Leaf δ¹⁵N as a physiological indicator of the responsiveness of N₂-fixing alfalfa plants to elevated [CO₂], temperature and low water availability

Idoia Ariz 1, Cristina Cruz 1, Tomé Neves 1, Juan J. Irigoyen 2, Carmen Garcia-Olaverri 3, Salvador Noguès 4, Pedro M. Aparicio-Tejo 5 and Iker Aranjuelo 6, 7*

1 Faculdade de Ciências, Centro Ecologia Evolução e Alterações Ambientais, Universidade de Lisboa, Lisboa, Portugal, 2 Grupo de Fisiología del Estrés en Plantas, Departamento de Biología Ambiental, Unidad Asociada al CSIC, EED, Zaragoza e ICSV, Logroño, Spain, 3 Departamento de Estadística e Investigación Operativa, Universidad Pública de Navarra, Pamplona, Spain, 4 Departamento de Biología Vegetal, Facultat de Biologia, Universitat de Barcelona, Barcelona, Spain, 5 Departamento de Ciencias del Medio Natural, Universidad Pública de Navarra, Pamplona, Spain, 6 Plant Biology and Ecology Department, Science and Technology Faculty, University of the Basque Country, Leioa, Spain, 7 Instituto de Agrobiotecnología (IADAB), Universidad Pública de Navarra-CSIC-Gobierno de Navarra, Mutxel Baja, Spain

The natural ¹⁵N/¹⁴N isotope composition (δ¹⁵N) of a tissue is a consequence of its N source and N physiological mechanisms in response to the environment. It could potentially be used as a tracer of N metabolism in plants under changing environmental conditions, where primary N metabolism may be complex, and losses and gains of N fluctuate over time. In order to test the utility of δ¹⁵N as an indicator of plant N status in N₂-fixing plants grown under various environmental conditions, alfalfa (Medicago sativa L.) plants were subjected to distinct conditions of [CO₂] (400 vs. 700 μmol mol⁻¹), temperature (ambient vs. ambient +4°C) and water availability (fully watered vs. water deficiency—WD). As expected, increased [CO₂] and temperature stimulated photosynthetic rates and plant growth, whereas these parameters were negatively affected by WD. The determination of δ¹⁵N in leaves, stems, roots, and nodules showed that leaves were the most representative organs of the plant response to increased [CO₂] and WD. Depletion of heavier N isotopes in plants grown under higher [CO₂] and WD conditions reflected decreased transpiration rates, but could also be related to a higher N demand in leaves, as suggested by the decreased leaf N and total soluble protein (TSP) contents detected at 700 μmol mol⁻¹ [CO₂] and WD conditions. In summary, leaf δ¹⁵N provides relevant information integrating parameters which condition plant responsiveness (e.g., photosynthesis, TSP, N demand, and water transpiration) to environmental conditions.

Keywords: alfalfa, climate change, growth, δ¹⁵N, physiology
**Introduction**

Considering the current rate of increase in CO₂ emissions (1.5 µmol mol⁻¹ year⁻¹), it is expected that atmospheric CO₂ concentrations ([CO₂]) will reach 550 µmol mol⁻¹ by 2050 and 700 µmol mol⁻¹ by 2100 (Myhre et al., 2013). The associated warming is expected to be greatest in summer in south-western Europe.

Although atmospheric [CO₂] is now limiting for C₃ photosynthesis and growth, the predicted increase in [CO₂] in coming decades could enhance photosynthetic rates and biomass production in C₃ plants (Farquhar et al., 1980a; Bowes, 1993; Amthor, 2001; Long et al., 2004). Nevertheless, the interaction of CO₂ with other limiting environmental factors, (e.g., higher temperature, lower water, and/or nitrogen availabilities) might decrease or eliminate the positive effect of elevated CO₂ on plant production (Ainsworth et al., 2004; Rogers et al., 2009; Aranjuelo et al., 2011).

Most experiments analysing the effects of climate change on plant growth have considered the variability of individual environmental factors (CO₂, temperature, water availability), keeping others at levels optimal for growth. However, analyses of the effect of CO₂ and its interaction with other environmental conditions are of great relevance. In the field, multiple stresses, such as high temperature and drought periods in semi-arid or drought-stricken areas, often occur simultaneously. Studies of field crops and model plants have shown that the combination of heat and drought stresses has a stronger detrimental effect on plants growth and productivity than either stress alone. Furthermore, many reports indicate that it is not possible to extrapolate plant responses to combined stresses based on the responses to single stresses (Rampino et al., 2012).

In recent decades, stable isotope techniques (Isotope Ratio Mass Spectrometry, IRMS, mostly with ¹³C and ¹⁸O) have been used as tools that provide useful information on parameters conditioning plant growth, such as transpiration efficiency, the ratio of net photosynthesis to water transpired, etc., and that integrate the period during which CO₂ is assimilated (Araus et al., 2002, 2003; Yousfi et al., 2010). Moreover, ¹³C isotope composition (δ¹³C) has been used as a breeding criterion for increasing yield in crops exposed to low water availability and salinity stresses (Yousfi et al., 2009, 2010; Araus et al., 2013). Variations in ¹⁵N isotope composition (δ¹⁵N) have also been proposed as a useful trait for crop screening (Pritchard and Guy, 2005; Yousfi et al., 2012). Robinson et al. (2000) proposed that the natural abundance of both ¹³C and ¹⁵N might indicate responses to stresses such as drought and nitrogen starvation. Moreover, δ¹³C and δ¹⁵N have been used to characterize the response of crops to salinity (Yousfi et al., 2009) and are widely used in plant ecophysiology to assess the effects of changing climatic conditions as both are sensitive to environmental constraints (Peuke et al., 2006). Three main factors have been described (Evans, 2001; Pritchard and Guy, 2005; Coque et al., 2006; Tcherkez, 2011) as determining plant δ¹⁵N: (i) morphophysiological differences (particularly in root systems); (ii) activity of principal enzymes involved in N assimilation and (iii) plant N demand and assimilation capacity.

However, it should be remembered that further ¹⁵N fractionation might take place as a result of N recycling, transport, exudation or volatilization (through stomata as ammonia and nitrous oxide) by the plants (Cernusak et al., 2009). Although δ¹⁵N has been previously determined in N₂-fixing plants (Arnone, 1999; Wanek and Arndt, 2002), with very few exceptions (Shearer et al., 1982; Unkovich, 2013) this parameter has been mostly determined in plants grown with both N sources: N₂ and NO₃⁻. The natural ¹⁵N abundance method has been widely used to provide semi-quantitative estimates of the relative contribution of atmospheric N₂ to N₂-fixing plants growing in natural and agricultural settings (Shearer and Kohl, 1988), where N is available in several forms (i.e., NO³⁻, NH₄⁺, N₂, etc.). Thus, despite recent advances in the interpretation of plant δ¹⁵N, there is still a lack of knowledge of δ¹⁵N in plants where N₂-fixation is the sole source of N.

Given that atmospheric N₂ is an unlimited N source, and that N₂-fixing legumes comprise the second most important group of agricultural crops worldwide (FAOSTAT, 2010¹), the use of δ¹⁵N as an integrative indicator of the responsiveness of N₂-fixing plants to climate change conditions may be of great interest. The study of δ¹⁵N gradients along plant axes (from N source to sinks) and their reaction to environmental stresses may provide valuable information on the transport and metabolism of C-N compounds (Peuke et al., 2006). To achieve this, exclusively N₂-fixing alfalfa (Medicago sativa L) plants, which are frequently exposed to high temperature and/or drought in field conditions, were studied. They were subjected to distinct levels of [CO₂] (400 vs. 700 µmol mol⁻¹), temperature (ambient vs. ambient +4°C) and water availability (fully watered vs. partially watered). In addition to growth, we characterized the N isotopic composition (δ¹⁵N) of whole plants and separate organs (leaves, stems, roots and nodules), and δ¹⁵N relationship with C-N related parameters.

**Materials and Methods**

**Plant Material and Experimental Design**

Alfalfa (Medicago sativa L. cv Aragon) plants were grown in 13 L plastic pots (five plants per pot) filled with 1:2 (v/v) vermiculite-perlite. At 2–4 weeks after planting, they were inoculated with Sinorhizobium meliloti strain 102F78 (The Nitragin Co., Milwaukee, WI, USA). One-month-old plants were transferred to the corresponding temperature gradient greenhouses (TGG; Figure S1). The experimental design and the use of the greenhouses were similar to that described by Morales et al. (2014). Half of the plants were placed at 700 µmol mol⁻¹ of [CO₂] in a TGG, whereas the other half was grown in a different TGG under ambient [CO₂] (400 µmol mol⁻¹). Within each TGG, one for each CO₂ concentration (400- and 700-µmol mol⁻¹), plants were separated into 4 treatments corresponding to all combinations of, temperature (ambient—around 19°C—and ambient +4°C) and water availability (control—fully irrigated- or drought -partially irrigated-). After 1 month development, at the

¹http://faostat.fao.org/site/291/default.aspx
corresponding growth conditions, gas exchange measurements and harvest were carried out (60 days—old plants).

**[CO₂] Control within the TGGs**

Ventilated [CO₂] temperature and humidity sensors (M22WHT4X transmitters, Rotronic Instrument Corp., Hauppauge, USA) and air probes connected to another CO₂ infrared gas analyser were placed at the center of each module 60 cm above the plants.

The [CO₂], concentration was monitored continuously at the outlet module by an infrared analyser (Guardian Plus gas monitor, Edinburgh Instruments Ltd, Livingston, UK) whose signal was fed into a proportional integrative differential controller that regulated the opening time (within a 10-s cycle) of a solenoid valve that injected CO₂ into both inlet fans because otherwise lateral mixing of CO₂ in the chambers was not complete. The data were continuously recorded by a computer through analog-digital converters (Microlink 751, Biodata Ltd, Manchester, UK) using Windmill software with the Test-Seq programming tool (Biodata Ltd). A subroutine of this software controlled solenoid valves that kept one of two sets of CO₂ cylinders open or closed (provided by Air Liquide, Bilbao, Spain) thus supplying the gas to the elevated CO₂ tunnel. When CO₂ concentration decreased below a fixed level, signaling that one of the cylinder sets was exhausted, the corresponding valve was closed and that of the other set opened.

**Temperature Control within the TGGs**

The measured temperature difference was used to set the required fan speed by altering the current: the gradient decreased or increased as the fan was sped or slowed, respectively. Two inlet fans (each 90 W, 0.5 m³ s⁻¹) mounted on the inlet module and an outlet fan (140 W, 0.54 m³ s⁻¹) mounted in the roof of the outlet compartment continuously circulated air through the tunnel at the speed required to maintain a difference of 4°C between the two extreme modules. The fan at the tunnel outlet was in the roof, rather than in the end wall of the outlet compartment, so that any external wind would not disrupt the temperature gradient (Morales et al., 2014). Air flow was continuously varied by changing the fan speed to achieve the end-to-end temperature difference. Three small fan heaters (variable 250–500 W), placed above plant level in the outlet compartment and facing the tunnel interior, were used to help maintain the temperature difference at night and whenever solar radiation was insufficient to raise the temperature.

**Water Treatment**

When analysing the interaction between [CO₂] and water availability, it should be remembered that plants grown at elevated [CO₂] deplete soil water at a lower rate than those grown with ambient [CO₂] (due to lower stomatal conductance and lower transpiration rates), so in many experiments, elevated [CO₂] increased the time to reach a particular water stress (De Luis et al., 1999; Aranjuelo et al., 2009). To test this, we designed an experiment in which all treatments were subjected to the same soil water content. Well-watered (WW) plants were irrigated until they reached maximum soil volumetric water content (θₑ), whereas partially irrigated plants (WD) were watered at 50% θₑ of WW plants. These θₑ levels were maintained throughout the experiment by daily measurement of transpired water (calculated by weighing the pots) and replenishing the lost water. In order to reduce evaporation from the soil, pots were covered with a plastic sheet perforated with very small holes to allow stems to pass through. In order to supply all treatments with the same amount of nutrients, WW plants were alternately watered with Evans N-free nutrient solution and distilled water, while WD plants were always watered with Evans solution. Pots were rotated weekly in each module to avoid edge effects. In order to avoid differences due to chamber effects, the plants were moved from one greenhouse to another every month. All the determinations listed below were made at the end of the experiment, when the plants were 60 days old, in apical fully expanded leaves.

**Plant Growth Determinations**

Plant growth in the TGGs under the aforementioned [CO₂], temperature and water availability conditions was determined by harvesting after 1 month of growth. Twenty plants were collected per treatment combination. The plants were divided into leaves, stems, roots and nodules, and the fresh weight of these components was recorded. After drying at 60°C for 48 h, their dry weight was determined. Leaf area was analyzed with an electronic planimeter (Li-3000 with LI-3050 conveyer accessory, LICOR, NE, USA). Total dry matter (DM) comprised leaf, stem, root and nodule DM.

**Total Soluble Protein (TSP) Content**

Proteins were extracted from frozen leaf subsamples and ground to a fine powder [in 50 mM Tricine buffer, pH 8.0, 1 mM EDTA, 5 mM 6-aminocaproic acid, 2 mM benzamidine, 8 mM β-mercaptoethanol, and 100 mM phenylmethylsulfonylfluoride (PMSF)]. This was kept on ice for 20 min and then centrifuged at 12,000 g and 4°C for 25 min. The total soluble protein content of the supernatant was determined according to the Bradford method (Bradford, 1976).

**Gas Exchange Analyses**

Fully expanded apical leaves from 50-day-old plants were individually enclosed in a leaf chamber (1010-M, Waltz, Effeltrich, Germany), and the gas exchange rate was measured with a portable photosynthesis system (HCM-1000, Waltz) under growth conditions. Net photosynthesis (A) and leaf conductance (g) were calculated as described by von Caemmerer and Farquhar (1981). The leaf internal CO₂ concentration (Ci) was estimated from net photosynthesis and conductance measurements according to Farquhar and Sharkey (1982). Fully expanded leaves were enclosed in a GFS-3000 portable gas exchange system (Waltz, Effeltrich, Germany). Gas exchange analyses were conducted in every plant grown at 400 and 700 μmol mol⁻¹ [CO₂] (A400 and A700 respectively), at the corresponding growth temperature and with a photosynthetic photon flux density of 1200 μmol m⁻² s⁻¹.

**C and N Isotope and Content Analysis**

A subsample of frozen leaf, stem, root and nodule from each plant was dried at 60°C for 48 h in small tin capsules and weighed.
The nitrogen and carbon isotope composition of the samples was determined using a Flash 1112 Elemental Analyzer (Carbo Erba, Milan) coupled to an IRMS Delta C isotope ratio mass spectrometer through a Conflo III Interface (Thermo-Finnigan, Germany).

Nitrogen results were expressed in parts per thousand (‰) in the δ notation (δ¹⁵N) using international secondary standards of known ¹⁵N/¹⁴N ratios (IAEA N₁ and IAEA N₂ ammonium sulfate and IAEA NO₃ potassium nitrate) relative to N₂ in air:

\[ \delta^{15}N = \left( \frac{R_{\text{sample}}}{R_{\text{standard}}} \right) - 1 \]  

where \( R \) is the ¹⁵N/¹⁴N ratio.

N and C contents were determined in three biological replicates of dried nodule, root and leaf samples, ground to powder, weighed (1.0 mg per sample) and stored in tin capsules.

TABLE 1 | Regression analysis between organ δ¹⁵N in 60-day-old nodulated alfalfa plants exposed to differing climate conditions.

| Factor          | [CO₂] (µmol CO₂ mol⁻¹) | Temperature (°C) | Water availability | Global |
|-----------------|------------------------|------------------|-------------------|--------|
|                 | 400                    | 700              |                   |        |
| Stem/Leaves     |                        |                  |                   |        |
| Slope           | 0.7*                   | 0.99             | 1.33*             | 1.02***|
| p-value         | 0.072                  | ns               | 0.089             | 0.009  |
| Root/Stem       |                        |                  |                   |        |
| Slope           | −0.83**                | 0.63             | 0.80              | −0.40  |
| p-value         | 0.011                  | ns               | ns                | 0.034  |
| Nodule/Root     |                        |                  |                   |        |
| Slope           | −0.2                   | −0.06            | −0.26             | −0.77**|
| p-value         | ns                     | ns               | ns                | 0.084  |

Nodule against root; root against stem; stem against leaves. The slopes from linear regression models [Model: \( Y = (a) + bX \), where \( Y \) corresponds to δ¹⁵N_{sink-organ} and \( X \) corresponds to δ¹⁵N_{source-organ}] are given with p-values and significances (ns, no significant differences, \( p > 0.1 \); * refer to significant differences where \( P \leq 0.1 \); ** refer to significant differences where \( P \leq 0.05 \); *** refer to significant differences where \( P \leq 0.01 \); **** refer to significant differences where \( P \leq 0.001 \)). Significant values are shown in bold text. For further details see legend to Figure 1.

**FIGURE 1** | Axial patterns of δ¹⁵N in 60-day-old nodulated alfalfa plants grown under differing conditions of CO₂ concentration [400 µmol CO₂ mol⁻¹, left panels (A,C), or 700 µmol CO₂ mol⁻¹, right panels (B,D)], temperature [ambient, upper panels (A,B), or +4°C, lower panels (C,D)] and water availability (well watered, WW, or water deficiency, WD). This figure summarizes data concerning δ¹⁵N values showed in Table 4 and Tables S1–S3. Data represent average values ± SE (n = 3).
TABLE 2 | Analysis of variance of the effect of [CO\(_2\)], water availability and temperature on plant growth, gas exchange and N fixation parameters.

| Factor       | Total biomass DM (g) | Root/Shoot Ratio | \(A_{\text{plant}}\) (\(\mu\)mol CO\(_2\) plant\(^{-1}\) s\(^{-1}\)) | Tr\(_{\text{plant}}\) (mmol H\(_2\)O plant\(^{-1}\) s\(^{-1}\)) | \(g_{\text{growth}}\) (mmol CO\(_2\) m\(^{-2}\) s\(^{-1}\)) | Total N\(_{\text{fixed}}\) (\(\mu\)mol N\(_{\text{fixed}}\) plant\(^{-1}\)) |
|--------------|----------------------|------------------|-------------------------------------------------|---------------------------------|---------------------------------|-------------------------------|
| [CO\(_2\)]   | *                    | ns               | ns                                              | *                               | ***                             | ns                            |
| H\(_2\)O     |                     |                  |                                                 | ns                               | ns                              | ns                            |
| T            | ns                   | ns               | ns                                              | ns                               | ns                              | ns                            |
| [CO\(_2\)]H\(_2\)O |                 | −                 | −                                               | −                                | +                               | ns                            |
| [CO\(_2\)]T  | −                    | −                 | −                                               | −                                | ns                              | ns                            |
| H\(_2\)O\(T\) | −                    | −                 | −                                               | −                                | −                               | −                             |

The effects of carbon dioxide concentration ([CO\(_2\)], water availability (H\(_2\)O), temperature (T) and their peer interactions ([CO\(_2\)]H\(_2\)O, [CO\(_2\)]T and H\(_2\)O\(T\)) were determined by (One- and Two-Way) ANOVA tests using SPSS software. Significant effects are shown with asterisks (* refer to significant differences where \(P \leq 0.1\); ** refer to significant differences where \(P \leq 0.01\); *** refer to significant differences where \(P \leq 0.001\); interaction between factors, +; no interaction between factors, −). Letters ns denote no significant differences (n = 3).

TABLE 3 | Analysis of variance of the effect of [CO\(_2\)], water availability and temperature on leaf C-N-related parameters.

| Factor       | Leaf area (cm\(^2\)) | TSP (mg prot g\(^{-1}\) DM) | N content (%) | C/N | \(\delta^{15}\)N (%) |
|--------------|-----------------------|-----------------------------|---------------|-----|---------------------|
| [CO\(_2\)]   | ns                    | ****                        | ****          | ****| ****                |
| H\(_2\)O     |                     | ns                           | ns            | ns  | ns                  |
| T            | ns                    | ns                           | ns            | ns  | ns                  |
| [CO\(_2\)]H\(_2\)O |                 | −                             | −             | −   | −                   |
| [CO\(_2\)]T  | −                    | +                            | −             | −   | +                   |
| H\(_2\)O\(T\) | −                    | −                            | −             | −   | −                   |

The effects of carbon dioxide concentration ([CO\(_2\)], water availability (H\(_2\)O), temperature (T) and their peer interactions ([CO\(_2\)]H\(_2\)O, [CO\(_2\)]T and H\(_2\)O\(T\)) were determined by (One- and Two-Way) ANOVA tests using SPSS software. Significant effects are shown with asterisks (* refer to significant differences where \(P \leq 0.1\); ** refer to significant differences where \(P \leq 0.01\); *** refer to significant differences where \(P \leq 0.001\); interaction between factors, +; no interaction between factors, −). Letters ns denote no significant differences (n = 3).

using an elemental analyser (EA1108, Series 1; Carbo Erba Instrumentazione, Milan, Italy).

Statistical Analysis

Statistical analyses were performed with the programs SPSS for Windows, version 15.0 (Sections Statistical analysis of physiological and C-N-related parameters in N\(_2\)-fixing alfalfa plants grown under various environmental conditions and Regression analyses of axial patterns of \(\delta^{15}\)N and Statistica 10, data analysis software system, version 10 (StatSoft, Inc. 2011; Section Statistical analyses of leaves: relationships among C-N natural isotopic abundances and physiological parameters.).

Statistical analysis of physiological and C-N-related parameters in N\(_2\)-fixing alfalfa plants grown under various environmental conditions

We examined results from eight treatments using analysis of variance (ANOVA) to test for effects and interactions of the various combinations of three environmental factors ([CO\(_2\)], temperature and water availability), and whether these results varied according to the organ tested. Besides analysis of whole plants (exploratory analysis, data not shown), each organ (nodule, root, stem, and leaves) was analyzed separately. Homoscedasticity was determined using the Levene test (Levene, 1960), then One- and Two-Way ANOVA tests, including interaction terms, were conducted using data displayed in Figure 2, Tables 2, 3 and Tables S1–S3.

Regression analyses of axial patterns of \(\delta^{15}\)N

Linear regression models (Table 1) were performed using the model: \(Y = (a) + bX\), where \(Y\) corresponds to \(\delta^{15}\)N\(_{\text{sink}}\)–organ and \(X\) corresponds to \(\delta^{15}\)N\(_{\text{source}}\)–organ.

Statistical analyses of leaves: relationships among C-N natural isotopic abundances and physiological parameters

Following an exploratory-inferential approach, data analysis revealed that leaves were the organs that were the most influenced by environmental factors, so several descriptive statistical analyses were conducted only on data from leaves. Simple regression models were estimated for \(\delta^{15}\)N and target parameters conditioning plant growth (e.g., plant biomass, plant level photosynthesis, TSP, leaf area, N content). Correlation and simple regression models for leaf parameters (Figures 3–5) were used to determine \(R^2\) and \(p\)-values for each analysis.

The results of this study were obtained for plants cultured in several independent series, at least one sample was analyzed for each of three independent series. Sample size varied depending on the analysis carried out, from 32 (for organ specific descriptive analysis) up to 192 (for exploratory-inferential analysis).

Results and Discussion

It is generally accepted that leaf \(\delta^{15}\)N reflects the \(15\)N abundance of plant main N source(s): available soil N for non-N\(_2\)-fixing plants and atmospheric N\(_2\) for N\(_2\)-fixing plants (Shearer and Kohl, 1988). Since, by definition, the \(\delta^{15}\)N of atmospheric N\(_2\) is 0, that of N\(_2\)-fixing plants growing without any other N source should also be around 0, but in fact it can be very distinct from zero (Unkovich, 2013). The precise value of the N\(_2\)-fixing plants \(\delta^{15}\)N depends, among other factors, on: (1) the physiological partition of the N metabolism between
FIGURE 2 | Plant growth (dry matter, DM, and root/shoot ration), total photosynthesis, total transpiration and total N fixed per plant of 60-day-old nodulated alfalfa plants exposed to differing environmental conditions: CO$_2$ concentration (400 µmol CO$_2$ mol$^{-1}$, left panels, or 700 µmol CO$_2$ mol$^{-1}$, right panels); temperature (ambient or +4°C); and water availability (well watered, WW, or water deficient, WD). Legend: (A,B)—relative and total plant growth; the relative bar areas represent the individual organ percentage relative to the total plant growth (black line); (C,D)—root/shoot ratio; (E,F)—total photosynthesis, $A_{plant}^{-1}$ (G,H)—total transpiration, $T_{plant}^{-1}$; (I,J)—total N fixed. Data represent average values ± SE ($n = 3–6$).
FIGURE 3 | Leaf N isotopic composition ($\delta^{15}N$; ‰) of nodulated alfalfa plants exposed to differing environmental conditions correlated with: (A–H) leaf biomass (grams); (I–L) $C_i$ (µmol CO$_2$ mol$^{-1}$); (M–P) transpiration rate (mmol H$_2$O m$^{-2}$ s$^{-1}$); (Q–T) leaf N content (%, w/w); and (U–X) leaf total soluble protein (TSP; mg prot g$^{-1}$ DM). Datasets were categorized in terms of environmental conditions: [CO$_2$], 400 or 700 ppm, left panels; water availability, well watered—WW, or water deficient—WD, right panels. The dataset displayed represents individual observations, at least $n = 3$ for each environmental combination. Significant $p$-values are shown in bold text. Significance: $p > 0.1$; *$p \leq 0.1$; **$p \leq 0.05$; ***$p \leq 0.01$; ****$p \leq 0.001$. 
shoot and root; (2) the N efflux; and (3) on the exudation of metabolites. The δ¹⁵N values of the distinct plant organs (nodules, roots, stems and leaves) show that leaf δ¹⁵N is the one more responsive to environmental factors (Figure 1; Table 1). The increase of ambient temperature by 4°C, did not significantly modify any leaf C-N related parameter (including leaf δ¹⁵N; Tables 1–3). The combined effect of increased [CO₂] and WD caused the more significant changes in leaf δ¹⁵N (Figure 1, Table 1).

δ¹⁵N as Affected by Stomatal Opening

Control plants (400 ppm CO₂, WW, environmental temperature) did not show differences between the δ¹⁵N values of nodules, roots or stems (δ¹⁵N ≈ −1.0), while leaves presented δ¹⁵N values closer to zero (Figure 1A). Theoretically this relative enrichment of the leaves in ¹⁵N may be due to NH₃ losses from the atmosphere (Farquhar et al., 1980b), and may be associated with two main factors: (1) the leaf NH₃ pool is predominantly originated through photorespiration and may have a δ¹⁵N as low as −40 ‰ (Handley et al., 1999; Peuke et al., 2006); and (2) the ¹⁴N is lost more readily through the stomata than ¹⁵N (O’Deen, 1989). In fact both environmental factors, [CO₂] and water availability lead to reduced stomatal conductance (Figures S2E,F) and transpiration rates (Figures S2G,H; Figures S2C,D; Table 2). As a consequence, the δ¹⁵N of leaves from plants grown at increased [CO₂] and/or WD tended to have lower leaf δ¹⁵N than those from plants grown at ambient [CO₂] or from WW plants (i.e., higher stomatal opening Figures 1, 3). However, the ranges of ¹⁵N depletion in leaves caused by both factors, WD and [CO₂], were not exactly the same (≈ −0.5 for [CO₂] and ≈ −1 to −1.5 for WD; Figure 1).

Leaf δ¹⁵N as an Indicator of Plant N Demand and Organ N Partitioning

Considering that the variability of δ¹⁵N in leaves reflects changes in N metabolic and metabolite fluxes, and/or environment-driven effects, leaf δ¹⁵N has been proposed as a good candidate for tracing these effects in plants (Tcherkez, 2011). Plants showing healthy physiological features (i.e., higher leaf and plant biomass, leaf area, leaf N content, and leaf TSP) had leaf δ¹⁵N values closer to that of their N source (δ¹⁵Natmosphere = 0; Figures 1–3). In contrast, plants affected by [CO₂] and water availability, with impaired growth (Figure 2, Table 2), had more negative leaf δ¹⁵N values (−2 to −0.5; Figure 1). These differences highlight the effect of environmental factors on transport and partitioning of N metabolism in N₂-fixing plants (Peuke et al., 2006). Correlation-regression analyses confirmed that both environmental factors ([CO₂] and water availability) influenced the correlations between leaf δ¹⁵N and biomass and several physiological parameters (leaf biomass, plant biomass, internal concentration of CO₂, transpiration, foliar N content and foliar TSP) (Figure 3). However, some other relationships were mostly influenced by [CO₂] (e.g., leaf area; Figure 4) or by water availability (e.g., stomatal conductance; Figure 5). The depletion of foliar δ¹⁵N under high [CO₂] has also been observed in a wide range of plant species (27 field-grown plant species) and ecosystem types (Bassirirad et al., 2003). However, there is no direct evidence that water availability influences foliar N isotope composition (Peuke et al., 2006).

This differential response of leaf δ¹⁵N to the combination of [CO₂] and water availability, together with the observed low correlations between leaf δ¹⁵N and plant transpiration associated with high [CO₂] and water deficiency (Figures 3N,P), suggest that other metabolic processes (different from stomatal conductance, see above) could be involved in such an isotopic effect. Higher [CO₂] and WD led to different C/N balances...
and N requirements (Figures 21, J; Tables 3, 4), which may be related to the observed differences in leaf δ15N. Despite the potentially increased C availability at higher [CO2], and the higher plant growth demonstrated by these plants (Figure 2; Table 2), they did not increase their total fixed-N2 (Figure 2), leading to unbalanced foliar N contents (%; ≈2% at 700 vs. ≈4–5% at 400 μmol mol−1) and C/N ratios (Table 4). The lower foliar N content at higher [CO2] indicates a higher N demand, limiting plant growth under such conditions. This concept is supported by the similarity of the Δ15N in leaves and nodules (WW plants, Figure 1), which suggests negligible losses of N and optimization of the N use efficiency (NUE) of the N2-fixing plants grown at high [CO2]. In other words, all fixed N is being used by the plants. In fact, plants containing increased leaf TSP contents had leaf δ15N values close to zero (δ15Natmosphere = 0; Figure 3), so the growth of N2-fixing plants exposed to higher [CO2] is determined by their N2 fixation capacity. Similar results were described by Bassirirad et al. (2003) with mycorrhizal plants exposed to elevated [CO2].

Plant N demand has been described as a key factor conditioning Δ15N (Tcherkez, 2011), so the higher N demand by alfalfa leaves exposed to higher [CO2] could lead to differential N partitioning between the plant’s above- and below-ground parts. On the other hand, translocation of organic N compounds rather than inorganic N (i.e., ammonium) from bacteroids to the plant (nodules, roots, stems, and finally leaves, mainly in the form of Asn in alfalfa plants; Kaspar et al., 2008) could also lead to a more 14N-enriched signature of plant organs, because the assimilated N organic pool in plants is generally 14N-enriched relative to the unassimilated N inorganic pool (Wermer and Schmidt, 2002; Kalcis and Guy, 2013).

Conclusion

Leaf δ15N was a sensitive integrator of such combined environmental stresses on N2-fixing alfalfa plants: plants affected by higher [CO2] and water deficiency, which displayed impaired growth features, had more negative leaf δ15N values than that of atmospheric N2. In contrast, physiologically healthy plants had leaf δ15N signatures close to those of their N source (δ15Natmosphere = 0). This observation, together with further investigation of isotope fractionation during transport and metabolic processes, may provide useful information on the metabolism, transport and allocation of N in N2-fixing plants exposed to combined environmental stresses.

Acknowledgments

This work was supported by the Spanish Economy and Competitiveness ministry (AGL-2012-37815-C05-05, AGL2011-30386-C02-02 and Ramón y Cajal research grant) and by the Portuguese FCT (PTDC/BLA-ECS/122214/2010). IA was supported by a postdoctoral Fellowship from the Government of Navarra (Anabasid outgoing Programme) and by a postdoctoral Fellowship from the Portuguese FCT (SFRH/BPD/90436/2012).

Supplementary Material

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fpls.2015.00574

References

Ainsworth, E. A., Rogers, A., Nelson, R., and Long, S. P. (2004). Testing the “source-sink” hypothesis of down-regulation of photosynthesis in elevated [CO2] in the field with single gene substitutions in Glycine max. Agric. For. Meteorol. 122, 85–94. doi: 10.1016/j.agrformet.2003.09.002

Amthor, J. S. (2001). Effects of atmospheric CO2 concentration on wheat yield: review of results from experiments using various approaches to control CO2 concentration. Field Crops Res. 73, 1–34. doi: 10.1016/S0378-4290(01)00179-4

Aranjuelo, I., Cabrera-Bosquet, L., Morcuende, R., Avice, J. C., Nogués, S., Araus, J. L., et al. (2011). Does ear C sink strength contribute to overcoming photosynthetic acclimation of wheat plants exposed to elevated CO2? J. Exp. Bot. 62, 3957–3969. doi: 10.1093/jxb/err095

Aranjuelo, I., Irigoyen, J. J., Nogués, S., and Sánchez-Díaz, M. (2009). Elevated CO2 and water-availability effect on gas exchange and module development in N2-fixing alfalfa plants. Environ. Exp. Bot. 65, 18–26. doi: 10.1016/j.envexpbot.2008.06.006

Araus, J. L., Cabrera-Bosquet, L., Serret, M. D., Bort, J., and Nieto-Taladriz, M. T. (2013). Comparative performance of 13C, 15O and 18O for phenotyping

TABLE 4 | Responsiveness of leaf C-N-related parameters of 60-day-old nodulated alfalfa plants exposed to different climate conditions.

| Treatments | Leaf area (cm²) | TSP (mg prot g⁻¹ DM) | N content (%) | C/N | δ15N (%) |
|------------|----------------|---------------------|---------------|-----|----------|
| 400–WW-Amb | 369 ± 13       | 5.3 ± 0.12          | 4.2 ± 0.06    | 10.8 ± 0.11 | −0.14 ± 0.06 |
| 400–WD–Amb | 158 ± 12       | 5.5 ± 0.23          | 4.8 ± 0.18    | 9.8 ± 0.35  | −0.60 ± 0.09  |
| 400–WW–4°C | 423 ± 60       | 4.8 ± 0.19          | 4.8 ± 0.03    | 9.8 ± 0.07  | −0.61 ± 0.06  |
| 400–WD–4°C | 149 ± 7        | 4.6 ± 0.11          | 4.6 ± 0.02    | 10.2 ± 0.05 | −0.91 ± 0.05  |
| 700–WW–Amb | 390 ± 39       | 4.5 ± 0.11          | 2.1 ± 0.01    | 20.8 ± 0.10 | −0.57 ± 0.02  |
| 700–WD–Amb | 171 ± 10       | 3.6 ± 0.09          | 2.3 ± 0.05    | 19.5 ± 0.40 | −1.76 ± 0.10  |
| 700–WW–4°C | 513 ± 42       | 5.4 ± 0.04          | 2.6 ± 0.01    | 17.4 ± 0.01 | −0.74 ± 0.13  |
| 700–WD–4°C | 183 ± 21       | 4.2 ± 0.18          | 2.5 ± 0.07    | 18.5 ± 0.49 | −1.45 ± 0.11  |

Parameters: leaf area (cm²); leaf total soluble proteins (TSP, mg prot g⁻¹ DM); leaf N content (%; m/m); leaf C/N ratio; and N natural isotopic signature of leaves (%). Environmental conditions: CO2 concentration (400 or 700 μmol CO2 mol⁻¹), temperature (ambient, Amb, or 4°C) and water availability (well watered, WW, or water deficient, WD). Data represent average values ± SE (n = 3–6).
durum wheat adaptation to a dryland environment. *Funct. Plant Biol.* 40, 595–608. doi: 10.1071/FP12254

Araus, J. L., Slafer, G. A., Reynolds, M. P., and Royo, C. (2002). Plant breeding and drought in C3 cereals: what should we breed for? *Ann. Bot.* 89, 925–940. doi: 10.1093/aob/mcf049

Araus, J. L., Villegas, D., Aparicio, N., Garcia del Moral, L. F., El Hani, S., Rharrabti, Y., et al. (2003). Environmental factors determining carbon isotope discrimination and yield in durum wheat under Mediterranean conditions. *Crop Sci.* 43, 170–180. doi: 10.2135/cropsci2003.1700

Arnone, J. A. III. (1999). Symbiotic N$_2$ fixation in a high Alpine grassland: effects of four growing seasons of elevated CO$_2$. *Funct. Ecol.* 13, 383–387. doi: 10.1046/j.1365-2435.1999.00325.x

Bassirirad, H., Constable, J. V. H., Lussenhop, J., Kimball, B. A., Norbys, R. J., Oechel, W. C., et al. (2003). Widespread foliage $^{15}$N depletion under elevated CO$_2$: inferences for the nitrogen cycle. *Global Change Biol.* 9, 1582–1590. doi: 10.1046/j.1365-2486.2003.00679.x

Bowes, G. (1993). Facing the inevitable: plants and increasing atmospheric CO$_2$. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* 44, 309–332. doi: 10.1146/annurev.pp.44.060193.001521

Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein–dye binding. *Ann. Biochem* 72, 248–254. doi: 10.1016/0003-2697(76)90527-3

Cernusak, L. A., Winter, K., and Turner, B. L. (2009). Plant $^{15}$N correlates with the transpiration efficiency of nitrogen acquisition in tropical trees. *Plant Physiol.* 151, 1667–1676. doi: 10.1104/pp.109.145870

Coque, M., Bertin, P., Hirel, B., and Gallais, A. (2006). Genetic variation and QTLs for $^{15}$N natural abundance in a set of maize recombinant inbred lines. *Field Crops Res.* 97, 310–321. doi: 10.1016/j.fcr.2005.11.002

De Luis, J., Iriogoyen, J. J., and Sánchez-Díaz, M. (1999). Elevated CO$_2$ enhances plant growth in droughted N$_2$-fixing alfalfa without improving water status. *Physiol Plantarum.* 107, 84–89. doi: 10.1111/j.1399-3054.1999.100112.x

Evans, R. D. (2001). Physiological mechanisms influencing plant nitrogen isotope composition. *Trends Plant Sci.* 6, 121–126. doi: 10.1016/S1360-1385(01)01889-1

Farquhar, G. D., Firth, P. M., Wetseelaar, R., and Weir, B. (1980b). On the gaseous exchange of ammonia between leaves and the environment: determination of the ammonia compensation point. *Plant Physiol.* 66, 710–714. doi: 10.1104/pp.66.4.710

Farquhar, G. D., and Sharkey, T. D. (1982). Stomatal conductance and photosynthesis. *Ann. Rev. Plant Physiol.* 33, 317–345. doi: 10.1146/annurev.pp.33.060182.001533

Farquhar, G. D., von Caemmerer, S., and Berry, J. A. (1980a). A biochemical model of photosynthetic CO$_2$ assimilation in leaves of C3 species. *Planta* 149, 78–90. doi: 10.1007/BF00380831

Handley, L. L., Austin, A. T., Robinson, D., Scrimgeour, C. M., Raven, J. A., Heaton, T. H. E., et al. (1999). The $^{15}$N natural abundance ($^{15}$N$_{nat}$) of ecosystem samples reflects measures of water availability. *Aust J. Plant Physiol.* 26, 185–199. doi: 10.1071/PP98146

Kalcits, L. A., and Guy, R. D. (2013). Whole-plant and organ-level nitrogen isotope discrimination indicates modification of partitioning of assimilation, fluxes and allocation of nitrogen in knockout lines of *Arabidopsis thaliana.* *Planta Physiol.* 151, 1491–1503. doi: 10.1002/pla.25080

Keeling, R. F., and Wherry, J. R. (1955). AtmosphericCO$_2$: a new climatic factor. *Science* 122, 152–156. doi: 10.1126/science.122.3195.152

Lambers, H., Pons, T. L., and Poorter, H. (2008). Plant Functional Types. London: Springer-Verlag.

Peuke, A. D., Gessler, A., and Rennenberg, H. (2006). The response of drought on C and N stable isotopes in different fractions of leaves, stems and roots of sensitive and tolerant beet ecotypes. *Plant Cell Enviro.* 29, 823–835. doi: 10.1111/j.1365-3040.2005.01452.x

Pritchard, E. S., and Guy, R. D. (2005). Nitrogen isotope discrimination in white spruce fed with low concentrations of ammonium and nitrate. *Trees Struct. Funct.* 19, 89–98. doi: 10.1007/s00468-004-0367-2

Rogers, A., Ainsworth, E. A., and Leakey, A. D. B. (2009). Will elevated carbon dioxide concentration amplify the benefits of nitrogen fixation in legumes? *Plant Physiol.* 151, 1009–1016. doi: 10.1104/pp.109.144113

Shearer, G., Feldman, L., Bryan, B. A., Skeeters, J. L., Kohl, D. H., and Amarger, N. (1982). $^{15}$N abundance of nodules as an indicator of N metabolism in N$_2$-fixing plants. *Plant Physiol.* 70, 465–468. doi: 10.1104/pp.70.2.465

Shearer, G., and Kohl, D. H. (1988). Natural $^{15}$N abundance as a method of estimating the contribution of biologically fixed nitrogen to N$_2$-fixing systems: potential for non-legumes. *Plant Soil.* 110, 317–327. doi: 10.1007/BF02226812

Tcherkez, G. (2011). Natural $^{15}$N/$^{14}$N isotope composition in C3 leaves: are enzymatic isotope effects informative for predicting the $^{15}$N-abundance in key metabolites? *Funct. Plant Biol.* 38, 1–12. doi: 10.1071/FP10091

Unkovich, M. (2013). Isotope discrimination provides new insight into biological nitrogen fixation. *New Phytol.* 198, 643–646. doi: 10.1111/nph.12227

von Caemmerer, S., and Farquhar, G. D. (1981). Some relationships between the biochemistry of photosynthesis and the gas exchange of leaves. *Planta* 153, 376–387. doi: 10.1007/BF00384257

Wanek, W., and Arndt, S. K. (2002). Difference in $^{15}$N signatures between nodulated roots and shoots of soybean is indicative of the contribution of symbiotic $^{15}$N fixation to plant N. *J. Exp. Bot.* 53, 1109–1118. doi: 10.1093/jxb/erq184

Werner, R. A., and Schmidt, H. (2002). The in vivo nitrogen isotope discrimination among organic plant compounds. *Phytochemistry* 61, 465–484. doi: 10.1016/S0031-9422(02)00242-2

Yousfi, S., Serret, M. D., and Araus, J. L. (2009). Shoot $^{15}$N gives a better indication than ion concentration or $\Delta^{13}$C of genotypic differences in the response of durum wheat to salinity. *Funct. Plant Biol.* 36, 144–155. doi: 10.1071/FP080135

Yousfi, S., Serret, M. D., Márquez, A. J., Voltas, J., and Araus, J. L. (2012). Combined use of $^{13}$C, $^{15}$O and $^{15}$N tracks nitrogen metabolism and genotypic adaptation of durum wheat to salinity and water deficit. *New Phytol.* 194, 230–244. doi: 10.1111/j.1469-8137.2011.04036.x

Yousfi, S., Serret, M. D., Voltas, J., and Araus, J. L. (2010). Effect of salinity and water stress during the reproductive stage on growth, ion concentrations, $\Delta^{13}$C, $\Delta^{15}$N of durum wheat and related amphiploids. *J. Exp. Bot.* 61, 3529–3542. doi: 10.1093/jxb/erq184

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.