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RESEARCH ARTICLE

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Cultivar-dependent increases in mycorrhizal nutrient acquisition by barley in response to elevated CO_2

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Societal Impact Statement

Modern agriculture is under pressure to meet yield targets while reducing reliance on finite resources to improve sustainability. Climate change represents an additional challenge—elevated atmospheric CO_2 concentrations may increase plant growth and boost yield, but the nutritional value of crops grown at elevated CO_2 is often reduced. Arbuscular mycorrhizal fungi (AMF) can improve plant nutrition, although how this symbiosis will be affected by climate change is unclear. Here, we demonstrate mycorrhizal contribution to nitrogen and phosphorus nutrition in barley under current and future CO_2 concentrations. In one cultivar, AMF substantially increased phosphorus uptake at elevated CO_2 and prevented phosphorus dilution, suggesting the symbiosis may become more important for crop nutrient uptake in the future.

Summary

- Globally important cereals such as barley (*Hordeum vulgare* L.) often engage in symbiosis with arbuscular mycorrhizal fungi (AMF). The impact of elevated atmospheric CO_2 on nutrient exchange between these symbionts remains unknown.
- In controlled environment experiments, we used isotope tracers (^{15}N, ^{33}P, ^{14}C) to quantify nutrient fluxes between two barley cultivars (Moonshine and Riviera) and their associated AMF at ambient (440 ppm) and elevated (800 ppm) CO_2.
- Elevated CO_2 reduced shoot N concentration in Moonshine, and shoot N and P concentration in Riviera. Elevated CO_2 substantially increased mycorrhizal ^{33}P acquisition in Moonshine. Mycorrhizal contribution to P uptake in Moonshine may have prevented dilution of tissue P concentration at elevated CO_2. In Riviera, AMF did not improve ^{33}P acquisition. Both cultivars received ^{15}N from their AMF symbionts, and this acquisition was not influenced by CO_2 concentration, although Moonshine received more ^{15}N than Riviera.
- Our results suggest that AMF may provide substantial contributions to barley nutrition at current and projected future CO_2 concentrations. This is especially noteworthy for barley, which is generally considered to have low mycorrhizal receptivity. AMF may help alleviate or avoid nutrient dilution normally observed at

elevated CO_2. Variation between cultivars indicates that mycorrhizal contribution to cereal nutrition could be improved through selective breeding practices.

KEYWORDS

arbuscular mycorrhiza, barley, carbon, carbon dioxide, climate change, mineral nutrients, sustainable agriculture

1 | INTRODUCTION

One of the greatest challenges facing humankind is that of generating enough food for the global population. Food production must be increased by an estimated 25%–70% to meet the demands of a projected human population of 9.8 billion people in 2050 (Alexandratos & Bruinsma, 2012; Hunter et al., 2017; Tilman et al., 2011) against the background of climate change (Parry et al., 2004; Smith & Gregory, 2013). During the "Green Revolution" of the 1950s and 60s, crop yield increases were achieved through intensive application of agrochemicals, irrigation and advances in plant breeding. These agricultural innovations improved nutrition globally, sparing the conversion of further natural ecosystems into agricultural land (Tilman et al., 2002). Phosphorus (P) fertilizer application increased 3.5-fold between 1960 and 1995 (Tilman et al., 2002), while nitrogen (N) addition increased ten-fold over the same period (Hinsinger et al., 2011) promoting crop growth and primary production (Čapek et al., 2018; Robertson & Vitousek, 2009). With increasing application, the efficiency with which crops acquire fertilizer has decreased (Hinsinger et al., 2011). At the same time, negative ecological effects due to excess fertilizer deposition, runoff and leaching into the wider environment have become a global phenomenon (Hautier et al., 2014). Serious environmental, economic and political issues associated with fertilizer production and usage mean that sustainable alternatives must now be sought (Carpenter, 2008; Cordell et al., 2009; Hinsinger et al., 2011).

Future food security faces additional challenges stemming from the impacts of global climate change. Increasing concentrations of greenhouse gases such as CO_2 in the atmosphere are linked to rising global temperatures (Stocker et al., 2013) and increasing frequency of extreme weather events (Wheeler & Von Braun, 2013). The agricultural sector is a significant producer of greenhouse gases, contributing *c*.10% of the UK's total GHG emissions (DEFRA, 2015), a significant proportion of which comes from fertilizer production (Goucher et al., 2017). Atmospheric CO_2 concentrations are currently 410–415 ppm (ESRL-NOAA Global Monitoring Division, 2020). If current emission rates are maintained, global CO_2 concentrations could rise to between 750 and 1,300 ppm by 2100 (Edenhofer, 2015). Field-based experiments have shown that crop growth in an elevated [CO_2] atmosphere (eCO_2) may initially increase photosynthetic C fixation and plant biomass, although the effects can decline over time as a result of CO_2 acclimation (Ainsworth & Long, 2005; Long et al., 2006). Increased plant C fixation and growth at eCO_2 is likely to increase plant demand for P under eCO_2 (Jin et al., 2015). Increased demand, together with the rapid depletion of the finite raw resources for P-based fertilizer production (Cordell et al., 2009), presents further problems for future crop production. Simply increasing agricultural productivity will not solve the problems associated with future food security if done in an unsustainable manner. Future increases in food production must be coupled with sustainable management practices geared towards minimizing or even removing carbon emissions from agriculture. In order to achieve this, alternative strategies which reduce agricultural reliance on synthetic fertilizer application must be sought.

Arbuscular mycorrhizal fungi (AMF), of the sub phylum Glomeromycotina (Spatafora et al., 2016), are found almost ubiquitously in agricultural soils and form intimate, intracellular associations with plant roots (Smith & Read, 2008). In exchange for plant carbon, AMF supply their hosts with phosphate, nitrogen and other mineral nutrients (Cavagnaro, 2008; Hodge & Storer, 2015; Parniske, 2008; Watts-Williams et al., 2017). AMF produce substantial hyphal networks that reach far beyond the rhizosphere, effectively extending the foraging range of the root system (Jansa et al., 2003; Puschel et al., 2016), and permitting the host plant access to soil pores which might otherwise be inaccessible (Allen, 2007). Integrating and exploiting AMF in more sustainable agricultural practices may potentially provide numerous benefits ranging from reduced fertilizer inputs, improved soil quality and increased plant nutrient uptake (Field et al., 2020; Thirkell et al., 2017).

The effects of eCO_2 in the future are likely to influence the dynamics of crop-AMF symbioses (Dong et al., 2018; Thirkell, Pastok, et al., 2019). Atmospheric CO_2 enrichment has been shown to affect carbon-for-nutrient exchange between AMF and plants, both non-domesticated and domesticated (Elliott et al., 2020; Field et al., 2012). Barley cultivars show a varied response to eCO_2, (Mitterbauer et al., 2017) but the extent to which this may be influenced or determined by AMF has not been investigated. In wheat, eCO_2 has been shown to have cultivar-specific effects on the function of associated AMF (Thirkell, Pastok, et al., 2019), suggesting that AMF receptivity, function and responsiveness to atmospheric [CO_2] could be key traits for future sustainable wheat breeding programmes. To date, research into the influence of abiotic factors (including eCO_2) on cereal-AMF symbiosis has largely focused on wheat (Cabral et al., 2016; Elliott et al., 2020; Mathur et al., 2019), although other crops species are recently being studied, including maize (Watts-Williams, Smith, et al., 2019). Barley is currently the world's 4th most commonly grown agricultural crop (FAO, 2018) and shows variable response to mycorrhizal colonization, from negative (Campos et al., 2018; Grace et al., 2009), through neutral (Khaliq & Sanders, 2000) to positive (Campos et al., 2018; Jakobsen

et al., 2005). Here, we investigated the effect of eCO_2 on the function of barley–AMF associations. Specifically, we address the following key questions:

1. Is there cultivar-specific variation in mycorrhizal receptivity and function in barley?
2. How is AM nutrient exchange in barley affected by increases in atmospheric [CO_2]?

2 | MATERIALS AND METHODS

2.1 | Barley pre-germination, AMF inoculation and plant growth conditions

Barley (*Hordeum vulgare* L. cv. Riviera and cv. Moonshine) seeds were surface sterilized using Cl_2 gas for 2 hr and incubated on damp filter paper (Whatman No.1, Cytiva – Little Chalfont, UK) for 5 days in controlled conditions (20˚C; 16 hr photoperiod). Twenty-four healthy seedlings were selected and transferred individually to 1.5 L pots which had been filled with a 3:1 mix of topsoil (J. Arthur Bowers Topsoil, Westland Horticulture Ltd.) and heat-sterilized (120 min at ≥120°C) silica sand (Figure 1). To supplement the resident AMF community of the topsoil (which was not heat-sterilized), seedlings were further inoculated with *Rhizophagus irregularis* (previously identified as *Glomus intraradices* [Schenck & Smith; isolate 09 collected in Spain by Mycovitro SL Biotechnología ecológica, Granada, Spain], and used by Kiers et al., 2011), a near-ubiquitous generalist species of AMF. *R. irregularis* inoculum was monoxenically cultured using Ri T-DNA transformed carrot (*Daucus carota* L.) root on MSR media (Cranenbrouck et al., 2005), and maintained at 21°C. Cultures containing fungal mycelium, spores and carrot roots were briefly blended (<20 s) in a benchtop food processor (Morphy Richards) with distilled water (d. H_2O) and added to the sterile sand/soil mix immediately prior to planting. Each pot received 15 ml of inoculum such that each plant was inoculated with 15,000 ± 1,500 *R. irregularis* spores. Spore density was quantified at 1,000 ± 100 spores per ml using a 100 µl aliquot placed on a microscope slide, inspected under a compound microscope at 40× magnification. Visual inspection of inoculum at this stage showed no physical damage to spores as a result of the brief homogenization process.

Plants were maintained in controlled environment growth cabinets (Snijder Labs) with the following conditions: 15 hr day (20°C and 70% humidity, day-time PAR, supplied by LED lighting, 225 µmol m^{-2} s^{-1} at canopy level) and 9 hr night (at 15°C and 70% humidity). CO_2 concentrations were 440 ppm (ambient atmospheric [CO_2] treatment, hereafter referred to as aCO_2) and 800 ppm (elevated [CO_2] treatment, hereafter referred to as eCO_2). Atmospheric [CO_2] was monitored using Vaisala sensors (Vaisala), maintained throughout by the addition of gaseous CO_2. The experiment took place within two growth chambers, such that there were 12 plants in each chamber. To mitigate for any unintended effects of growth chamber usage, plants were transferred between growth cabinets every 4 weeks and CO_2 concentrations amended accordingly. Each week, plants within each growth chamber were moved to randomize any possible effect of unintended

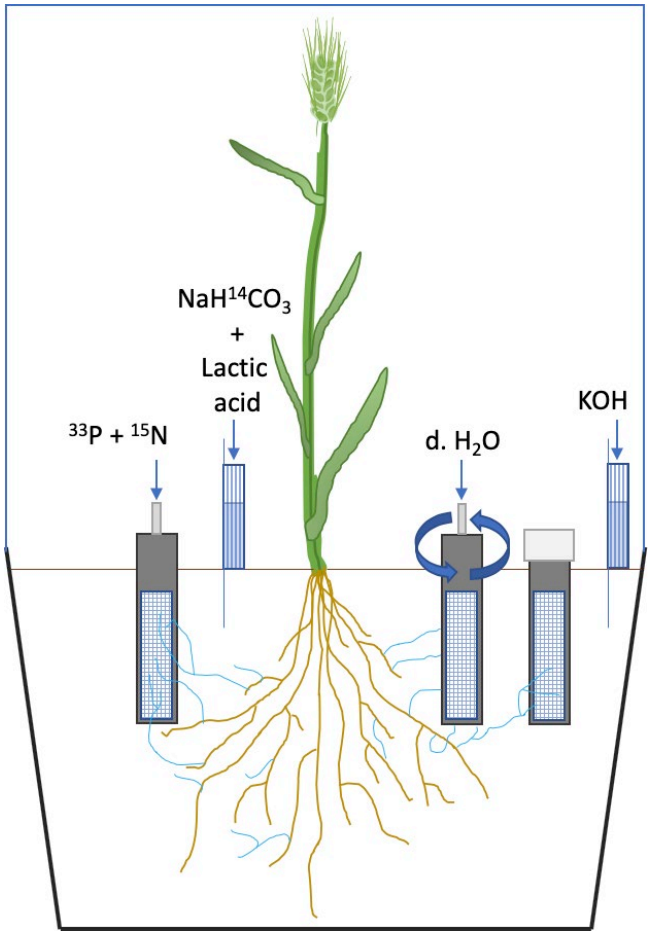

FIGURE 1 Diagram of experimental setup. Barley (*Hordeum vulgare* L. cv Moonshine, Riviera) were grown in a 3:1 mixture of topsoil and autoclaved silica sand in 1.5 L pots. Pots received a supplementary inoculum of the arbuscular mycorrhizal fungus *Rhizophagus irregularis*. Plants were grown in two CO_2 concentration treatments—ambient (440 ppm) and elevated (800 ppm). Isotope tracing was used to quantify barley N and P acquisition; a labeling solution (^{15}N and ^{33}P) was added via capillary tubing to a mesh-walled core in each pot, into which AMF hyphae could grow but roots could not. In control pots (no fungal access to isotope tracers), mesh-walled cores were rotated through 90˚ every 48 hr to sever AMF hyphae. Cores which did not receive labeling solution received autoclaved, distilled H_2O. Eleven days after ^{15}N and ^{33}P addition, plant allocation of carbon to fungi was quantified using a $^{14}CO_2$ pulse label. Plants were enclosed in polypropylene bags to create an airtight labeling chamber. A solution of $NaH^{14}CO_3$ was added to a spectrophotometer cuvette before the labeling chamber was sealed. A 10% solution of lactic acid was then added through the polypropylene bag using a hypodermic syringe to evolve $^{14}CO_2$. Below-ground $^{14}CO_2$ was sampled by use of a third, glass wool-filled core. After 20–22 hr of exposure, the remaining $^{14}CO_2$ in the headspace was captured by addition of 2M KOH to another cuvette. Gas sampling and KOH addition was done through the polypropylene bags using a hypodermic syringe. Plants were destructively harvested 24 hr after $^{14}CO_2$ liberation

environmental gradients (e.g. light, heat, humidity) within the cabinets. Starting 4 weeks after the experiment was established, plants were given 25 ml of a low P (25% of the original P quantity) Long Ashton Solution (LAS) twice weekly (Table S1). Plants were watered with tap water as required.

2.2 | ^{33}P, ^{15}N and ^{14}C isotope tracing

Assimilation of N and P via AMF was quantified using ^{33}P and ^{15}N tracers when plants were 11 weeks old and were at anthesis. Based on the methods of Thirkell, Pastok, et al. (2019), three cores constructed from PVC tubing (length 80 mm, diameter 18 mm), with windows (50 mm × 12 mm) cut in the lower two-thirds of each side were inserted into each of the plant pots (Figure 1). These windows and the bottom of each core were covered in a 20 μm nylon mesh which prevented penetration by plant roots but allowed AMF hyphal ingrowth. Mesh windows represented *c*. 70% of the area of the lower two-thirds of the cores. Plastic capillary tubing, 140 mm in length and 1.02 mm in diameter, perforated along the entire length with holes (*c*. 0.5 mm diameter) was positioned and glued using AquaMate silicone sealant (Everbuild Building Products) to the base inside the cores. Two of the cores were filled with the same soil and sand substrate as the bulk soil, plus 3 g/L crushed basalt (particle size <1 mm), to act as AMF "bait" (Quirk et al., 2012). Each pot also contained a third mesh-windowed core, loosely packed with glass wool (Acros Organics) and then the top was sealed with an airtight septum (SubaSeal® Perkin Elmer) through which gas sampling can be conducted with a hypodermic syringe, in order to measure below-ground respiration throughout the course of the experiment.

To ensure only AMF-mediated ^{33}P and ^{15}N tracer movement was measured, one of the mesh-windowed soil cores in each pot was rotated by 90° immediately prior to isotope tracer additions. This rotation severed the fungal connections between the plant and the core contents, preventing direct transfer of the isotope tracers to the host plants via extraradical mycorrhizal fungal mycelium. In half of pots ($n = 6$ per cultivar), ^{15}N and ^{33}P labeling solution was added to the static core, and in the remaining microcosms ($n = 6$ per cultivar), to the rotated core. Core rotation was repeated every 48 hr between isotope tracer addition and harvest to prevent hyphal re-entry to cores. The second core in each pot remained static, thereby preserving the hyphal connections between the core contents and the host plant. After 10 weeks of growth, 150 μl labeling solution, containing 1 MBq ^{33}P (as $H_3{}^{33}PO_4$, specific activity = 111 TBq mmol^{-1}; Hartmann Analytic) and 46.26 μg ^{15}N (as >98 atom% $^{15}NH_4Cl$; Sigma Aldrich) was introduced to each pot via the pierced capillary tubing. Cores which did not receive tracer solution were given 110 μl autoclaved d. H_2O, also added through pierced capillary tubing. Adding ^{15}N and ^{33}P labelling solution (or H_2O in controls) to the cores via capillary tubing ensured homogenous dispersal within the cores. By comparing the amount of isotope tracers detected in plants from pots with severed hyphal connections to the core containing ^{33}P and ^{15}N labeling solution (rotated core treatment) to those where the AMF hyphal connections remained intact (static core treatment), we are able to account for movement of isotopes caused by dissolution and diffusion and alternative soil microbial nutrient cycling processes in our assessment of AMF-mediated nutrient transfer to the plants. Movement of ^{33}P into plant shoots was tracked daily using a Geiger monitor (Series 900 mini monitor – ThermoFisher Scientific). As each pot contained one static and one rotated core, the levels of disturbance to the bulk soil and rhizosphere were consistent across all treatments. It is possible that core rotation may have influenced root uptake of isotope labels, but an experimental testing of the method suggests that such effects are likely be minimal (Leifheit et al., 2014).

Ten days after the addition of the ^{33}P and ^{15}N labeling solution, pots were prepared for labeling with $^{14}CO_2$ to track the movement of carbon from plant to AMF. 110 μl of $NaH^{14}CO_3$ (Perkin Elmer) containing 1.0175 MBq ^{14}C (specific activity = 1.621 GBq mmol^{-1}) was added to a 4.5 ml polystyrene spectrophotometer cuvette (ThermoFisher Scientific) within each pot, fastened to a plant label secured in the soil (Figure 1). The tops of all cores were sealed using gas-tight rubber septa (SubaSeal®, Merck, Gillingham) to minimize diffusion of $^{14}CO_2$ into the soil within the cores. Plant pots were then enclosed in airtight polypropylene bags (305 × 406 mm, 350 gauge; Polybags Ltd.) to allow $^{14}CO_2$ labeling. Immediately before dawn, $^{14}CO_2$ gas was liberated from $NaH^{14}CO_3$ within cuvettes by addition of 1 ml of 10% lactic acid, generating a 1.0175 MBq pulse of $^{14}CO_2$. Lactic acid was added to cuvettes through the polypropylene bag using a syringe and hypodermic needle, and resultant needle holes sealed using insulation tape immediately after the syringe was withdrawn. Using further syringes and needles, samples of 1 ml aboveground gas and 1 ml below-ground gas (via the glass wool-filled core) were taken 1 hr after release of $^{14}CO_2$, and every 4 hr thereafter to monitor the drawdown, respiration and flux of ^{14}C through the plant-AMF network. As before, needle holes were sealed with insulation tape to prevent loss of headspace gas from labeling chambers. For analysis, extracted samples were injected into gas-evacuated scintillation vials containing 10 ml of Carbosorb® (Perkin Elmer). To this, 10 ml of Permafluor® scintillation cocktail (Perkin Elmer) was added, and the ^{14}C content of each sample was quantified by liquid scintillation counting (Tri-Carb 3100TR scintillation counter, Perkin Elmer). Pots were maintained under cabinet conditions until maximum below-ground ^{14}C flux (20–22 hr after release of $^{14}CO_2$) was detected, at which point 4 ml of 2M KOH was added to cuvettes within each microcosm to capture remaining gaseous $^{14}CO_2$.

2.3 | Plant harvest and sample preparation

Plants were destructively harvested 24 hr after $^{14}CO_2$ labeling, 88 days after planting. Each microcosm was separated into shoots, roots, bulk soil, static core soil, and rotated core soil before being freeze-dried for 48 hr and weighed. A small sub-sample of roots was separated out before freeze-drying and stored for assessment of

colonization by AMF. After weighing, plant materials were homogenized and stored in airtight desiccators prior to analysis.

2.4 | Assessment of mycorrhizal colonization

Plant roots were stained using the "ink and vinegar" staining method described by Vierheilig et al. (1998), modified for use with barley roots. Briefly, 1 cm root sections were cleared in 10% (w/v) KOH for 60 min at 70°C, washed in distilled water, immersed in staining solution (5% Pelikan "Brilliant Black" ink [Pelikan Holding AG], 5% acetic acid and 90% d.H_2O) for 25 min at 20°C, then de-stained for 24 hr in 1% acetic acid at 20°C. Stained roots were mounted using PVLG (8.33 g polyvinyl alcohol, 50 ml d.H_2O, 50 ml lactic acid, 5 ml glycerol), dried in a 65˚C oven for 18 hr. For each plant, 10 sections of root (comprising a minimum of 100 root intersections) were assessed for mycorrhizal colonization using the methods of McGonigle et al. (1990) at 10× magnification.

2.5 | Movement of ^{33}P and ^{15}N from AMF to plant tissues

Fifty to seventy mg dry weight (DW) samples of bulk, labeled and unlabeled soil and 20–30 mg DW milled ear, shoot and root material from each plant were weighed directly into acid washed digest tubes. Samples were digested in 1 ml of concentrated (96% v/v) sulphuric acid (H_2SO_4) for 2 hr at room temperature, before being heated at 350˚C for 15 min (BT5D Dry Block Heater, Grant Instruments, Shepreth). Once cooled, 100 µl of 30% hydrogen peroxide (H_2O_2) was added to each sample before reheating to 365˚C for 1 min to clear. Each sample was then diluted to 10 ml with distilled water. For analysis, 2 ml of each sample digest was mixed with 10 ml of the liquid scintillant Emulsify Safe® (Perkin Elmer). ^{33}P radioactivity within each sample was measured using liquid scintillation counting (Packard Tri-carb 3100TR Liquid Scintillation Analyser; Isotech). ^{33}P content was quantified using the following formula, from Cameron et al. (2007):

$$M^{33}\mathrm{P}\left\{\left[\frac{c\mathrm{DPM}}{60}\right]\frac{\mathrm{Mwt}}{S\mathrm{Act}}\right\}\mathrm{Df},$$

where $M^{33}P$—mass of ^{33}P (mg), *c*DPM—counts as disintegrations per min, *S*Act—specific activity of the source (Bqmmol − 1), Df—dilution factor, Mwt—molecular mass of P.

4 mg (± 2 mg) of shoot and root tissue from all plants was weighed for analysis of ^{15}N content by continuous-flow mass spectrometry (PDZ Europa 2020 Isotope Ratio Mass Spectrometer coupled to PDZ ANCA GSL preparation unit [both Sercon Ltd]). Data were collected as Atom% ^{15}N and %N using unlabeled plants for background detection.

To quantify movement of ^{14}C from plant to AMF, 15–20 mg dry plant and soil material were weighed into Combusto-Cones (Perkin Elmer). Samples were combusted (Model 307 sample oxidiser - Packard Sample Oxidiser; Isotech) and subsequent $^{14}CO_2$ released by oxidation was trapped with 10 ml of Carbosorb (Perkin Elmer) and mixed with 10 ml of Permafluor (Perkin Elmer). The radioactivity of each sample was measured by liquid scintillation counting (Packard Tri-carb 3100TR Liquid Scintillation Analyser; Isotech). Enrichment of ^{14}C in core soil was used to extrapolate total fixed carbon (^{14}C and ^{12}C) transferred to the mycelial network from each plant. It was assumed that ^{13}C enrichment did not differ between treatments, and ^{13}C was not included in calculations owing to its negligible contribution to total CO_2 in atmospheric air. Total fixed carbon was calculated as a function of total CO_2 in each labeling system and proportion of $^{14}CO_2$ which had been fixed by each plant. Total ^{14}C and ^{12}C assimilated by plants and transferred to AMF was calculated using the following formulae (Cameron et al., 2006):

$$T_{\mathrm{fp}}\left(\left(\frac{A}{A_{\mathrm{sp}}}\right)m_a\right)+\left(P_r\times m_c\right),$$

where T_{fp} is the total C transferred from plant to AMF (g), A is sample radioactivity (Bq), A_{sp} is the specific activity of the source (Bqmol^{-1}), m_a is the atomic mass of ^{14}C (14), P_r is the proportion of the total supplied ^{14}C present in plant tissue and m_c is the mass of C in the CO_2 present within the labeling system (g) (using the ideal gas law, below).

$$m_{cd}=M_{cd}\left(\frac{\mathrm{P}V_{cd}}{RT}\right)\therefore m_c=m_{cd}\times 0.27292,$$

where m_{cd} is the mass of CO_2 (g), M_{cd} is the molecular mass of CO_2 (44.01 g/mol), P is pressure (kPa), V_{cd} is volume of CO_2 in the system (0.003 m^3), m_c is mass of unlabeled C (^{12}C) in the labeling system (g), M is the molar mass of C (12.011 g), R is the universal gas constant (JK^{-1}/mol), T is the absolute temperature (K), m_c is the mass of C in the CO_2 present in the labeling system (g), where 0.27292 is proportion of C in CO_2 (27.292%; Cameron et al., 2008).

2.6 | Plant nutrient content

Total phosphorus content within plant and soil material (i.e., non-tracer P) was quantified following the colorimetric determination of phosphorus methods adapted from Murphy and Riley (1962). Briefly, 0.5 ml of H_2SO_4 digest samples (from ^{33}P analysis, above) were combined with 0.2 ml of 0.1 M L-ascorbic acid ($C_6H_8O_6$), 0.2 ml 3.44 M NaOH, and 0.5 ml of a developer solution prepared by dissolving 4.8 g of ammonium molybdate ($(NH_4)_6Mo_7O_{24}.4H_2O$) and 0.1 g of antimony potassium tartrate ($C_6H_4O_7SbK$) in 250 ml 2 M H_2SO_4. Absorbance was measured at 882 nm using a Jenway 6300 spectrophotometer (Cole-Palmer) 45 min after mixing. Phosphorus concentration of digested samples was calculated from a calibration curve, prepared using a standard P solution (10 ppm $NaH_2PO_4.H_2O$).

2.7 | Statistics

Statistical analyses were carried out using the "RStudio" interface of R statistical software, version 3.4.3. (R Core Team, 2020; RStudio Team, 2015). For tissue nutrient content, biomass and mycorrhizal colonization, data were tested by two-way ANOVA (using base R functions), where cultivar and [CO_2] were used as predictor variables. For ^{15}N, ^{33}P and ^{14}C enrichment, data were analyzed separately by cultivar so that CO_2 concentration and core rotation treatment were predictor variables. Where ANOVA gave $p < .05$ for interaction or main effects, Tukey's honestly significant difference tests were used to identify statistical differences between groups and performed using the *emmeans* package in R (Lenth, 2020). Prior to running analyses, data were tested for normality using Shapiro–Wilk test, by visual inspection of residual plots and model fit was compared using Akaike information criterion (AIC) testing. Where relevant assumptions were not met, data were $\log_{10}$ transformed. Data were plotted using the packages *ggplot2* (Wickham, 2016) and *multcompView* (Graves et al., 2019). The data that support the findings of this study are available from the corresponding author upon reasonable request.

3 | RESULTS

3.1 | Barley growth stimulated by elevated eCO_2

Barley shoot biomass was significantly greater when plants were grown in eCO_2 compared to aCO_2 (Figure 2a; Tables S2 and S3; $F_{2,44} = 20.39$, $p < .001$), a trend seen in both cv. Moonshine (Tukey $p = .0263$) and cv. Riviera (Tukey $p = .0065$). However, root biomass was not significantly influenced by [CO_2] (Figure 2b, $F_{2,44} = 0.472$, $p > .05$). Barley cultivar significantly influenced shoot dry weight (Figure 2a, $F_{2,44} = 49.49$, $p < .001$), and root dry weight (Figure 2b, $F_{2,44} = 9.614$, $p < .01$), with cv. Riviera biomass being greater for both shoots and roots than cv. Moonshine. No significant interactions between [CO_2] and cultivar influenced plant biomass (Figure 2; Table S3).

3.2 | Mycorrhizal colonization in barley was unaffected by eCO_2 or variety

All plants of both cultivars were colonized by AMF (Figure 3a–c), and the extent of fungal proliferation in roots was not affected by [CO_2] (Figure 3a; $F_{2,44} = 0.977$, $p > .05$) or variety (Figure 3a, $F_{2,44} = 2.016$, $p > .05$). Mean root length colonization ranged from just over 10% in Riviera at aCO_2 to 18% in Moonshine at aCO_2 (Table S2). Overall, arbuscule frequency was low; ranging from around 2% in Riviera at aCO_2 to around 4% in Riviera at eCO_2 and was not significantly influenced by CO_2 or cultivar (Figure 3b). Similarly, vesicle frequency was low across treatment groups, ranging from 0.18% in Riviera at aCO_2 to 0.68% in Moonshine at eCO_2, although the majority of plants sampled had no vesicles recorded (Table S2).

3.3 | Elevated CO_2 dilutes mineral nutrition of barley

Aboveground phosphorus content was significantly greater in cv. Riviera than Moonshine (Figure 4a; Table S2; $F_{2,42} = 173.7$, $p < .01$), but was unaffected by [CO_2] in either cultivar (Figure 4a; Table S3; $F_{2,42} = 3.475$, $p > .05$). There was no interaction between [CO_2] and variety (Table S3). Phosphorus concentration ([P]) was significantly affected by CO_2, by variety and there was a significant interaction between these factors (Figure 4b). Most notably, Moonshine [P] was not affected by [CO_2] (Tukey $p > .05$) while Riviera shoot [P] was significantly lower at eCO_2 than at aCO_2 (Tukey $p < .01$). Riviera shoot [P] was higher than in Moonshine, following the trend of P content (Figure 4b).

Mycorrhizal P uptake in Moonshine was strongly dependent upon CO_2 concentration—there was a significant interaction between

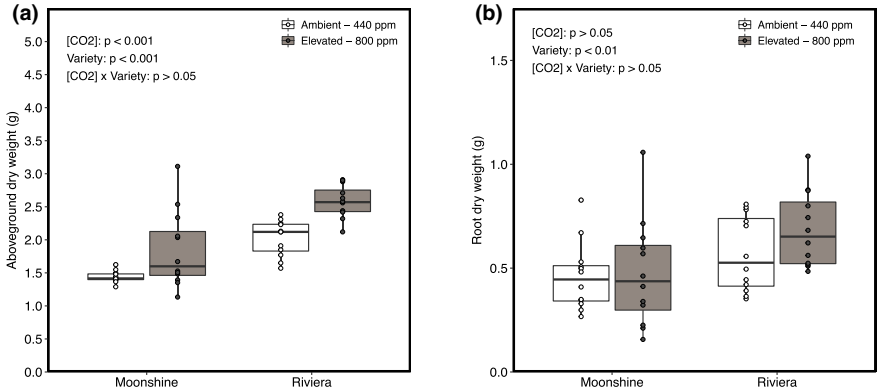

FIGURE 2 Shoot (a) and root (b) dry weight (g) of spring barley (*Hordeum vulgare* L. cv. Moonshine and Riviera) grown in ambient (440 ppm, white boxes) and elevated (800 ppm, gray boxes) atmospheric CO_2, $n = 12$. Boxes sharing letters do not significantly differ, $p > .05$ (ANOVA and Tukey post-hoc tests). "N.S.D." denotes no significant statistical difference between treatments. ANOVA *p*-values are included for main effects and interaction between main effects. Data were log10 transformed where assumptions for statistical tests were not satisfied

CO_2 and variety (Figure 4c; Table S3; $F_{2,20} = 16.90$, $p < .001$). While there was no difference between static and rotated treatments for aCO_2 (Tukey $p > .05$), under eCO_2, there was significantly more ^{33}P in Moonshine shoots of the static treatment than the rotated treatment (Tukey $p < .001$), indicating substantial contribution of AMF to barley P nutrition (Figure 4c). By contrast, there was no clear evidence of mycorrhizal P uptake in cv. Riviera, as the ^{33}P content of static core treatment was not different from the rotated core treatment (Figure 4d; Figure S1).

Riviera had significantly higher N content (Figure 4a) and concentration (Figure 5b) than Moonshine, and eCO_2 caused significantly reduced N concentration ($F_{2,43} = 21.48$, $p < .001$), a trend which was stronger in Riviera (Tukey $p = .002$) than it was in Moonshine (Tukey $p = .069$). ^{15}N uptake was significantly enhanced by AMF in both Moonshine (Figure 5c; Figure S1) and Riviera (Figure 5d; Figure S1), demonstrated by higher ^{15}N content in static core plants compared to rotated core plants. There was reduced ^{15}N uptake in eCO_2 compared to aCO_2, a trend seen across all treatments (Figure 5c,d). Core rotation did not affect shoot N or P concentration (Figure S2a–d).

3.4 | Carbon transfer from plants to fungi

All treatments showed similar amounts of C transfer from plants to fungi in both varieties, quantified as plant-fixed C detected in static and rotated cores (Figure 6a,b; Tables S2 and S3). Root length colonization data (Figure 3a–c) corroborate the pattern seen in carbon allocation data, that neither [CO_2] nor cultivar significantly affect carbon allocation to AMF.

4 | DISCUSSION

As future atmospheric CO_2 concentrations are projected to continue rising (Le Quéré et al., 2015), crop growth is also expected to increase, due to enhanced photosynthetic C assimilation (Ainsworth & Long, 2005; Dong et al., 2018; Mitterbauer et al., 2017; Terrer et al., 2016). Our data support this trend, as both cultivars had greater shoot biomass at eCO_2 compared to ambient [CO_2], although root biomass appeared to be less affected (Figure 2). While the biomass response to eCO_2 was similar in both cultivars examined here, it is important to note that significant variation in barley growth responses to eCO_2 has been demonstrated elsewhere (Mitterbauer et al., 2017). The mechanisms responsible for this variation are not entirely clear; crop genetic diversity will prove critical when adapting agriculturally important species to climate change factors such as drought and eCO_2. Crop cultivars have demonstrated differing susceptibility to photosynthetic acclimation to eCO_2, where predicted increases in photosynthesis are not observed (Tausz-Posch et al., 2020). The extent to which this occurs in cereals remains to be resolved, although it is worth noting that photosynthetic acclimation to eCO_2 may depend on nitrogen availability and water use efficiency—two factors which themselves may be influenced by AMF.

Concerns have been raised that any "CO_2 fertilisation" effect on crop growth may exacerbate problems of malnutrition; despite potential increases in yield, the nutritional quality of the grains is often decreased at eCO_2 (Myers et al., 2014). This is largely because carbohydrate assimilation accounts for the majority of the yield increases observed at eCO_2, thus the relative concentrations of mineral nutrients and protein become "diluted" (Cotrufo et al., 1998; Manderscheid et al., 1995).

4.1 | AMF may mediate barley P assimilation response to eCO_2

Substantial ^{33}P enrichment in Moonshine shoots at eCO_2 (Figure 4c,d) indicates that increased transfer of P at eCO_2 by AMF helped maintain tissue P concentrations across [CO_2] treatments for this cultivar. In contrast, cv. Riviera received no more ^{33}P from AMF symbionts at eCO_2 than at ambient [CO_2], and P concentration became diluted as biomass increased. Although little is known about the mechanisms underpinning the effects of eCO_2 on mycorrhizal cereal crop nutrient acquisition, a variety of responses are evident in the literature. In general, eCO_2 tends to enhance P uptake in mycorrhizal plants (Dong et al., 2018); although it is not clear whether any additional P assimilation is directly acquired via AMF rather than the plants' own root epidermal P transporters. Isotope tracer experiments have shown mycorrhizal P uptake is generally unresponsive to eCO_2, as seen in a number of plant species including pea (*Pisum sativum*; Gavito et al., 2002; Gavito et al., 2003), medic (*Medicago truncatula*), brome grass (*Brachypodium distachyon*; Jakobsen et al., 2016) and wheat (*Triticum aestivum* L.; Charters et al., 2020). Our results suggest that AMF may allow crops to maintain critical levels of mineral nutrients while growing at eCO_2, thereby avoiding the nutrient dilution effect which is usually observed (Cotrufo et al., 1998). Such an effect could have significant implications for crop nutrition (Myers et al., 2014) and warrants further experimental testing. As our experimental plants were grown until anthesis (but not yield), assessing how eCO_2 and AMF interact to affect the nutrient qualities of the grain produced by the barley cultivars tested here will be an important next step. From the perspective of human nutrition and health, the dilution of mineral nutrients (especially micronutrients) in grain of staple crops grown at eCO_2 is potentially very significant (Soares et al., 2019). Dietary deficiencies of micronutrients such as Zn and Fe represent a hidden hunger for millions worldwide (Soares et al., 2019), and it is likely that nutrient dilution in grains caused by increasing atmospheric [CO_2] (Loladze, 2014; Myers et al., 2014) may exacerbate this problem.

4.2 | Insufficient mycorrhizal contributions to N uptake to prevent eCO_2 dilution

N concentrations were reduced in barley grown at eCO_2 compared to ambient [CO_2], as increases in biomass outpaced N acquisition. Low N availability in the substrate may partly explain the decreased

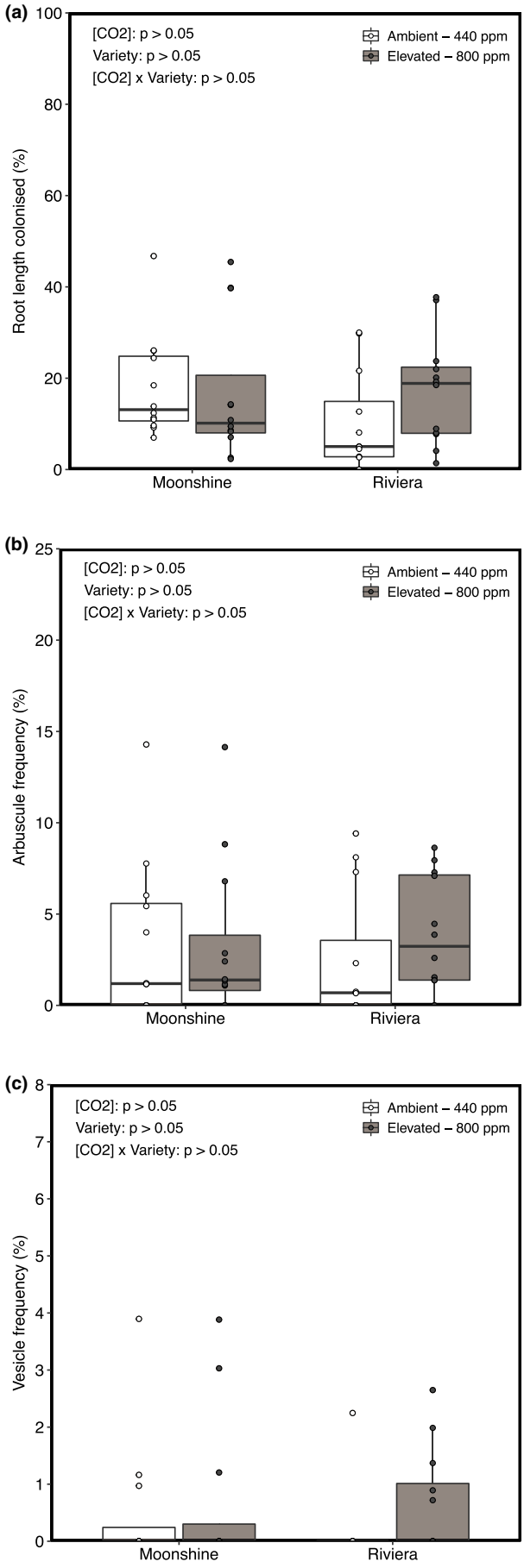

FIGURE 3 Arbuscular mycorrhizal colonization—total fungal biomass (a) arbuscule frequency (b) and vesicle frequency (c) of spring barley (*Hordeum vulgare* L. cv. Moonshine and Riviera) grown in ambient (440 ppm, white boxes) and elevated (800 ppm, gray boxes) atmospheric CO_2, $n = 12$. ANOVA p-values are included for main effects and interaction between main effects. Data were log10 transformed where assumptions for statistical tests were not satisfied

N concentrations at eCO_2, as both cultivars here were grown in nutrient-limited conditions. N and P dilution was probably more pronounced in cv. Riviera than Moonshine at eCO_2 because Riviera achieved a greater biomass, and as such had a higher nutrient demand. Previously, N availability has been identified as the most significant limitation for eCO_2 fertilization in AM plants (Terrer et al., 2016). In both cultivars, allowing AMF access to the labeled core resulted in greater ^{15}N label assimilation in barley shoots, suggesting AMF contributed to N uptake. Mycorrhizal acquisition of N has been demonstrated in barley (Wilkinson et al., 2019), and wheat (Miransari et al., 2009; Thirkell, Pastok, et al., 2019; Zhu et al., 2016), where it has been shown to increase under eCO_2 (Zhu et al., 2018). By contrast, there was no effect of eCO_2 (Figure 5c,d) on mycorrhizal ^{15}N uptake here. Notably, mycorrhizal acquisition of ^{15}N in cv. Moonshine was around double that in cv. Riviera (Figure 5c,d). Variation in mycorrhizal functioning among cultivars of crops is well-known from the literature (Hetrick et al., 1992; Sawers et al., 2017; Watts-Williams, Emmett, et al., 2019; Zhang et al., 2019). Indeed, cultivar specificity in mycorrhizal ^{15}N uptake has been shown previously in a barley field study (Thirkell, Cameron, et al., 2019). It is clear from our data that cv. Riviera receives less nutritional contribution from its mycorrhizal symbionts than Moonshine does (Figures 4c,d and 5c,d), although it is not clear why this is the case. A recent meta-analysis of AMF influence over grain yields suggests that older varieties typically benefit more from AMF than do modern varieties (Zhang et al., 2019). Similarly, an experimental comparison of five barley cultivars showed that modern cultivars generally responded more negatively to AMF inoculation than older ones (Al Mutairi et al., 2020). cv. Riviera is indeed a newer cultivar than Moonshine, however both cultivars were developed relatively recently (2010 vs. 1994, SASA, 2020). As such, cultivar age is unlikely to be a contributory factor here.

4.3 | Mycorrhizal nutrient acquisition patterns are uncoupled in barley

Our data suggest that mycorrhizal acquisition of N and P are not intrinsically linked, i.e., plants which receive N from their AMF symbionts do not necessarily also receive P (Figures 4c,d and 5c,d). As N and P transfer from fungi to a plant host occurs via transporters specific for ammonium (Guether et al., 2009; Kobae et al., 2010; Perez-Tienda et al., 2011) and phosphate (Harrison et al., 2002, 2010), this is not surprising. Uncoupled fungal transfer of N and P may reflect plant demand, as both cultivars appeared more N-limited than P-limited

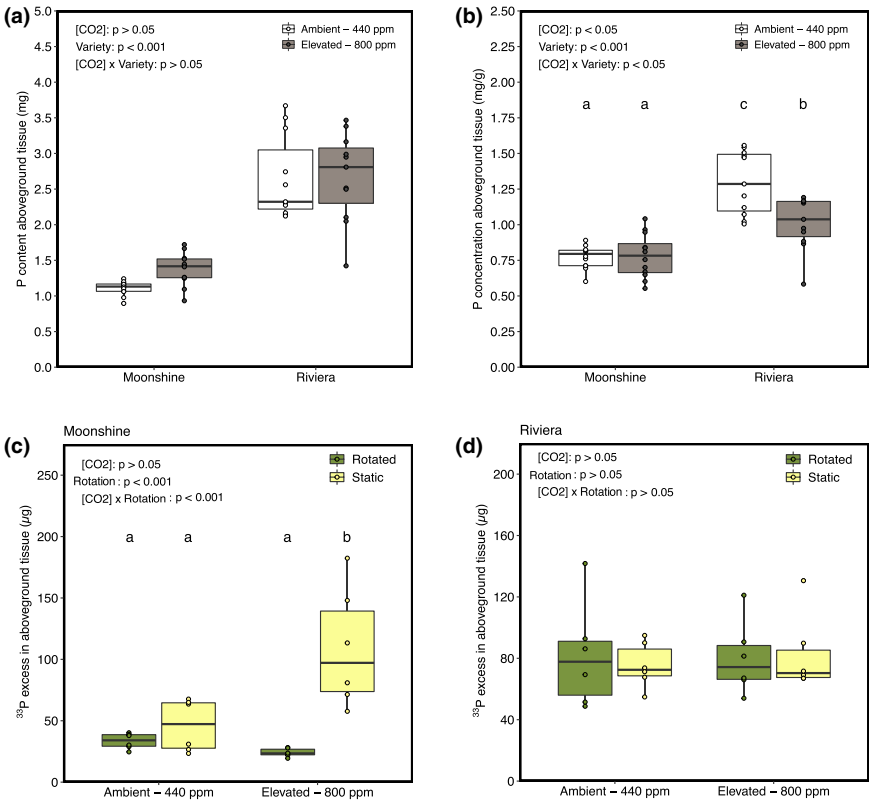

FIGURE 4 Phosphorus content (a) and concentration (b) of aboveground tissue of spring barley (*Hordeum vulgare* L. cv. Moonshine and Riviera) grown in ambient (440 ppm, white boxes) and elevated (800 ppm, gray boxes) atmospheric CO_2, $n = 12$. Panels (c and d) show ^{33}P content in aboveground tissue of Moonshine and Riviera varieties, respectively. Green boxes denote plants with rotated isotope cores, yellow boxes denote plants with static isotope cores, $n = 6$. Boxes sharing letters do not significantly differ, $p > .05$ (ANOVA and Tukey post-hoc tests). ANOVA p-values are included for main effects and interaction between main effects. Data were log10 transformed where assumptions for statistical tests were not satisfied

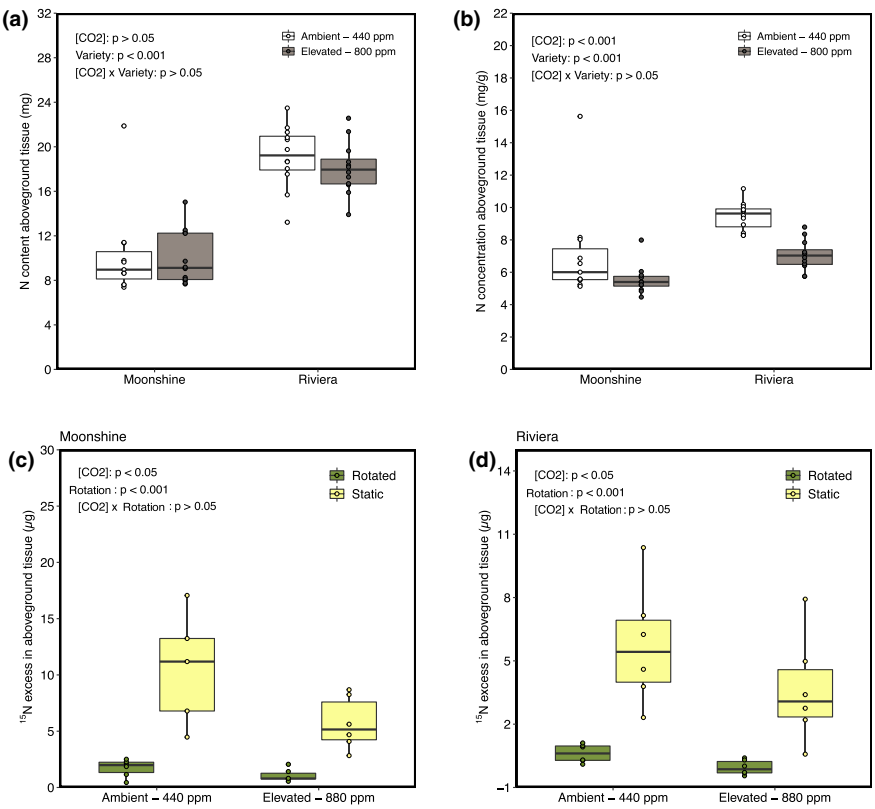

FIGURE 5 Nitrogen content (a) and concentration (b) of aboveground tissue of spring barley (*Hordeum vulgare* L. cv. Moonshine and Riviera) grown in ambient (440 ppm, white boxes) and elevated (800 ppm, gray boxes) atmospheric CO_2, $n = 12$. Panels (c) and (d) show ^{15}N content in aboveground tissue of Moonshine and Riviera varieties, respectively. Green boxes denote plants with rotated isotope cores, yellow boxes denote plants with static isotope cores, $n = 6$. ANOVA p-values are included for main effects and interaction between main effects. Data were log10 transformed where assumptions for statistical tests were not satisfied

in our experiments (Figures 4b and 5b), given the typical demand from cereals for these macronutrients (Maathuis & Diatloff, 2013; Marschner, 2011). It is unclear from the literature how mycorrhizal benefit would be affected by higher nutrient availabilities; some evidence suggests that limitation in either N or P is sufficient to stimulate plant hosts to rely on mycorrhizal nutrient uptake (Nouri et al., 2014).

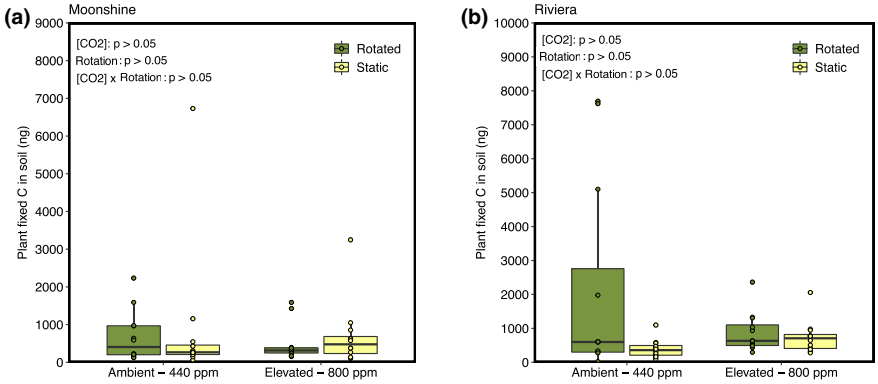

FIGURE 6 Plant-fixed carbon content of rotated (green boxes) and static (yellow boxes) isotope labeling cores for Moonshine (a) and Riviera (b) spring barley (*Hordeum vulgare* L.) at ambient and elevated [CO_2], $n = 12$. ANOVA p-values are included for main effects and interaction between main effects. Data were log10 transformed prior to analysis where assumptions for statistical tests were not satisfied

Further evidence suggests the contrary, that limited P and sufficient (or luxury) N supply should promote the greatest transfer of P and N from fungi to hosts (Johnson et al., 2015). Without experimental testing, it may not be possible to determine how to maximize the contribution of the AMF to P and N nutrition of these cultivars, given the substantial functional diversity in mycorrhizal functioning arising from plant genotype (Baon et al., 1993; Hetrick et al., 1992; Sawers et al., 2017; Watts-Williams, Emmett, et al., 2019).

4.4 | Mycorrhizal C acquisition largely unresponsive to cultivar or CO_2 concentration

Using ^{14}C tracing, we found no evidence that plant-to-fungus carbon allocation was affected by eCO_2, counter to the results of other experimental studies (Drigo et al., 2013; Field et al., 2012) and meta-analyses (Alberton et al., 2005; Treseder, 2004). However, our results are consistent with data previously reported in wheat in similar experimental systems (Thirkell, Pastok, et al., 2019). Our root length colonization data suggest that allocation to intraradical fungal structures was likewise unaffected by eCO_2 (Figure 3a–c). The amounts of C transferred to fungi here were perhaps too low to detect by the ^{14}C labeling, a technique which can create noisy data (Figure 6; Table S2). Although AMF have been shown to acquire more than 30% of recent photosynthate from their host plants (Drigo et al., 2010), many studies show far lower C allocation to fungi, usually under 10% (or even 5%) of recently fixed C (Calderón et al., 2012; Drigo et al., 2010; Grimoldi et al., 2006; Konvalinková et al., 2017). As the root length colonization in our plants was low, it is not surprising that there was little C acquisition by the extraradical mycelium of the AMF.

In a meta-analysis of 112 studies, Dong et al. (2018) found that plant biomass increased on average by 33% in plants grown at eCO_2 while associated AMF biomass increased by only 6%. It is possible that fungal C acquisition may become limited by the availability of further mineral nutrients such as N when a luxury quantity of C is available. Terrer et al. (2016) demonstrated that N availability largely limits AM plant growth increases in response to eCO_2; further experimental work may determine whether N similarly limits the growth of the extraradical mycelium of AMF. Future work quantifying the abundance of AMF biomass in the soil under different treatments would strengthen the results we have presented here. Sequential harvests of plants at different growth stages up to yield would provide an understanding of mycelial growth through the lifespan of the plant, which we cannot infer from our data.

4.5 | Future perspectives

Despite the ubiquity of AMF in agricultural systems, the mechanisms regulating mycorrhizal functioning in crops remain unclear (Rillig et al., 2019; Ryan & Graham, 2018; Smith & Smith, 2011). Moreover, how rising atmospheric [CO_2] will affect AM symbioses is also uncertain (Cotton, 2018). As the combined pressures of climate change, population growth and environmental accountability mount, and demand for sustainable food production increases through the 21st century, innovative agricultural solutions must be found. Exploiting the soil microbial community has been suggested as one potential tool which could be used to achieve sustainable intensification in agriculture (Rillig et al., 2016; Thirkell et al., 2017).

As we used an unsterilized farm soil in our growth media, it is possible that the differences we observed in carbon-for-nutrient exchange in our experiments were a result of changes in AMF fungal community structure and composition as a result of the [CO_2] treatments in our experiments (Cotton et al., 2015; Panneerselvam et al., 2020). Little is currently known about how or why these changes may occur (Cotton, 2018) but it is clear that different AMF isolates and species show strongly contrasting symbiotic phenotypes (Mensah et al., 2015; Munkvold et al., 2004). As such, [CO_2]-induced changes in AMF community structure and composition may affect C-for-nutrient exchange with host plants. Unfortunately, we did not investigate changes in AMF community composition between [CO_2] treatments in our experiments, but

this is certainly worth future investigation, particularly within the context of future climate change.

Our results, together with those in previous research (Thirkell, Cameron, et al., 2019; Thirkell, Pastok, et al., 2019) suggest that cultivar identity is an important factor in regulating the response of mycorrhizal cereal nutrient acquisition in barley to eCO_2. Our finding that AMF might limit, or even prevent, [CO_2]-induced dilution of P in barley shoots is intriguing, and must be validated in further trials, including those which grow plants to yield, before ultimately being tested in the field (Lekberg & Helgason, 2018). With a greater understanding of the factors regulating carbon-for-nutrient exchange between mycorrhizal symbionts, it should be possible using existing breeding techniques to maximize the benefit of cereal mycorrhizas.

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AUTHOR CONTRIBUTIONS

MC, JD, TJT, and KJF designed the research. TJT, MC, JD, DP, and BM carried out the experimental work. TJT led the manuscript writing and all authors approved of the submitted version.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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