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A reduced fraction of plant N derived from atmospheric N (%Ndfa) and reduced rhizobial nifH gene numbers indicate a lower capacity for nitrogen fixation in nodules of white clover exposed to long-term CO_2 enrichment

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Abstract

Using the δ^{15}N natural abundance method, we found that the fraction of nitrogen derived from atmospheric N (%Ndfa) in field grown white clover (*Trifolium repens* L.) plants was significantly lower (72.0 % vs. 89.5 %, $p = 0.047$ in a grassland exposed to elevated CO_2 for 13 yr using Free Air Carbon Dioxide Enrichment (FACE). Twelve months later we conducted an experiment to investigate the reasons behind the reduced N fixation. We took cuttings from white clover plants growing in the FACE and established individual plants in a glasshouse using soil from the appropriate ambient or elevated CO_2 treatments. The established plants were then transplanted back into their "rings of origin" and sampled over a 6 week period. We used molecular ecological analyses targeting nifH genes and transcripts of rhizobia in symbiosis with white clover (*Trifolium repens* L.) to understand the potential mechanisms. Shoot biomass was significantly lower in eCO_2 but there was no difference in nodule number or mass per plant. The numbers of nifH genes and gene transcripts per nodule were significantly reduced under eCO_2 but the ratio of gene to transcript number and the strains of rhizobia present were the same in both treatments.

We conclude that the capacity for biological nitrogen fixation was reduced by eCO_2 in white clover and was related to the reduced rhizobia numbers in nodules. We discuss the finding of reduced gene number in relation to factors controlling bacteroid DNA amount which may imply an influence of nitrogen as well as phosphorus.

1 Introduction

In the temperate zone, food production from grasslands relies heavily on the input of nitrogen (N) by legumes (Ledgard and Steele, 1992; Whitehead, 1995); white clover (*Trifolium repens* L.) is the predominant species and is estimated to fix about 100–350 kg N ha^{-1} yr^{-1} with a maximum of 670 kg N ha^{-1} yr^{-1} in grasslands in New Zealand (Whitehead, 1995). Consequently, any factor that might modify biological N fixation

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(BNF) has direct interest for agricultural production as well as environmental issues such as N emissions and carbon storage (De Deyn et al., 2009). One such factor is the increasing concentration of CO_2 in the atmosphere (Dolman et al., 2010) which has been shown to have the potential to modify BNF (Lam et al., 2012; Reich et al., 2006; van Groenigen et al., 2006). BNF is arguably of increasing importance under elevated CO_2 (eCO_2) as additional N (through changes in N cycling, fertilization or BNF) is required to maintain C/N ratios in the ecosystem and ensure functions such as photosynthesis and decomposition continue or are increased (Hartwig and Sadowsky, 2006; van Groenigen et al., 2006; Zanetti et al., 1997).

BNF in fertilized grassland is expected to increase under eCO_2 as a result of greater nodule number and/or size and, more rarely, increased specific activity i.e. the amount of N fixed per unit nodule and time (Lam et al., 2012; Rogers et al., 2009) or through a greater proportion of legumes in the system (Newton et al., 2006; Reich et al., 2006). In a long running Free Air Carbon Dioxide Enrichment (FACE) experiment in Switzerland (the Swiss FACE) there was a strong stimulation of BNF in the early years of enrichment with the sum of the physiological and species proportions effects resulting in a 50 % increase in BNF (Hartwig and Sadowsky, 2006). However, deficiencies of other nutrients such as phosphorus (P) have been shown to remove the stimulation of BNF by eCO_2 (Almeida et al., 2000; Edwards et al., 2006; Sa and Israel, 1998) although Nguyen et al. (2006) found the reverse effect where eCO_2 removed the negative effects of P deficiency on BNF of two *Acacia* species. Rogers et al. (2009) concluded that under eCO_2 and adequate nutrients (particularly P), nodule biomass and N_2 fixation per plant will increase (but not specific nitrogenase activity) however there will be no change if nutrients are limiting. The meta-analysis of Van Groeningen et al. (2006) produced a similar conclusion with the strength of the BNF response under eCO_2 mediated by nutrient status. Under nutrient limitation, the effect of eCO_2 is most commonly to return BNF to the same level as at ambient CO_2 (aCO_2) although we have found two reports of negative effects of eCO_2 on BNF (Hungate et al., 2004; West et al., 2005).

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In this paper we describe the effects of aCO_2 and eCO_2 on white clover plants and their associated rhizobia after long-term enrichment of a grazed grassland with elevated CO_2 in a FACE experiment. The system is typical of medium-low input temperate systems where the only *de novo* source of N is BNF. Fertiliser was used to supply P, potassium (K) and sulphur (S) (Newton et al., 2010) but applications were suspended 3 yr prior to the experiment reported here and fertility was at the lower end of the spectrum for managed grassland (see Discussion). Long-term field experiments are important for understanding BNF responses to eCO_2 as results from the field can differ from those found in the laboratory where nutrient supply is constant and the rhizobium strain is the same across treatments (Hartwig and Sadowsky, 2006). Examples of changes in the BNF response to eCO_2 over time can be found in Hungate et al. (2004) and West et al. (2005) referring to earlier work on the same experiment reported by Lee et al. (2003).

Because total soil N has increased in this FACE system (Newton et al., 2010; Ross et al., 2013) we expected that changes could be evident in N inputs through BNF. In 2010, after 13 yr of exposure to eCO_2, we investigated the capacity of white clover (*Trifolium repens* L.), the dominant legume in our system, to fix nitrogen at aCO_2 and eCO_2. We used the ^{15}N natural abundance ($\delta^{15}N$) technique (Shearer and Kohl, 1987; Kerley and Jarvis, 1999) to estimate BNF and found reduced BNF in plants growing under eCO_2. Twelve months later we made a detailed examination of white clover plants and rhizobia to investigate the mechanisms responsible for the change in BNF. This paper describes both the initial study and the subsequent molecular analyses which focussed on rhizobial nifH genes and their transcripts (mRNA). The nifH gene codes for nitrogenase reductase and thus acts as a marker for BNF (Bürgmann et al., 2003); changes in transcript number represent changes in the capacity to fix nitrogen implying a positive relationship between nifH expression and BNF activity and this has indeed been shown to be the case for free-living diazotrophs in soil (Bürgmann et al., 2003) and marine (Zehr et al., 2007) environments and symbiotic rhizobia in nodules (Resendis-Antonio et al., 2011).

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2 Materials and methods

2.1 The FACE experiment

Details of the FACE experiment can be found in (Edwards et al., 2001; Newton et al., 2006; Newton et al., 2010). Briefly, the experiment is on grassland that has not been resown for at least 50 yr. The vegetation includes about 25 species drawn from all the major functional groups of grassland viz. C3 and C4 grasses, forbs and legumes. The site is on the west coast of the North Island of New Zealand (40°1′ S, 175°16′ E). The experimental design pairs ambient and elevated CO_2 rings into three blocks; each ring is 12 m in diameter and is fenced to contain sheep during the grazing periods. Enrichment, to 475 ppm CO_2, started in October 1997 and has been continuous since that time. Grazing is applied using a protocol where animals are introduced when the herbage mass reaches 180–200 g m^{-2} dry weight and removed when the residual herbage is grazed down to 50–70 g m^{-2}. Average annual temperature at the site is 12.9 °C with average annual rainfall of 870 mm.

The soil is a Pukepuke black sand (Mollic Psammaquent) with a 0.25 m black loamy fine-sand topsoil (Cowie and Hall, 1965) that is hydrophobic (Newton et al., 2003). Soil properties are given in Ross et al. (2004) and changes in properties over time in Ross et al. (2013). Fertiliser was applied to maintain adequate levels of P, K and S based on annual soil sampling and established guidelines (Cornforth and Sinclair, 1984) from 1997–2009 (Newton et al., 2010). No N fertiliser was used – the N inputs coming from the legume component of which white clover was the dominant species (Newton et al., 2006). Atmospheric N deposition in this region is minimal (RA Carran unpublished data).

2.2 Determination of %Ndfa

We used the ^{15}N natural abundance ($\delta^{15}N$) technique (Shearer and Kohl, 1987; Kerley and Jarvis, 1999) to estimate the proportion of N in clover plants that could be ascribed

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to BNF (the %Ndfa). We used ryegrass (*Lolium perenne* L.) grown adjacent to the white clover plants as reference plants to estimate δ^{15}N of the N uptake from soil; we used an average *B* value (−1.8) for New Zealand soils (Steele et al., 1983). The *B* value is the δ^{15}N of the legume when completely dependent on N_2 and accounts for any internal fractionation of legume plants (Amarger et al., 1979; Högberg, 1997). The %Ndfa was estimated using the following equation:

$$\%\text{Ndfa} = \frac{\delta^{15}\text{N Ryegrass} - \delta^{15}\text{N white clover}}{\delta^{15}\text{N Ryegrass} - B} \times 100 \quad (1)$$

In the spring of 2010 we harvested undamaged, mature shoot material (leaflet and petiole) from three patches of white clover in each ring and harvested leaves from ryegrass plants growing adjacent to these patches. Each patch constituted a sample giving a total of nine samples for each treatment. The plant material was dried at 60 °C and ground using a pestle and mortar which were thoroughly cleaned between samples. The tissue δ^{15}N was analysed at the Lincoln University Soil Plant Analysis Centre using a mass spectrometer (Anco 20–20 stable isotope analyser).

2.3 Design of experiment to study mechanisms underlying changes in BNF

The experimental protocol was designed to remove potentially confounding variation in the plant and soil material in order to maximize the chance of finding differences between CO_2 treatments. White clover in a permanent pasture consists of clonal fragments with varying proportions of root, stolon and leaf material making an "individual" plant difficult to define. In order to standardize the plant material we took uniform cuttings ("ramets" i.e. a rooted node plus associated leaf and axillary bud) from white clover in the rings in the middle of September 2011 (15 cuttings from each ring) and then allowed them to establish as individual plants in 200 mL pots in a greenhouse under ambient atmospheric CO_2. The soil used was taken from the corresponding rings (i.e. matched with the origin of the cuttings); this soil had previously been sieved (2 mm)

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to remove heterogeneity in nutrient availability introduced by the return of animal excreta and stored at 4 °C until required. After 15 days, the pots were transferred back to the rings with each pot sunk into the ground to leave 10 mm above the surface. The pots were grouped in a 0.5 m × 1 m area within each ring which allowed them to be protected by an exclusion cage during periods when the areas were grazed by sheep. Three plants from each ring were harvested at day 0 to provide an initial above-ground dry weight. Further harvests of six plants were taken 20 d (3 weeks) and 43 d (6 weeks) later. The pots were lifted and placed in plastic bags which were sealed with the air from the sampled ring in an attempt to maintain the atmospheric treatment conditions for as long as possible during the 40 min journey back to the laboratory. Then the plants were removed from the soil, washed with distilled water and divided into shoots and roots. Root samples were immediately frozen in liquid nitrogen and stored at −80 °C until needed for DNA and RNA extractions (see below). Shoots were dried at 60 °C and soil samples were stored at −80 °C until needed for subsequent analysis. Inorganic elements were determined on shoot and soil samples harvested after 6 weeks. The analyses were conducted in a commercial laboratory; briefly the analytical techniques were for plant shoots: N by Dumas combustion, all other elements were extracted using nitric acid/hydrogen peroxide digestion followed by ICP-OES with the exception of molybdenum (Mo) where ICP-MS was used. Soil N used a Kjeldahl digestion followed by FIA colorimetry (the result excludes NO_3-N); P used an Olsen extraction and colorimetry; K and calcium (Ca) were extracted with ammonium acetate and analysed using flame emission spectrophotometry; sulphur (SO_4) had a phosphate extraction followed by ion chromatography; magnesium (Mg) an ammonium acetate extraction and AA spectrophotometry; iron (Fe), manganese (Mn) zinc (Zn) and copper (Cu) were extracted with 0.02 M EDTA and measured by ICP-OES; boron (B) was extracted with 0.01 % $CaCl_2$ followed by ICP-OES and Mo was digested with HNO_3/$HClO_4$ followed by colorimetry.

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2.4 DNA and RNA extraction from nodules

Nodules from three of six replicates from each ring (two of three replicates for day 0) were used for molecular analyses. All nodules were excised with a razor, immersed in 200–600 µL of RNA*later* solution (Ambion, Life Technologies, Carlsbad, CA, USA) in a 2 mL tube with screw cap containing a ball for homogenization and incubated overnight at 4 °C to prevent degradation of RNA. Fresh weights and numbers of the nodules were recorded.

DNA and RNA were extracted simultaneously from the same nodule by TRIZOL reagent (Invitrogen, Life Technologies) by following the standard instructions with minor modifications. In brief, 1000 µL of TRIZOL were added to a nodule sample in a 2 mL tube with screw cap after removal of RNA*later* solution. Then, the nodules were crushed with a Fastprep-24 Instrument (MP Biomedicals, Solon, OH, USA) at a speed of 6.5 for 20 s. After incubating at room temperature for 5 min, 200 µL of Chloroform: Isoamylalcohol (CIA, 24 : 1; Fluka, Sigma-Aldrich, St. Louis, MO, USA) was added and the tubes were vigorously shaken for 15 s. The mixture was centrifuged at maximum speed for 15 min at 4 °C, and then the upper (water) layer was transferred to a new RNase-free 1.5 mL tube for RNA purification. Remaining residues (intermediate and phenol-chloroform layers) were subjected to DNA purification.

For RNA purification, 400 µL of isopropanol was added to the water layer and vigorously mixed. RNA was precipitated by centrifugation at maximum speed for 20 min at 4 °C after cooling at −20 °C for 15 min. The supernatant was removed and the RNA pellet rinsed in 800 µL of 75 % ethanol by centrifugation at maximum speed for 5 min at 4 °C. The pellet was then air-dried at room temperature and dissolved in 100 µL of RNase-free ultrapure water. The extracted RNA was further purified by an RNeasy kit (Qiagen, Hilden, Germany) by following the manufacturer's instructions and finally dissolved in 30–50 µL of RNase-free ultrapure water. An RNAsecure reagent (Ambion) was added to RNA solution and incubated at 60 °C for 10 min to inactivate ribonuclease (RNase) completely. Deoxyribonuclease (DNase) I (Amplification grade; Invitro-

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gen) was added to approximately 0.5–1 µg of extracted RNA to remove any contaminating DNA.

For DNA purification, 240 µL of 99.7 % ethanol was added to the residue and vigorously mixed. DNA was precipitated by centrifugation at maximum speed for 15 min at 4 °C after incubation for 5 min on ice. The supernatant was removed and the DNA pellet was washed with 800 µL of 0.1 M sodium citrate dissolved in 10 % ethanol. After incubation for 30 min at room temperature, the tubes were centrifuged again and the supernatant was discarded. The DNA was dissolved in 300 µL of TE buffer and then purified by phenol : chloroform : isoamylalcohol (25 : 24 : 1; Fluka, Sigma-Aldrich) and CIA to separate the DNA from plant debris. Finally, the DNA was collected by ethanol precipitation and rinsed in 70 % ethanol. The DNA pellet was then air-dried at room temperature and dissolved in 50–60 µL of Tris-ethylenediaminetetraacetic acid (EDTA) buffer (TE buffer; 10 mM Tris-HCl, 1.0 mM EDTA).

We confirmed there was no contamination by DNA in DNase I-treated RNA extracts using polymerase chain reaction (PCR) without the reverse transcription reaction using a primer set PolFI/AQER-G30 targeting nifH genes. The quantity and quality of extracted DNA and RNA solutions were measured with NanoDrop ND-1000 (Thermo Fisher Scientific, Waltham, MA, USA).

2.5 cDNA synthesis by reverse transcription reaction

cDNA was synthesized from purified RNA samples by a SuperScript VILO cDNA Synthesis kit (Invitrogen), following the manufacturer's instructions. Approximately 0.5–1 µg of RNA was used in the reaction.

2.6 PCR-DGGE analysis of nifH genes

Partial nifH gene was polymerase chain reaction (PCR)-amplified by primers PolFI/AQER-GC30 (Poly et al., 2001; Wartiainen et al., 2008). Platinum Taq polymerase (Invitrogen) was used for amplification. DNA and synthesized cDNA samples,

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which were diluted to approximately 0.5–1.5 ng μL^{-1} by TE buffer, were used as PCR templates. The PCR program had an initial denaturation at 94 °C for 15 min, followed by 35 cycles of denaturation at 94 °C for 1 min, annealing at 55 °C for 1 min and extension at 72 °C for 1 min, and final extension at 72 °C for 10 min. PCR amplicons were checked by agarose gel electrophoresis with ethidium bromide.

Denaturing gradient gel electrophoresis (DGGE) analysis was carried out with a TTGE/DGGE system (model TTGE-2401; C.B.S. Scientific Inc., Del Mar, CA, USA). Amplified nifH gene fragments were applied onto an 8 % polyacrylamide gel with 45–60 % denaturing gradient, in which 100 % of denaturant contained 7 M urea and 40 % (v/v) formamide. Electrophoresis was carried out at 50 V for 20 h at 60 °C in 1 × Tris-acetate-EDTA buffer (TAE buffer; 40 mM Tris-acetate, 1 mM EDTA). After electrophoresis, gel was stained by 1 : 10 000 diluted SYBR green I for 30 min and photographed with the GelDoc system (Biorad, Hercules, CA, USA) under ultra violet light.

2.7 Real-time PCR

Real-time PCR analysis of nifH genes and transcripts was carried out with a primer set PolF/AQER by Lightcycler 480 (Roche, Penzberg, Germany) and SYBR master I kit (Roche), according to the manufacture instructions. A clone of nifH gene derived from *Rhizobium* sp. R1–1, which was isolated from white clover in an eCO_2 ring (see below), was used as the standard reference. The PCR amplicon of the nifH gene was ligated into pCR2.1-TOPO vectors (Invitrogen) and transformed into competent cells of *Escherichia coli* Mach1-T1 (Invitrogen). Positive clones were obtained by blue-white selection and confirmed by colony-PCR with PolF/PolR. Plasmid DNA was extracted with a QIAprep Spin Miniprep kit (Qiagen) after incubation with Luria–Bertan overnight. Copy number of the plasmid DNA was calculated from absorbance at 260 nm (50 ng μL^{-1} when A_{260} is 1.0). Serial dilutions of nifH gene-cloned plasmid DNA ($1.88 \times 10^1 - 1.88 \times 10^7$ copies μL^{-1}) were used as standard references. The program for the real-time PCR had an initial denaturation at 95 °C for 5 min, followed by 45 cy-

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cles of denaturation at 94 °C for 10 s, annealing at 55 °C for 20 s and extension at 72 °C for 30 s. Fluorescence signal was detected at every extension step. No amplification of nonspecific DNA fragments was confirmed by dissociation curve analysis.

2.8 Isolation of rhizobia from nodules

In situ white clovers were sampled from each ring on 13 October 2011. The roots were washed in distilled water by sonication and four pinkish nodules were selected from each ring. The nodules were surface sterilized by immersing in 96 % ethanol for 1 min and 1 % sodium hypochloride for 5 min. The sterilized nodules were washed 5 times with sterilized water and crushed with a sterilized rod in 200 µL of sterilized water. The suspensions were inoculated on yeast extract-mannitol (YM) agar plates, which contained 1 g of yeast extract, 10 g of mannitol, 0.5 g of K_2HPO_4, 0.41 g of $MgSO_4 \cdot 7H_2O$, 0.1 g of NaCl, 1 g of $CaCO_3$ and 15 g of agar in one liter and incubated at 28 °C in the dark. Formation of white and/or creamy colonies was confirmed after 3–7 days of incubation. A single colony was repeatedly transferred to new YMA plates for purification.

Nodulation of isolated strains on white clover was confirmed by the following procedure. White clover seeds were surface-sterilized by immersing in 96 % ethanol for 10 s and 3 % hydrogen peroxide for 5 min. After 6 washings in sterilized water, the seeds were immersed in sterilized water for 4 h to imbibe. The seeds were washed twice more in sterilized water and spread on 0.75 % water agar plates for incubation at 25 °C for 3–5 days. Germinated seeds were transplanted onto 1.5 % water agar slants with N-free nutrient solution, which contained 0.4 M $CaCl_2 \cdot H_2O$, 0.2 M KH_2PO_4, 4 mM Fe-citrate, 0.1 M $MgSO_4 \cdot 7H_2O$, 0.1 M K_2SO_4, 0.4 mM $MnSO_4 \cdot H_2O$, 0.8 mM H_3BO_3, 0.2 mM $ZnSO_4 \cdot 7H_2O$, 0.08 mM $CuSO_4 \cdot 5H_2O$, 0.04 mM $CoSO_4 \cdot 7H_2O$ and 0.04 mM $Na_2MoO_2 \cdot 2H_2O$ (Somasegaran and Hoben, 1985). Each isolated strain was picked up from a single colony, suspended in sterilized water and the germinated seeds inoculated. The seeds were then incubated at 25 °C in the light. Nodulation was confirmed after 10 days of incubation.

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2.9 Sequencing analysis of DGGE bands and isolated rhizobial strains

The nucleotide sequences of some DGGE bands and nifH and 16S rRNA genes of different types of isolated strains were determined by direct sequencing as described by Watanabe et al. (2004). For the isolated strains, a single colony was suspended in sterilized water and directly subjected to PCR as a template. Partial nifH and 16S rRNA genes were amplified with PolF/PolR (Poly et al., 2001) and 27f/1492r (Lane, 1991), respectively. The PCR program for both nifH and 16S rRNA genes was the same as the program above. PCR amplicons were checked by 2 % of agarose gel electrophoresis with ethidium bromide and purified by a QIAquick PCR purification kit (Qiagen).

A BigDye terminator cycle sequencing kit (Applied Biosystems, Life Technologies) and an ABI 3130 genetic analyzer (Applied Biosystems) were used for sequencing analysis. Nucleotide sequences of nifH genes were translated to deduced amino acid sequences by the European Molecular Biology Open Software Suite (EMBOSS) Transeq program (Rice et al., 2000). Closest relatives of the 16S rRNA genes and deduced amino acid sequences of nifH genes were searched with the basic local alignment search tool (BLAST) program (Altschul et al., 1990).

2.10 Accession numbers

Sequences of nifH and 16S rRNA genes determined in the present study have been deposited in the DDBJ/EMBL/GenBank nucleotide sequence databases under accession no. AB721420- AB721425, AB721426-AB721431 and AB721432-AB721444 for 16S rRNA genes and nifH genes of isolated rhizobia and nifH genes of DGGE bands, respectively.

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2.11 Statistical analysis

There was more than one sample (pseudoreps) from each ring for %Ndfa, shoot dry weight, nodule number and dry weight and nifH gene and transcript numbers; these samples were averaged to give a single value per ring so that $n = 3$ in all cases.

Data for %Ndfa and inorganic elements were analyzed by one-way ANOVA in Genstat (Payne et al., 2010). The %Ndfa data were arcsine transformed before analysis to normalize the distribution.

Other data were analyzed with a mixed effects model with the random effect "Ring" nested within "Block" and the fixed effects "CO_2", "Time" (as a factor) and their interaction using "R" (Pinheiro et al., 2012). Variance was stabilized using a square root transformation for the variables nodule weight, gene and gene transcript numbers per plant and a natural log transformation for shoot weight, gene and transcript number per nodule. For the analysis of the nodule number per plant we used a mixed effects model with the same structure as above but with error term following a Poisson distribution. Fisher's LSD test was used for post-hoc comparison between treatment means.

3 Results

3.1 %Ndfa

The %Ndfa under aCO_2 was 89.8 ± 3.8 (mean ± sem) and under eCO_2 was 72.0 ± 7.2. These values were significantly different ($p = 0.047$).

3.2 Aboveground biomass, number and weight of nodules

During the 6 weeks of growth in the FACE rings shoot biomass in aCO_2 increased from 57.5 to 226.4 mg and from 71.3 to 146.0 mg in eCO_2. There was a significant Time x CO_2 interaction ($p = 0.003$) resulting from significantly greater biomass under aCO_2 compared to eCO_2 after 6 weeks but not at the start of the experiment or after 3 weeks

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(Fig. 1a). Nodule number and nodule fresh weight per plant increased over time in both treatments but there was no effect of CO_2 (Fig. 1b, c).

3.3 Real-time PCR analysis of nodule nifH genes and their transcripts

The numbers of nifH genes and gene transcripts per nodule remained the same over time in aCO_2 but decreased in eCO_2 leading to a significant CO_2 × time interactions in both cases ($p = 0.001$ for genes Fig. 2a) ($p = 0.016$ for transcripts Fig. 2b). By six weeks there were significantly fewer genes and transcripts per nodule under eCO_2 compared to aCO_2 (LSD exceeded by the difference between means, Fig. 2a, b). There was an increase in nifH gene number per plant in aCO_2 but a decline in eCO_2 leading to significantly fewer genes under eCO_2 after 6 weeks (Fig. 2c). Transcript number per plant was relatively constant over time in aCO_2 but declined in eCO_2 leading to a marginally significant CO_2 × time interaction ($p = 0.069$, Fig. 2d).

3.4 Inorganic elements in shoot and soil samples

There were minor differences between treatments in the elemental composition of plant and soil samples (Table 1). S and Cu were both significantly higher under eCO_2 in the leaves (Table 1a) and B significantly lower in the soil; soil Zn was marginally significantly lower under aCO_2 and Mg marginally higher (Table 1b). There was no difference in N or P concentrations in shoot or soil although there was a trend for lower soil P under eCO_2. Neither Mo or Fe, which are essential metals for nitrogenase enzyme function, were significantly different in plant or soil between aCO_2 and eCO_2.

3.5 Phylogenetic relationships of isolated rhizobial strains

In total, 19 strains were isolated from eCO_2 and aCO_2 rings (9 and 10 strains, respectively). DGGE analysis of nifH genes of isolated strains showed that isolates were divided into 5 different types depending on the band mobility although two bands were observed from each isolate probably because of degenerate primers (Fig. 3a). The mo-

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bility of type B was same as that of type E, but we judged that these types were different because the growth of type-E strains was slower than that of type-B strains. Sequencing analysis of 16S rRNA and nifH genes of these five types of rhizobia showed that all types of strains were closely related to *Rhizobium leguminosarum* bv. *trifolli* (Table 2). Sequences of 16S rRNA genes and deduced amino acid sequences of nifH genes were identical among the strains except for R5–1. Nucleotide sequences of nifH genes between the strains R1-1 and R2-1 (Type A) and between R3-1 and R6-1 (Type B and E) were identical (Fig. 3a).

3.6 DGGE analysis of nifH genes and transcripts of nodules

DGGE band patterns of nifH genes and transcripts in white clover nodules in aCO_2 and eCO_2 rings during incubation are shown in Fig. 3b–f. All bands were closely related to the sequences from *Rhizobium leguminosarum* bv. *trifolli* (Fig. 3c, Table 2). Four different types of nifH genes were identified, that is, the nucleotide sequences of the band 1, 2 and 13 were identical to those we have called Type-A nifH gene. The sequences were the same among bands 3, 4, 5, 6 and 7 and among bands 8, 9, 10 and 11. The nucleotide sequence of band 12 was identical to our Type-B (E) nifH genes. Type-A *Rhizobium* sp. were dominant in both aCO_2 and eCO_2 rings and during the whole incubation period. Type-B (E) species and non-isolated types were also observed in some plants and their occurrence ratio among samples increased during the incubation. Transcripts of nifH genes also showed similar tendency with some exceptions (the patterns of nifH transcripts in R6-2 at 0 day and R1-2 at 6 weeks were different from those of nifH genes). But, distinct differences between aCO_2 and eCO_2 rings were not observed in DGGE band patterns of nifH genes and their transcripts.

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4 Discussion

Our measurement of ^{15}N natural abundance showed that BNF in white clover (measured as %Ndfa) was lower under eCO_2. In the subsequent experiment looking at mechanisms we found the same number of nodules per plant in eCO_2 as in aCO_2 (Fig. 1c) but each nodule had significantly fewer nifH genes (Fig. 2a) and transcripts (Fig. 2b) resulting in fewer nifH genes per plant (Fig. 2c) and a trend for reduced transcript numbers per plant (Fig. 2d). We did not measure nitrogen fixation per se in this experiment but evidence for a positive relationship between expression of nifH genes and fixation (Bürgmann et al., 2003; Resendis-Antonio et al., 2011; Zehr et al., 2007) implies reduced BNF under eCO_2 which is consistent with the earlier finding of a lower %Ndfa at eCO_2.

It is generally thought that elevated CO_2 will result in increased N fixation unless the supply of nutrients (particularly P) is inadequate in which case the effect on BNF is removed (Edwards et al., 2006; Rogers et al., 2009; van Groenigen et al., 2006). There are only a few examples of negative effects of eCO_2 on BNF; Hungate et al. (2004) found reductions in the vine *Galactia elliottii* and West et al. (2005) found negative responses in some species of grassland legumes. We are not aware of previous reports of reduced BNF in white clover growing under eCO_2.

There was no difference in nutrient status in the plant or soil for Mo or Fe (Table 1a, b), elements essential for BNF, or for any nutrients likely to be involved in an indirect effect on BNF such as P and K. However, if inadequate P can remove a BNF response to CO_2 then we need to ask at what level of P does this occur? Høgh-Jensen et al. (2002) have shown that below 0.27 % P in shoots of white clover there are changes that reduce BNF; these include reductions in nodule mass but not changes in the nitrogenase activity per unit of nodule mass. This does not answer the question at what level of P does any BNF response to CO_2 cease but it does show our plant with 0.17 % P under aCO_2 and 0.16 % P under eCO_2 (Table 1a) were growing in a low P environment. In an experiment on white clover under different N and P supply, Edwards et al. (2006)

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found there was no difference in BNF between aCO_2 and eCO_2 at the low nutrient supply where leaf concentration of P was ≈ 0.13 % but a 31 % increase in BNF at eCO_2 when P was added giving a leaf P concentration of ≈ 0.26 %. The corresponding N/P ratios were 21.4 at low P and 11.8 at high P which compare to our values of 16.2 at eCO_2 and 14.7 for aCO_2 (calculated from data in Table 1a). So, based on the leaf P concentrations, we can conclude that the P availability was in the range where nutrient limitation might occur. In a fertiliser experiment using soil from this FACE experiment in a controlled environment, Gentile et al. (2012) found no difference in leaf P concentrations for aCO_2 and eCO_2 in *Lolium perenne* (ryegrass) plants but found a P limitation to growth under eCO_2 implying a higher demand for P in this treatment. To put our P status in some context, relationships drawn from fertiliser trials in New Zealand with ryegrass/white clover swards indicate a clover P concentration of 0.26 % is required to gain 90 % of the maximum response to P (Sinclair et al., 1997). In the Swiss FACE, where fertiliser was added, the P concentration of the white clover averaged 0.43 % (Lüscher et al., 2006). Natural systems without fertiliser have much lower P concentrations, for example, in the serpentine grassland in California used for the mini-FACE experiments in the Jasper Ridge reserve the *Avena* sp. had P levels of 0.05–0.10 % (Menge and Field, 2007). The N/P ratios we found (16.2 for eCO_2 and 14.7 for aCO_2) were below the level of 20 suggested to indicate P limitation (Güsewell, 2004) and fell in the range that would suggest a contribution from BNF (%Ndfa) of about 60 % (see Fig. 4 Almeida et al., 2000). So while we are certainly working in a low P system by managed grassland standards which can achieve P concentration of > 30 % we still have a P level above those of many natural systems.

One potential source of differences in BNF is the identity of the rhizobium strain. Laboratory experiments frequently inoculate with the same strain e.g. (Almeida et al., 2000; Edwards et al., 2006) thus removing potential treatment differences in strain selection and potential differences in N fixation efficiency that might be strain related (Montealegre et al., 2000). In the early years of the 10 yr's operation of the Swiss FACE experiment, Montealegre et al. (2000) found changes in the population struc-

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ture of *Rhizobium leguminosarum* bv. *trifolli* with the strains that dominated at eCO_2 producing 17 % more nodules under eCO_2 conditions than those isolated from aCO_2. However, the differences in strain identity disappeared over time (Hartwig and Sadowsky, 2006) which is consistent with the results we found that showed no differences between treatments in nifH gene sequences (Table 2) or in strain identity as shown by DGGE banding patterns (Fig. 3).

The process of N fixation involves multiple steps and thus offers a number of potential opportunities for modification. N fixation starts with rhizobial infection and nodule formation which occur after rhizobia are attracted to plant roots by flavonoids exuded from the roots. Rhizobia release species-specific lipochitooligosaccharides (nod factors) and nodule development is then initiated (Ferguson et al., 2010). The rhizobia invade the plant, most usually through root hairs, and then become incorporated in the root surrounded by a membrane of plant-derived tissue (Ferguson et al., 2010). The rhizobia continue to divide and then differentiate into bacteriods which perform the N fixation. In galegoid legumes, such as *Trifolium* spp., the differentiation of the bacteroids involves genome amplification through endoreduplication (Mergaert et al., 2006) resulting in "dramatic" increases in DNA content (Prell et al., 2009). The processes of differentiation and endoreduplication are now known to be mediated by the plant (Prell et al., 2009) specifically by the supply of amino acids (particularly glutamate) to the amino acid-starved bacteroids (Prell et al., 2009; Mergaert et al., 2006). Our results show there was no effect of CO_2 on nodule number (Fig. 1c) suggesting no interference with infection or nodulation. There was a reduction in the number of nifH genes per nodule (Fig. 2a) and in the number of transcripts per nodule (Fig. 2b) but no change in the ratio of transcripts/gene number (mean aCO_2 13.7, eCO_2 10.2 $p = 0.336$, data not shown) suggesting that the efficiency of fixation was unchanged. Nutrient factors such as P insufficiency frequently alter nodulation (nodule number) as well as nodule weight (Almeida et al., 2000). As we found no effect on nodule number (Fig. 1c) and a non-significant reduction in nodule weight after 6 weeks in eCO_2

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(Fig. 1b) we did not have results consistent with the effects of inadequate P supply although we cannot exclude a CO_2 effect mediated by nutrient status.

The variable that is driving our results is the reduced number of nifH genes under eCO_2. We are not aware of any literature on elevated CO_2 and rhizobia that has considered this variable although nodule weight presumably is related to DNA amount. Because, in the galegoid legumes, the plant controls the DNA increase of the bacteroids through the transfer of amino acids (Prell et al., 2009) it is relevant to consider whether elevated CO_2 might have any effects that might modify amino acid transfer particularly the supply of glutamatae. In their study of leaf amino acid contents and photorespiration, Novitskaya et al. (2002) showed that increasing CO_2 concentration led to reduced 2-oxoglutarate (2-OG) – which provides the C skeleton necessary for ammonia assimilation (Lancien et al., 2000), where 2-OG is deficient the ratio of glutamine/glutamate increases (Novitskaya et al., 2002). We conclude that the factors modifying BNF in eCO_2 in this experiment might well include N interactions that are not currently well described.

An increased input from BNF under eCO_2 is highly desirable if C/N ratios are to be maintained in plants and soils and thus functions such as photosynthesis and decomposition be maintained or enhanced (Hartwig and Sadowsky, 2006; Reich et al., 2006; Soussana and Hartwig, 1996; Zanetti et al., 1997). The fate of BNF under eCO_2 is also of central importance for future productivity of pastoral systems that rely on BNF for N input. Consequently, our finding of a negative effect of eCO_2 on BNF capacity is of concern. In the early years of this experiment we inferred that BNF was increased due to a greater proportion of legumes under eCO_2 (Newton et al., 2006). However, as the experiment has continued the absolute amount of legume has remained higher under eCO_2 but the proportion has returned to be similar to that under aCO_2 (Paul Newton, unpublished data). If a lower capacity for BNF under eCO_2 in the longer-term is correct then we can say that although the proportion of legume is unchanged the relative input of N from fixation will be less under eCO_2 with implications for sustained growth responses to CO_2. The finding of increased total soil N under eCO_2 in this ex-

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periment (Newton et al., 2010; Ross et al., 2013) can therefore only be ascribed in part to additional N inputs through a greater legume proportion in the early years of the experiment but must also likely include other changes in N cycling such as the observed enhancement of mineralisation-immobilisation turnover (Rütting et al., 2010). In the three other long-term CO_2 enrichment experiments where BNF has been studied there is clear evidence of declining responsiveness to eCO_2. In the Swiss FACE, under both high and low N fertiliser treatments, the initial stimulation in the proportion of plant N yield derived from BNF at eCO_2 declined over 10 yr to be the same or less than that measured at ambient (Richter, 2003). During the first 14 months of enrichment in a scrub-oak ecosystem BNF was stimulated in *G. elliottii* by eCO_2 (Hungate et al., 1999) but by the 5–7th years of treatment BNF was significantly reduced under eCO_2 (Hungate et al., 2004). In the BioCON FACE experiment, in the treatment without added N fertiliser, BNF in *Lupinus perennis* added twice as much N per unit area in eCO_2 compared to aCO_2 after one year of enrichment (Lee et al., 2003) but after 4 yr N fixation in this species was significantly lower under eCO_2 (West et al., 2005). As BNF is a critical process in controlling the response of ecosystems to elevated CO_2 (Lüscher et al., 2000; Reich et al., 2006; Rogers et al., 2009) the impact of long-term exposure to CO_2 enrichment on BNF deserves further attention.

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Table 1. Elemental analysis of white clover shoot (leaf + petiole) and (b) soil sampled after 6 weeks of the experiment. Values are means ($n = 3$). p values ≤ 0.05 are in bold, values ≤ 0.1 are in italic.

(a)

PLANT		N g 100 g^{-1}	P g 100 g^{-1}	K g 100 g^{-1}	S g 100 g^{-1}	Ca g 100 g^{-1}	Mg g 100 g^{-1}	Na g 100 g^{-1}	Fe mg kg^{-1}	Mn mg kg^{-1}	Zn mg kg^{-1}	Cu mg kg^{-1}	By mg kg^{-1}	Mo mg kg^{-1}
aCO_2	mean	2.48	0.17	1.88	0.19	0.99	0.19	0.14	557	50	53	5.5	23	0.20
	sem	0.20	0.03	0.27	0.01	0.11	0.02	0.02	71	2	4	0.6	1	0.06
eCO_2	mean	2.92	0.16	2.28	0.25	0.88	0.24	0.20	561	49	61	6.8	21	0.33
	sem	0.28	0.01	0.37	0.02	0.08	0.01	0.05	156	8	8	0.4	2	0.07
	p	0.424	0.691	0.529	**0.036**	0.402	0.211	0.225	0.985	0.888	0.267	**0.015**	0.347	0.264

(b)

SOIL		N g 100 g^{-1}	P µg mL^{-1}	K mg kg^{-1}	S mg kg^{-1}	Ca mg kg^{-1}	Mg mg kg^{-1}	Na mg kg^{-1}	Fe mg kg^{-1}	Mn mg kg^{-1}	Zn mg kg^{-1}	Cu mg kg^{-1}	B mg kg^{-1}	Mo mg kg^{-1}
aCO_2	mean	0.31	23	46	2.2	613	62	20	754	34	5.1	4.2	0.36	0.54
	sem	0.02	4	9	0.1	13	6	1	52	5	0.8	0.2	0.03	0.04
eCO_2	mean	0.30	15	56	2.5	647	73	20	706	31	4.6	4.5	0.57	0.58
	sem	0.02	1	11	0.2	30	6	2	123	5	0.7	1.3	0.02	0.05
	p	0.762	0.223	0.370	0.195	0.532	*0.081*	0.930	0.789	0.633	*0.054*	0.855	**0.040**	0.440

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Table 2. Phylogenetic closest relatives of (a) isolated rhizobial 16SrRNA gens, (b) of deduced amino acid sequences of isolated rhizobial nifH genes, and (c) deduced amino acid sequences of nifH genes from DGGE bands (see Fig. 3). R1-3 are eCO_2 rings and 4-6 aCO_2 rings.

(a) Phylogenetic relatives of isolated rhizobial 16S rRNA genes.

Strain	nifH type	Seq bp	Identity	Closest relative	Accession No.	Isolation source
R1-1	A	1403	1403/1403 (100 %)	*Rhizobium leguminosarum* bv. trifolii WSM1325	CP001622	annual clover plant in Greece
R2-1	A	1403	1403/1403 (100 %)	*Rhizobium leguminosarum* bv. trifolii WSM1325	CP001622	annual clover plant in Greece
R3-1	B	1403	1403/1403 (100 %)	*Rhizobium leguminosarum* bv. trifolii WSM1325	CP001622	annual clover plant in Greece
R4-2	C	1403	1403/1403 (100 %)	*Rhizobium leguminosarum* bv. trifolii WSM1325	CP001622	annual clover plant in Greece
R5-1	D	1403	1403/1403 (100 %)	*Rhizobium leguminosarum* bv. trifolii Rlt E11	U73208	*Trifolium alexandrinum*
R6-1	E	1403	1403/1403 (100 %)	*Rhizobium leguminosarum* bv. trifolii WSM1325	CP001622	annual clover plant in Greece

(b) Phylogenetic relatives of deduced amino acid sequences of isolated rhizobial nifH genes.

Strain	nifH type	Amino acid	Identity	Closest relative	Accession No.	Isolation source
R1-1	A	106	106/106 (100 %)	*Rhizobium leguminosarum* bv. trifolii WSM1325	AC5 59148	annual clover plant in Greece
R2-1	A	106	106/106 (100 %)	*Rhizobium leguminosarum* bv. trifolii WSM1325	AC5 59148	annual clover plant in Greece
R3-1	B	106	106/106 (100 %)	*Rhizobium leguminosarum* bv. trifolii WSM1325	AC5 59148	annual clover plant in Greece
R4-2	C	106	106/106 (100 %)	*Rhizobium leguminosarum* bv. trifolii WSM1325	AC5 59148	annual clover plant in Greece
R5-1	D	106	103/106 (97 %)	*Rhizobium leguminosarum* bv. trifolii WSM1325	AC5 59148	annual clover plant in Greece
R6-1	E	106	106/106 (100 %)	*Rhizobium leguminosarum* bv. trifolii WSM1325	AC5 59148	annual clover plant in Greece

(c) Phylogenetic relatives of deduced amino acid sequences of nifH genes of DGGE bands.

Band	nifH type	Amino acid	Identity	Closest relative	Accession No.	Isolation source
1	A	99	99/99 (100 %)	*Rhizobium leguminosarum* bv. trifolii WSM1325	AC5 59148	annual clover plant in Greece
2	A	99	99/99 (100 %)	*Rhizobium leguminosarum* bv. trifolii WSM1325	AC5 59148	annual clover plant in Greece
3	unknown	99	98/99 (99 %)	*Rhizobium leguminosarum* bv. trifolii WSM1325	AC5 59148	annual clover plant in Greece
4	unknown	99	98/99 (99 %)	*Rhizobium leguminosarum* bv. trifolii WSM1325	AC5 59148	annual clover plant in Greece
5	unknown	99	98/99 (99 %)	*Rhizobium leguminosarum* bv. trifolii WSM1325	AC5 59148	annual clover plant in Greece
6	unknown	99	98/99 (99 %)	*Rhizobium leguminosarum* bv. trifolii WSM1325	AC5 59148	annual clover plant in Greece
7	unknown	99	98/99 (99 %)	*Rhizobium leguminosarum* bv. trifolii WSM1325	AC5 59148	annual clover plant in Greece
8	unknown	99	99/99 (100 %)	*Rhizobium leguminosarum* bv. trifolii WSM1325	AC5 59148	annual clover plant in Greece
9	unknown	99	99/99 (100 %)	*Rhizobium leguminosarum* bv. trifolii WSM1325	AC5 59148	annual clover plant in Greece
10	unknown	99	99/99 (100 %)	*Rhizobium leguminosarum* bv. trifolii WSM1325	AC5 59148	annual clover plant in Greece
11	unknown	99	99/99 (100 %)	*Rhizobium leguminosarum* bv. trifolii WSM1325	AC5 59148	annual clover plant in Greece
12	B (E)	99	99/99 (100 %)	*Rhizobium leguminosarum* bv. trifolii WSM1325	AC5 59148	annual clover plant in Greece
13	unknown	99	99/99 (100 %)	*Rhizobium leguminosarum* bv. trifolii WSM1325	AC5 59148	annual clover plant in Greece

nifH types correspond to the DGGE band mobility (Fig. 3a)

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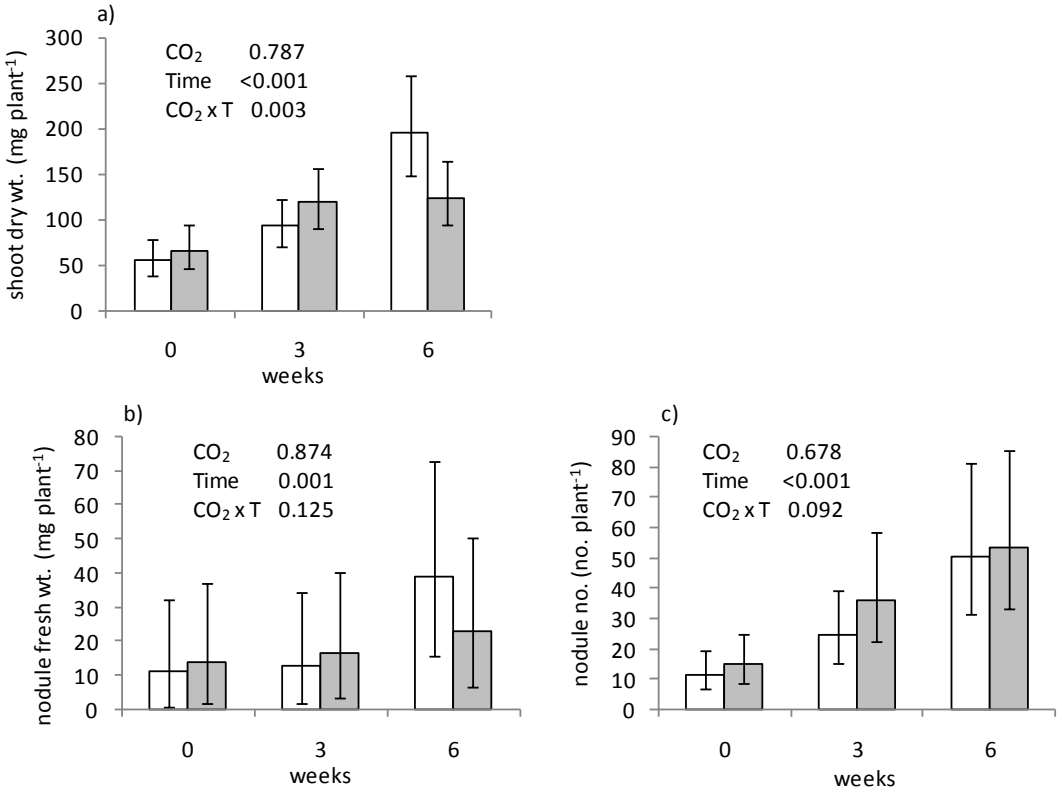

Fig. 1. (a) shoot (leaf + petiole) dry weight of white clover plants grown under aCO_2 (open bars) or eCO_2 (filled bars) over 6 weeks; **(b)** nodule fresh weight per plant and **(c)** nodule number per plant. Values are means (n = 3) and bars are 95 % confidence intervals.

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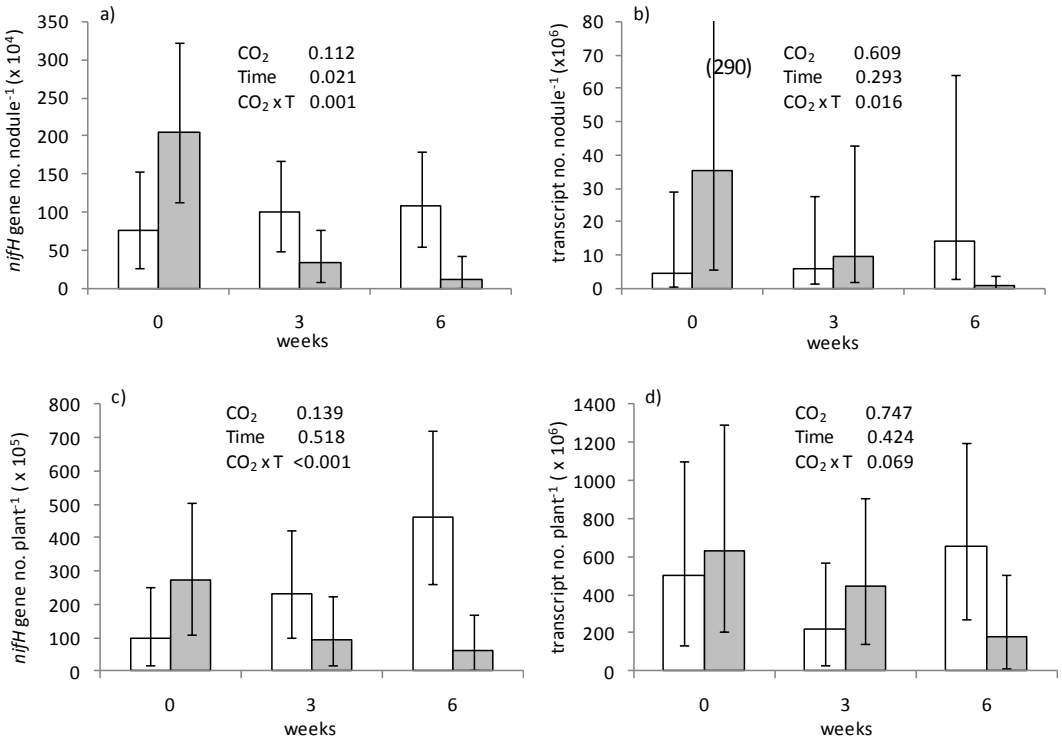

Fig. 2. (a) nifH gene number and **(b)** nifH gene transcript number per nodule and **(c)** nifH gene number and **(d)** nifH gene transcript number per white clover plant grown under aCO_2 (open bars) or eCO_2 (filled bars). Values are means (n = 3) and bars are 95 % confidence intervals.

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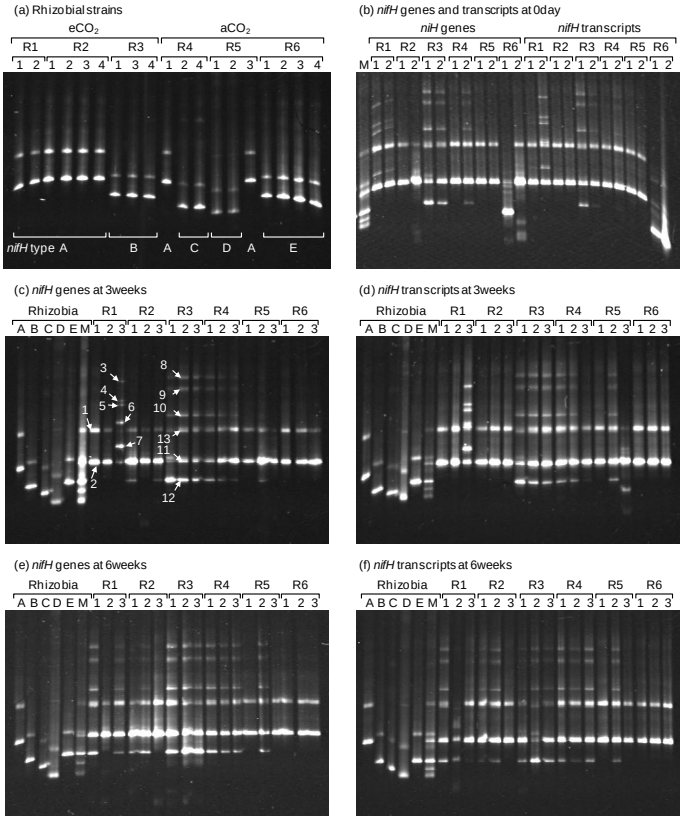

Fig. 3. DGGE band patterns of nifH genes and their transcripts from isolated rhizobial strains **(a)** and nodules **(b–f)**. Denaturing gradients range from 45–60 %. R1–R3 and R4–R6 are patterns from eCO_2 and aCO_2 rings respectively. A–E indicates nifH gene types of isolated strains. M indicates a mixture of nifH gene fragments derived from type A–E strains. Arrows with numbers show the bands that were used for sequencing.

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