soil origin and net C exchange in a mesocosm were associated with foliar P in seedlings. This could be a result of potentially distinct ECM fungal communities from seedlings grown in home and family soils in this experiment, which is supported by previous findings (Bogar & Kennedy, 2013). Such an effect could be achieved either by (a) specialist ECM fungal species that exhibit greater P acquisition activity and potentially receiving higher reward in terms of C (Johnson et al., 2010; Revillini et al., 2016) occurring solely in home soils (Bogar & Kennedy, 2013) or by (b) the abiotic characteristics of the home soil promoting an environment that favours P acquisition activity or more efficient ECM fungi (Courty et al., 2016). Ectomycorrhizal fungi vary in their nutrient acquisition abilities (e.g. Courty et al., 2016; Walker et al., 2014), and rare species may often contribute disproportionately to total enzymatic activity (Rineau & Courty, 2011; Walker et al., 2014), indicating the potential importance of less dominant taxa for plant nutrition and ecosystem functioning.

We found that alder seedlings grown in home soils exhibited greater variation in productive traits, such as biomass and height, than other soils; indeed, two seedlings from home soil performed as poorly as those grown in away soils. Surprisingly, these poor performing seedlings on home soil were nodulated and mycorrhizal and therefore likely benefited from nutrient exchange with *Frankia* and ECM fungi. A possible explanation for this phenomenon is that the interacting feedback effects range from positive—driven by symbiont availability and compatibility—to negative, potentially driven by accumulation of antagonists in the home soil (i.e. the Janzen–Connell effect).

Overall, our findings provide insight into the mechanisms driving plant–soil feedbacks in plants forming tripartite symbioses. This study demonstrates the importance to alder performance of the availability and formation of symbioses with *Frankia* and ECM fungi in nutrient-poor soils, which enhances tissue nutrient stoichiometry. These effects likely influence plant–soil feedback through increasing the ability of plants to capture of C and allocate recent photosynthate below-ground to facilitate nutrient acquisition.

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AUTHORS' CONTRIBUTIONS

D.J., J.K.M.W. and A.F.S.T. designed the study; J.K.M.W. and U.K. performed the experiment; J.K.M.W., U.K. and A.A. conducted the laboratory analyses; A.A. analysed the data; A.A. led the writing of the manuscript together with D.J.; All co-authors contributed to the discussion and development of the manuscript. D.J. and A.F.S.T. obtained the funding.

DATA AVAILABILITY STATEMENT

Data deposited in the Dryad Digital Repository https://doi.org/10.5061/dryad.98sf7m0j0 (Ardanuy et al., 2021).

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