Running page heading: Forage quality and carbohydrates in mycorrhizal alfalfa under high CO₂

Title: Changes in alfalfa forage quality and stem carbohydrates induced by arbuscular mycorrhizal fungi (AMF) and elevated atmospheric CO₂

Marouane Baslam¹, M Carmen Antolín¹, Yolanda Gogorcena², Fernando Muñoz³ & Nieves Goicoechea^{1*}

¹Departamento de Biología Ambiental. Grupo de Fisiología del Estrés en Plantas (Unidad Asociada al CSIC, EEAD, Zaragoza e ICVV, Logroño). Facultades de Ciencias y Farmacia, Universidad de Navarra, Irunlarrea 1, 31008, Pamplona, Spain. Telephone +34 948 425600; Fax + 34 948 425649

²Dpto. Pomología. Estación Experimental de Aula Dei, Consejo Superior de Investigaciones Científicas (CSIC), P.O. Box 13034, 50080 Zaragoza, Spain.

³Centro de Investigación y Tecnología Agroalimentaria de Aragón, P.O. Box 727, 50080 Zaragoza, Spain.

*** Corresponding author:** Nieves Goicoechea. Departamento de Biología Ambiental. Grupo de Fisiología del Estrés en Plantas (Unidad Asociada al CSIC, EEAD, Zaragoza e ICVV, Logroño). Facultades de Ciencias y Farmacia, Universidad de Navarra, Irunlarrea 1, 31008, Pamplona, Spain. Telephone +34 948 425600; Fax + 34 948 425649. e-mail: niegoi@unav.es

Abstract

Alfalfa is a widely distributed forage legume whose leaves are high in protein content and whose stems are suitable for bioethanol production. However, alfalfa forage digestibility, quality and yield may vary under future climate change scenarios. This legume can establish double symbiosis with nitrogen fixing bacteria and arbuscular mycorrhizal fungi (AMF). The presence of AMF can modify the evolution of biomass production and partitioning during the vegetative growth of alfalfa. We hypothesized that mycorrhizal symbiosis may vary the quantity and/or quality of carbohydrates and lignin in leaves and/or stems of alfalfa, these changes being dependent on the atmospheric CO₂ concentration at which plants are grown. Results showed that mycorrhizal alfalfa plants exposed to elevated CO₂ had improved leaf, stem and root biomass, enhanced amount of hemicellulose and decreased concentration of lignin in cell walls of leaves as well as increased levels of glucose and fructose in stems compared with non-mycorrhizal alfalfa. These results indicated improved forage quality (leaves) and enhanced potential for bioethanol conversion (stems) in mycorrhizal alfalfa cultivated under elevated CO₂. Moreover, the potential of stems for producing CH₄ reinforced their suitability for the conversion of biomass into bioethanol.

Keywords: Carbon dioxide enrichment, cell wall, *Medicago sativa*, methane, mycorrhizal symbiosis, soluble carbohydrates

Abbreviations Used: AMF = arbuscular mycorrhizal fungi; BRM = Batavia Rubia Munguía; CP = crude protein; CW = cell wall; DM = dry matter; MV = Maravilla de Verano; M = mycorrhizal plants; NM = non-mycorrhizal plants.

Introduction

The European Union (EU) Nitrate Directive (Council Directive 91/676/EEC) and the EU Drinking Water Directive (Council Directive 98/83/EC) are focused on reducing the eutrophication of water resources by agricultural practices. In this context, forage legumes are becoming attractive for grassland utilization, particularly in dairy farming, due to their ability for fixing atmospheric N₂, which allows the reduction or avoidance of N fertilizer application (Gierus *et al.*, 2012). Together with white (*Trifolium repens* L.) and red (*Trifolium pratense* L.) clover, alfalfa (*Medicago sativa* L.) is one of the most important forage crops in Europe. Alfalfa is also widely distributed around the world and is highly persistent in comparison to most common field crops (Bagavathiannan & Van Acker, 2009). In a recent work, Gierus *et al.* (2012) concluded that grazing-type alfalfa mixed with perennial ryegrass and subjected to high cutting frequency may be a reliable combination of species and management to provide forage with increased net energy for lactation (NEI) content, while keeping high dry matter (DM) yields in the first production year. Leaves of alfalfa can be marketed as animal feed because they are high in protein content (26-30%). However, an unavoidable product of rumen fermentation is methane (CH₄), which acts as an important greenhouse gas (GHG). The loss of CH₄ from ruminant livestock is a problem for efficient nutrition of animals and for emissions of GHG, being the amount of CH₄ emission related to the type of carbohydrate in the diet (Wang *et al.*, 2007).

Moreover, alfalfa has a great potential as a feedstock for biofuel production because of its high biomass production and perennial nature. Thus, different studies proposed alfalfa (especially stems) as a sustainable crop for second-generation bioethanol production (Samac *et al.*, 2006; González-García *et al.*, 2010; Dien *et al.*, 2011). Alfalfa

forage would be fractionated into stems and leaves. The stems would be processed to generate energy or a biofuel, and the leaves would be sold as a high-protein livestock feed supplement (DeLong *et al.*, 1995; Lamb *et al.*, 2003). Stems contain greater amounts of lignin and cellulose and less crude protein than leaves. Several studies have been focused on reducing lignin content of alfalfa by genetic engineering. For example, Chen & Dixon (2007) doubled the sugar yield in alfalfa by reducing lignin content in the cell walls. Other authors (Dien *et al.*, 2011) have focused their work on altering lignin composition in order to enhance alfalfa conversion efficiencies for sugar recovery and ethanol production. One disadvantage of lowering lignin content as a strategy for improving conversion quality is that the production of total biomass may diminish (Simmons *et al.*, 2010).

Alfalfa can be associated not only with N₂ fixing bacteria, but also with arbuscular mycorrhizal fungi (AMF). The presence of AMF colonizing roots can improve photosynthetic rates when alfalfa are growing under either optimal (Sánchez-Díaz *et al.*, 1990) or restricted (Goicoechea *et al.*, 1997a) irrigation and benefit mineral nutrition of host plants (Goicoechea *et al.*, 1997b). In a recent study in which alfalfa plants were grown either under ambient or elevated atmospheric CO₂ in a greenhouse, Baslam *et al.* (2012a) concluded that the presence of AMF associated with roots of alfalfa can modify the evolution of both biomass production and partitioning during the vegetative growth of this legume. This fact may be a consequence of two factors: (1) an increased sink of C from host plants to mycorrhizal fungus in roots and (2) the shorter vegetative period of mycorrhizal plants compared to that of non-mycorrhizal controls. At the end of the vegetative stage, AMF significantly increased the leaves to stems ratio in alfalfa grown at ambient CO₂ and enhanced root biomass in plants exposed to elevated CO₂.

In addition, AMF colonization can alter or enhance the production of phenolics (the group of secondary metabolites in which lignin is included) within the host plant. Some studies have reported increased phenolic content in olive trees inoculated with a mixture of three different AMF, *Glomus invermaium*, *Acaulospora laevis* and *Scutellospora calospora* (Ganz *et al.*, 2002), in mycorrhizal plants of “sweet” basil (Toussaint *et al.*, 2007; Lee & Scagel, 2009), in strawberry inoculated with *G. intraradices* (Castellanos-Morales *et al.*, 2010) as well as in the outer leaves of lettuce plants (var. Longifolia) associated with *G. fasciculatum* or inoculated with a mixture of *G. mosseae* and *G. intraradices* (Baslam *et al.*, 2011). However, the accumulation of secondary metabolites (including phenolics) induced by AMF may diminish or even disappear when plants are exposed to elevated CO₂ as a consequence of different partitioning of resources at ambient and under elevated CO₂. Results obtained by Baslam *et al.* (2012b) in alfalfa suggested that a relevant quantity of photoassimilates were used for improving shoot growth and spreading mycorrhizal colonization in detriment to the secondary metabolism when mycorrhizal plants were grown under elevated CO₂.

Taking together all these findings, we hypothesize that the association of alfalfa with AMF may vary the quantity and/or quality of carbohydrates and lignin in leaves and/or stems, these changes being dependent on the atmospheric CO₂ concentration at which plants are grown. Therefore, the aims of this work were (1) to test if the association of alfalfa with AMF can induce metabolic changes that may modify the quality of this forage legume for feeding livestock and/or its potential as sustainable crop for second-generation bioethanol production; and (2) to assess the influence of the atmospheric CO₂ concentration on the possible effects of AMF on the alfalfa forage digestibility and methane emission.

Material and Methods

Plant material and growth conditions

Seeds from alfalfa (*Medicago sativa* L. cv. Aragón) were surface disinfected in a 0.1% (w/v) HgCl₂ solution for 10 min, washed five times with sterile water to remove any trace of chemicals and placed in Petri dishes to germinate. One week later, seedlings were transferred to 2 L pots (four plants per pot) filled with a mixture of vermiculite-sand- light peat (2.5:2.5:1, v:v:v). Peat (Floragard, Vilassar de Mar, Barcelona, Spain) was previously sterilized at 100°C for 1 h on three consecutive days. At transplanting, half of the plants (15 pots) were inoculated (13 g per pot) with the mycorrhizal inoculum AEGIS Endo Gránulo commercialized by Atens (Tarragona, Spain). The inoculum was a mixture of *Glomus intraradices* (Schenck and Smith) (recently reclassified as *Rhizophagus intraradices* (Schenck and Smith) Walker & Schüßler comb. nov., Krüger *et al.* 2012) and *Glomus mosseae* (Nicol. and Gerd.) Gerd. and Trappe (recently reclassified as *Funneliformis mosseae* (Nicol. and Gerd.) Walker & Schüßler comb. nov., Krüger *et al.* 2012) that contained around 100 spores and other infective propagules (mycelium, spores and roots) per gram of product. A filtrate was added to pots (15 pots) that did not receive the mycorrhizal inoculum in an attempt to restore other soil free-living microorganisms accompanying AMF. The filtrate for each pot was obtained by passing 13 g of mycorrhizal inoculum in 20 mL of distilled water through a layer of 15-20 µm filter papers (Whatman, GE Healthcare, UK). During the first month after transplanting, all plants were inoculated three times with *Sinorhizobium meliloti* 102F78 (The Nitragin Co. Milwaukee, WI, USA) maintained on yeast extract mannitol agar (YEMA; 0.5 g L⁻¹ K₂HPO₄, 0.2 g L⁻¹ MgSO₄ 7 H₂O, 0.1 g

L⁻¹ NaCl, 10 g L⁻¹ mannitol, 0.4 g L⁻¹ yeast extract and 15 g L⁻¹ agar in distillate water, pH 6.8-7.0).

Plants were grown at 25/15°C d/n temperatures, 50/85% d/n relative humidity and received natural daylight supplemented with irradiation from Son-T-Agro high-pressure sodium lamps (Philips Nederland B.V., Eindhoven) that provided a minimum photosynthetic photon flux of around 300-400 $\mu\text{mol m}^{-2} \text{s}^{-1}$ during a 14 h photoperiod. Four weeks after transplanting three pots inoculated with AMF (M) and three non-inoculated (NM) pots were harvested for evaluating mycorrhizal establishment. Intensity of mycorrhizal colonization reached around 10% in roots of inoculated plants and no mycorrhizal structures were observed in roots of non-inoculated plants. Also four weeks after transplanting (Erice *et al.*, 2006a, b), half of the remaining pots (six non-mycorrhizal, NM, and six mycorrhizal, M, with four plants in each pot) were transferred to a greenhouse where the atmospheric CO₂ concentration was set at 700 $\mu\text{mol mol}^{-1}$ (measured experiment mean $710 \pm 22 \mu\text{mol mol}^{-1}$) (elevated CO₂). Other six non-mycorrhizal (NM) and six mycorrhizal (M) pots (four plants in each pot) were always cultivated at ambient CO₂ concentration (set at 392 $\mu\text{mol mol}^{-1}$, measured experiment mean $395 \pm 20 \mu\text{mol mol}^{-1}$). Atmospheric CO₂ concentrations in both greenhouses were constantly monitored by using a ‘Guardian Plus Infra-Red Gas Monitor’ (Edinburgh Instruments Limited, Livingston, UK). CO₂ treatments were switched weekly between the greenhouses and the pots were randomized within treatments to eliminate edge effects. Harvest was performed four weeks after starting different atmospheric CO₂ treatments (at the end of the vegetative period, nine-week old plants). All plants were alternately irrigated with 200 mL of distilled water and 200 mL of nutrient solution (Goicoechea *et al.*, 1997a, b). Water was added to avoid salt

accumulation and ensure optimal irrigation. The basal nutrient solution was N-free so that N source mainly relied on N₂ fixation.

Mycorrhizal analyses, nodule biomass and plant growth parameters

Root samples were cleared and stained (Phillips & Hayman, 1970) and mycorrhizal colonization was determined by examining 1 cm root segments (n = 45 per pot) under the microscope. Extension (E), incidence (I) and intensity (Int) of mycorrhizal colonization were calculated for each pot. The E of mycorrhizal colonization was firstly determined for every root segment and it was calculated as the product between value of mycorrhizal colonization in width (W) and value of mycorrhizal colonization in length (L). Values of mycorrhizal colonization in width (W) and length (L) were ascribed according a scale in which 0 meant complete absence of fungal structures and 10 meant that fungal structures occupied the full length or width of the root segment. Afterwards, total E per pot was calculated as $E = \sum (W \times L) / n$, where 'n' was the total number of root segments observed under the microscope (n = 45 per pot) and it was expressed as a percentage. Incidence (I) of mycorrhizal colonization per pot was calculated as the ratio between number of root segments with fungal structures (arbuscules, vesicles and/or hyphae) and total number of root segments observed under the microscope (n = 45 per pot). Finally, the intensity (Int) of mycorrhizal colonization per pot was calculated as the product between E and I ($Int = E \times I$) and results were expressed as percentage of infection (Hayman *et al.*, 1976). The presence or absence of arbuscules was also assessed as the ratio between root segments that had arbuscules among fungal structures and total root segments with mycorrhizal structures; results were also expressed as percentages. Dry matter (DM) of nodules, leaves, stems and roots was determined after

drying plant material in the oven at 80°C until weight was constant.

Determination of soluble carbohydrates by HPLC

Samples (0.2 g of leaves or stems) for soluble carbohydrate analyses were freeze crushed and polar compounds were extracted into 1 mL aqueous 80% ethanol at 80°C, in three steps, each lasting 20 min (Jiménez *et al.*, 2011). The mixture of each step was centrifuged for 5 min at 14,000 x g and slurries were pooled. Ethanol was evaporated under vacuum in a speed vac system (Thermo Fisher Scientific Inc., Waltham, MA, USA) and dry extracts were solubilized in 500 µL double-distilled water. The soluble carbohydrates of the samples were purified using about 3.5 g g⁻¹ plant material ion exchange resins (Bio-Rad AG 50 W-X8 Resin 200-400 mesh hydrogen form, Bio-Rad AG 1-X4 Resin 200-400 chloride form). The samples were concentrated to 400 µL, filtered through a 0.22 µm filter and 20 µL was injected and analyzed by high-performance liquid chromatography (HPLC), using Ca-column (Aminex HPX-87C 300 mm x 7.8 mm column Bio-Rad) flushed with 0.6 mL min⁻¹ double distilled water at 85°C with a refractive index detector (Waters 2410). Concentrations of the main carbohydrates, stachyose, raffinose, sucrose, galactinol, glucose, xylose, fructose and sorbitol were calculated for each sample using manitol as an internal standard since it was not present in alfalfa samples. Carbohydrate quantification was performed with the Empower Login software, Waters (Millford, Mass, USA) using standards of analytical grade from Panreac Quimica S.A. (Barcelona, Spain) and Sigma-Aldrich (Schnelldorf, Germany). Concentrations of carbohydrates were expressed as mg g⁻¹ DM.

Leaves and stem fibre analysis

Fresh leaves and stems were oven dried at 60°C for 48 h and ground in a Fritsch pulverisette mill (Fritsch GmbH, Idar-Oberstein, Germany) through a 1 mm screen. Neutral detergent fibre (NDF), acid detergent fibre (ADF) and acid detergent lignin (ADL) content were determined according to Van Soest *et al.* (1991) using the Ankom Filter Bag method. NDF was used as an estimate of cell wall (CW) concentration. Cellulose was calculated as ADF minus ADL and hemicellulose as the difference between NDF and ADF values after removing ash. Lignin, hemicellulose, and cellulose were expressed as a proportion of CW (Halim *et al.*, 1989)

Crude protein (CP) content

Leaf and stem samples were dried at 60°C for 48 h and analysed for percentage of nitrogen (%N) in the total organic matter. One milligram of ground sample was used for each determination. Leaf and stem N was determined using an elemental analyser (EA1108, Series 1, Carlo Erba Instrumentazione, Milan, Italy). Crude protein (CP) content was calculated as nitrogen content $\times$ 6.5 (Licitra *et al.*, 1996).

Methane production

Ground samples (0.5 g DM) of alfalfa leaves or stems were incubated as described by Sallam *et al.* (2010) in diluted rumen fluid in serum bottles. Ten mL gas from each bottle (2 mL every time) was collected at 3, 6, 9, 14 and 24 h of incubation using a syringe and pooled at the vacutainer tubes for methane analyses. Methane (CH₄)

detection was realized with flame ionization detector (FID, gas chromatograph HP-4890), equipped with a capillary column GS-DB-WAX (J&N Scientific), 30 m, 0.25 mm id and 0.25 micron film thickness. The carrier gas was helium and the flow rate was 2 mL min⁻¹, H₂ flow rate 35 mL min⁻¹, and synthetic air flow rate 350 mL min⁻¹. The temperature of the inlet and detector was maintained at 200 and 250°C, respectively. Oven temperature was 70°C and the temperature program was isothermal (70°C temperature of column flow). Injection volume was 200 µL splitless and was realized manually with a Hamilton gas-tight syringe. CH₄ identification was based on retention times as compared with the standard CH₄. Analysis time was 2 min (included equilibration time). The standard curve was established by plotting a linear regression of the standard quantities injected of each CH₄ gas volume *versus* area of the peak obtained. The amount of gas in a sample was determined using the regression equation: $\text{Area} = 2420.3 \times \text{amount} (\mu\text{mol } 10^{-2}) + 168.7$ (correlation = 0.99945).

Statistical analysis

Data were subjected to a two-way analysis of variance (ANOVA) (SPSS v. 15.0). Mycorrhizal symbiosis (AMF) and atmospheric CO₂ concentration (CO₂) were used as first and second factor, respectively. Data on percentages of mycorrhizal roots showing arbuscules were subjected to arc-sin transformation before applying Chi-square (χ^2) test. Significant differences between factors were calculated at 5%.

Results

Nodule biomass, mycorrhizal analyses and plant growth parameters

The highest nodule DM per plant was observed in NM plants grown under elevated CO₂ (Figure 1A). The percentage of mycorrhizal colonization was similar in roots of alfalfa grown at ambient or under elevated CO₂ (Figure 1B). However, the percentage of mycorrhizal roots that showed arbuscules was lower in plants subjected to elevated CO₂ than in those always grown at ambient CO₂ (Figure 1B).

Elevated CO₂ increased the leaf DM (LDM) in NM plants (Figure 2A); however, mycorrhizal symbiosis (AMF) had stronger influence ($P \leq 0.001$) than the factor 'CO₂' ($P \leq 0.01$) in enhancing leaf biomass. Increased CO₂ in the atmosphere improved stem DM, regardless of whether the alfalfa plants were inoculated with AMF or not (Figure 2B). In M plants, elevated CO₂ also enhanced root DM (Figure 2C).

Soluble carbohydrates

Neither mycorrhizal symbiosis nor CO₂ and the interaction between both factors showed significant effect on the concentrations of total soluble sugars in leaves (Table 1). However, AMF slightly influenced the amount of total sugars in stems and individual carbohydrate composition differed according to mycorrhizal presence and CO₂ level assayed. Table 1 shows the soluble carbohydrates detected in leaves and stems of alfalfa associated or not with AMF and grown under either ambient or elevated CO₂ in the atmosphere. The main soluble carbohydrates identified in both leaves and

stems were stachyose, raffinose, sucrose, galactinol, glucose, xylose, fructose and sorbitol, sucrose and xylose being the predominant sugars in both organs.

The inoculation of alfalfa with AMF significantly influenced the levels of several sugars in leaves (raffinose, sucrose, galactinol and xylose) and stems (sucrose, glucose and fructose). The CO₂ concentration in the atmosphere also exerted a significant effect on the concentrations of stachyose and galactinol in leaves, as well as on the amounts of glucose, xylose, fructose and sorbitol in stems. The interaction between AMF and atmospheric CO₂ increased the levels of raffinose, galactinol and glucose in leaves (AMF × CO₂, $P \leq 0.001$ for raffinose; $P \leq 0.01$ for galactinol and glucose) as well as the content of glucose in stems (AMF × CO₂, $P \leq 0.001$) of M plants compared with those found in M plants cultivated at ambient CO₂. In contrast, the lowest concentrations of sorbitol in stems were found in M plants subjected to elevated CO₂ (AMF × CO₂, $P \leq 0.05$).

Cell wall components in leaves and stems

Cell wall components of leaves and stems are shown in Table 2. The factor AMF significantly increased the concentration of CW in leaves at the end of the vegetative period. In contrast, elevated CO₂ decreased the concentration of cellulose in leaves, this effect being more evident for M than for NM plants. Mycorrhizal plants always had greater content of hemicellulose in leaves than NM controls, regardless of the atmospheric CO₂ concentration at which they had been grown. The amount of lignin in leaf cell walls was higher in M than in NM plants grown at ambient CO₂; however, lignin concentration sharply decreased in leaves of M alfalfa subjected to elevated CO₂.

Elevated CO₂ had not a significant effect on the total weight or composition of CW in stems of NM alfalfa. In contrast, the concentration of cellulose decreased in stems of plants associated with AMF when subjected to elevated CO₂ compared with that found in M plants at ambient CO₂ (AMF × CO₂, $P \leq 0.001$). Amount of lignin and hemicellulose was similar in stems of all plants independent of mycorrhizal symbiosis and atmospheric CO₂ concentration.

Crude protein (CP) content

When plants were cultivated at ambient CO₂, the concentration of CP was higher in leaves of M alfalfa than in leaves of NM plants (Table 3). The exposure of plants to elevated CO₂ had a negative effect on the CP content in leaves; however, values of CP remained higher in leaves of M plants than in those of NM plants (Table 3). The CP contents in stems were similar in all alfalfa plants regardless of whether they were or not associated with AMF (Table 3). The amounts of CP in stems (Table 3) were always lower than those in leaves (Table 3).

Methane production

Elevated CO₂ increased the potentiality of alfalfa leaves for producing methane (CH₄), regardless plants were or not associated with AMF (Table 3). When grown at ambient CO₂, the production of CH₄ was slightly higher from leaves (Table 3) and significantly higher from stems (Table 3) of M plants in comparison with NM plants. In fact, mycorrhizal association was the only factor that significantly influenced the production of CH₄ from stems ($P \leq 0.01$).

Discussion

Rising atmospheric CO₂ produces increased potential net photosynthesis in C3 plants with subsequent improved yield over short-term exposures (Oliveira *et al.*, 2010). This phenomenon has also been observed in alfalfa grown under optimal irrigation and associated or not with AMF (Aranjuelo *et al.*, 2009; Baslam *et al.*, 2012a). Improved photosynthesis of alfalfa under elevated CO₂ can be due to both increased CO₂ exchange rate per unit area and enhanced total photosynthetic rates per plant (Baslam *et al.*, 2012a). An enhanced photosynthetic rate may have allowed increased translocation of sugars from leaves to roots thus favouring nodule development in NM plants under elevated CO₂ (Figure 1A) and, *viceversa*, the presence of N₂ fixing bacteria associated with roots of alfalfa could have enhanced the rate of photosynthesis as a consequence of the increased sink: source ratio due to C costs of N₂ fixation (Antolín *et al.*, 2010). In contrast, the nodule DM per plant was similar in M plants grown at ambient CO₂ to those exposed to elevated CO₂ (Figure 1A), suggesting that the two symbionts (nitrogen fixing bacteria and mycorrhizal fungi) would be competing for plant carbohydrates.

Although the concentrations of total soluble sugars were similar among treatments (Table 1), individual carbohydrate composition was different depending on the presence or absence of mycorrhizal symbiosis and the level of atmospheric CO₂ (Table 1). The larger concentration of monosaccharides (glucose and fructose) in stems of M plants exposed to elevated CO₂ might be advantageous for a more efficient bioethanol production because hexoses can be converted at higher yields to ethanol than most other carbohydrates (Dien *et al.*, 2006, 2011). Thus, in stems of NM plants subjected to high atmospheric CO₂, amounts of glucose and fructose accounted 12.5% of total soluble

carbohydrates whereas in M plants, the contribution of glucose and fructose to total soluble carbohydrates in stems was 15.4%.

Sanz-Sáez *et al.* (2012) concluded that changes in the amounts of cellulose, hemicellulose and lignin in cell walls (CW) of alfalfa (not associated with AMF) grown under elevated CO₂ depended on the strain of nitrogen fixing bacteria in symbiosis with plants. These authors found enhanced cellulose and lignin content in shoots when plants inoculated with the strain of *Sinorhizobium* used in our study (102F78) were subjected to elevated CO₂. However, when leaves and stems were separately analysed, we observed that only in leaves CO₂ had significant effect on the levels of cellulose, hemicellulose and lignin (Table 2). The association of alfalfa with AMF also exerted a clear influence on most CW components in leaves and so did the interaction between AMF and CO₂ on the amounts of hemicellulose and lignin. At ambient CO₂, M plants had greater amount of lignin in CW than NM ones, which may indicate that mycorrhizal symbiosis stimulated the phenylpropanoid pathway, a phenomenon widely observed (Ganz *et al.*, 2002; Toussaint *et al.*, 2007; Lee & Scagel, 2009; Castellanos-Morales *et al.*, 2010; Baslam *et al.*, 2011). However, the concentration of lignin strongly decreased in leaves of M plants subjected to high CO₂, which supports the idea that, under elevated CO₂, a relevant quantity of photoassimilates produced by M plants would probably be used for improving plant growth in detriment to the secondary metabolism (Baslam *et al.*, 2012b). Decreased level of lignin in CW of M plants grown under elevated CO₂ would mean an improved quality of alfalfa as forage because lignin is indigestible by ruminants (Milchunas *et al.*, 2005). Another factor positively related to the quality of forage is the quantity of crude protein (CP) (Milchunas *et al.*, 2005; Larson & Mayland, 2007). Crude protein is highly digestible and non-degradable protein that passes through the rumen and can be efficiently utilised in the lower digestive tract providing additional

soluble protein to rumen microorganisms, thus enhancing *in vitro* dry matter digestibility (Casler, 2001). The concentration of CP was significantly higher in leaves of M alfalfa than in NM plants independent of CO₂ concentration in the atmosphere (Table 3). Similar to the results obtained by Sanz-Sáez *et al.* (2012), the levels of CP decreased under elevated CO₂. However, our results indicate that the decrease in CP observed by Sanz-Sáez *et al.* (2012) in shoots was mainly due to decreased concentrations of CP in leaves.

There are several compounds that may influence the CH₄ potential of lignocellulosic materials (Monlau *et al.*, 2012). Among them, condensed tannins clearly diminish the production of CH₄ (Vargas *et al.*, 2012). In alfalfa, however, the levels of condensed tannins are very low (Sallam *et al.*, 2010). Lignin decomposition has been correlated with CH₄ formation due to cracking of methoxy groups of lignin molecules (Liu *et al.*, 2008). Although Monlau *et al.* (2012) found negative correlations between CH₄ potentials and lignin contents in various lignocellulosic residues, Pasangulapati *et al.* (2012) concluded that higher lignin content in eastern redcedar was implied in its significantly high CH₄ concentration in comparison with switch-grass and wheat straw. In our study, the production of CH₄ was always higher in stems than in leaves (Table 3), stems being the plant organ showing the greatest amount of lignin (Table 2). Another factor that may be implied in the different CH₄ potential between leaves and stems is the presence of saponins. These compounds can reduce the production of CH₄ (Vargas *et al.*, 2012). Alfalfa has relatively high levels of saponins (Golawska *et al.*, 2012; Vargas *et al.*, 2012), the levels being higher in leaves than in stems. When analyzed, the production of CH₄ in leaves was found to be similar in M plants subjected to elevated CO₂ to that found in the other treatments. Despite the low lignin content in leaves of M plants under elevated CO₂ the production of CH₄ in leaves was found to be similar

across all of the treatments. This finding reinforces the idea that lignin was not the only factor affecting CH₄ production. It is possible that the increased amount of hemicellulose in leaves of M plants under elevated CO₂ may have enhanced the production of CH₄ in these organs (Monlau *et al.*, 2012).

In summary, AMF inoculated alfalfa plants exposed to elevated CO₂ showed improved leaf, stem and root biomass, enhanced amount of hemicellulose and decreased concentration of lignin in cell walls of leaves as well as increased levels of glucose and fructose in stems. Together these results indicate improved forage quality (leaves) and enhanced potential for bioethanol conversion (stems) in alfalfa associated with AMF and cultivated under high atmospheric CO₂ concentration. In addition, the potential of stems for producing CH₄ reinforces their suitability for the conversion of biomass into bioethanol.

Acknowledgments

The authors wish to thank Adriana Hernández (Atens, Tarragona, Spain) for kindly providing the mycorrhizal inoculum; María Laura Fiasconaro, Amadeo Urdiáin and Rosa Giménez for technical support. This study has been supported by the ‘Ministerio de Ciencia e Innovación’ and ‘Gobierno de Aragón’ (Spain) (BFU2011-26989, A-44). Marouane Baslam received a grant from Asociación de Amigos de la Universidad de Navarra (ADA).

References

- Antolín M.C., Fiasconaro M.L., Sánchez-Díaz M. (2010) Relationship between photosynthetic capacity, nitrogen assimilation and nodule metabolism in alfalfa (*Medicago sativa*) grown with sewage sludge. *Journal of Hazardous Materials*, **182**, 210-216.
- Aranjuelo I., Irigoyen J.J., Nogués S., Sánchez-Díaz M. (2009) Elevated CO₂ and water-availability effect on gas exchange and nodule development in N₂-fixing alfalfa plants. *Environmental and Experimental Botany*, **65**, 18-26.
- Bagavathiannan M.V., Van Acker R.C. (2009) The biology and ecology of feral alfalfa (*Medicago sativa* L.) and its implications for novel trait confinement in North America. *Critical Reviews in Plant Sciences*, **28**, 69-87.
- Baslam M., Garmendia I., Goicoechea N. (2011) Arbuscular mycorrhizal fungi (AMF) improved growth and nutritional quality of greenhouse grown lettuce. *Journal of Agricultural Food Chemistry*, **59**, 5504-5515.
- Baslam M., Erice G., Goicoechea N. (2012a) Impact of arbuscular mycorrhizal fungi (AMF) and atmospheric CO₂ concentration on the biomass production and partitioning in the forage legume alfalfa. *Symbiosis*, **58**, 171-181.
- Baslam M., Garmendia I., Goicoechea N. (2012b) Elevated CO₂ may impair the beneficial effect of arbuscular mycorrhizal fungi (AMF) on the mineral and phytochemical quality of lettuce. *Annals of Applied Biology*, **161**, 180-191.
- Casler M.D. (2001) Breeding forage crops for increased nutritional value. *Advances in Agronomy*, **71**, 51-107.
- Castellanos-Morales V., Villegas J., Wendelin S., Vierheilig H., Eder R., Cárdenas-Navarro R. (2010) Root colonisation by the arbuscular mycorrhizal fungus *Glomus*

470 *intraradices* alters the quality of strawberry fruits (*Fragaria ananassa* Duch.) at
 471 different nitrogen levels. *Journal of the Science of Food and Agriculture*, **90**, 1774-
 472 1782.

473 Chen F., Dixon R.A. (2007) Lignin modification improves fermentable sugar yields for
 474 biofuel production. *Nature Biotechnology*, **25**, 759-761.

475 Delong M.M., Swanberg D.R., Oelke E.A., Hanson C., Onischak M., Schmid M.R.,
 476 Wiant B.C. (1995) Sustainable biomass energy production and rural economic
 477 development using alfalfa as a feedstock. In *Second Biomass Conference of the*
 478 *Americas: Energy, Environment, Agriculture, and Industry*, pp. 21-24. Ed D.L.
 479 Klass. Portland OR. National Renewable Energy Laboratory, Golden CO.

480 Dien B.S., Jung H.G., Vogel K.P., Casler M.D., Lamb J.F.S., Weimer P.J., Iten L.,
 481 Mitchell R.B., Sarath G. (2006) Chemical composition and response to dilute-acid
 482 pretreatment and enzymatic saccharification of alfalfa, reed canarygrass, and
 483 switchgrass. *Biomass Bioenergy*, **30**, 880-891.

484 Dien B.S., Miller D.J., Hector R.E., Dixon R.A., Chen F., McCaslin M., Reisen P.,
 485 Sarath G., Cotta M.A. (2011) Enhancing alfalfa conversion efficiencies for sugar
 486 recovery and ethanol production by altering lignin composition. *Bioresource*
 487 *Technology*, **102**, 6479-6486.

488 Erice G., Irigoyen J.J., Pérez P., Martínez-Carrasco R., Sánchez-Díaz M. (2006a) Effect
 489 of elevated CO₂, temperature and drought on dry matter partitioning and
 490 photosynthesis before and after cutting of nodulated alfalfa. *Plant Science*, **170**,
 491 1059-1067.

492 Erice G., Irigoyen J.J., Pérez P., Martínez-Carrasco R., Sánchez-Díaz M. (2006b) Effect
 493 of elevated CO₂, temperature and drought on photosynthesis of nodulated alfalfa
 494 during a cutting-regrowth cycle. *Physiologia Plantarum*, **126**, 458-468.

495 Ganz T.R., Kailis S.G., Abbott L.K. (2002) Mycorrhizal colonization and its effect on
 496 growth phosphorus uptake and tissue phenolic content in the European olive (*Olea*
 497 *europaea* L.). *Advances in Horticultural Science*, **16**, 109-116.

498 Gierus M., Kleen J., Loges R., Taube F. (2012) Forage legume species determine the
 499 nutritional quality of binary mixtures with perennial ryegrass in the first production
 500 year. *Animal Feed Science and Technology*, **172**, 150-161.

501 Goicoechea N., Antolín M.C., Sánchez-Díaz M. (1997a) Gas exchange is related to the
 502 hormone balance in mycorrhizal or nitrogen-fixing alfalfa subjected to drought.
 503 *Physiologia Plantarum*, **100**, 989-997.

504 Goicoechea N., Antolín M.C., Sánchez-Díaz M. (1997b) Influence of arbuscular
 505 mycorrhizae and *Rhizobium* on nutrient content and water relations in drought
 506 stressed alfalfa. *Plant and Soil*, **192**, 261-268.

507 Golawska S., Lukasik I., Wójcicka A., Sytykiewicz H. (2012) Relationship between
 508 saponin content in alfalfa and aphid development. *Acta Biologica Cracoviensia*
 509 *Series Botanica*, **54**, 1-8.

510 González-García S., Moreira M.T., Feijoo G. (2010) Environmental performance of
 511 lignocellulosic bioethanol production from alfalfa items. *Biofuels, Bioproducts and*
 512 *Biorefining*, **4**, 118-131.

513 Halim R.A., Buxton D.R., Hattendorf M.J., Carlson R.E. (1989) Water stress effects on
 514 alfalfa forage quality after adjustment for maturity differences. *Agronomy Journal*,
 515 **81**, 189-194.

516 Hayman D.S., Barea J.M., Azcón R. (1976) Vesicular-arbuscular mycorrhiza in
 517 southern Spain: its distribution in crops growing in soil of different fertility.
 518 *Phytopathologia Mediterranea*, **15**, 1-6.

519 Jiménez S., Ollat N., Deborde C., Maucourt M., Rellán-Álvarez R., Moreno M.A.,
 520 Gogorcena Y. (2011) Metabolic response in roots of *Prunus* rootstocks submitted to
 521 iron chlorosis. *Journal of Plant Physiology*, **168**, 415-423.

522 Krüger M., Krüger C., Walker C., Stockinger H., Schübler A. (2012) Phylogenetic
 523 reference data for systematics and phylotaxonomy of arbuscular mycorrhizal fungi
 524 from phylum to species level. *The New Phytologist* **193**, 970–984.

525 Lamb J.F.S., Sheaffer C.C., Samac D. (2003) Population density and harvest maturity
 526 effects on leaf and stem yield in alfalfa. *Agronomy Journal*, **95**, 635-641.

527 Larson S.R., Mayland H.F. (2007) Comparative mapping of fibre, protein, and mineral
 528 content QTLs in two interspecific *Leymus* wildrye full-sib families. *Molecular*
 529 *Breeding*, **20**, 331-347.

530 Lee J.M., Scagel C.F. (2009) Chicoric acid found in basil (*Ocimum basilicum* L.)
 531 leaves. *Food Chemistry*, **115**, 650-656.

532 Licitra G., Hernández T.M., Van Soest P.J. (1996) Standardization of procedures for
 533 nitrogen fractionation of ruminant feeds. *Animal Feed Science and Technology*, **57**,
 534 347-358.

535 Liu Q., Wang S., Zheng Y., Luo Z., Cen K. (2008) Mechanism study of wood lignin
 536 pyrolysis by using TG-FTIR analysis. *Journal of Analytical and Applied Pyrolysis*,
 537 **82**, 170-177.

538 Milchunas D.G., Moiser A.R., Morgan J.A., LeCain D.R., King J.Y., Nelson J.A. *et al.*
 539 (2005) CO₂ and defoliation effects on shortgrass steppe: forage quality *versus*
 540 quantity for ruminants. *Agriculture Ecosystems & Environment*, **111**, 166-184.

541 Monlau F., Sambusiti C., Barakat A., Guo X.M., Latrille E., Trably E., Steyer J.-F.,
 542 Carrere H. (2012) Predictive models of biohydrogen and biomethane production

543 based on the compositional and structural features of lignocellulosic materials.
544 *Environmental Science & Technology*, **46**, 12217-12225.

545 Oliveira V.F., Zaindan L.B.P., Braga M.R., Aidar M.P.M., Carvalho M.A.M. (2010)
546 Elevated CO₂ atmosphere promotes plants growth and inulin production in the
547 cerrado species *Vernonia herbacea*. *Functional Plant Biology*, **37**, 223-231.

548 Pasangulapati V., Ramachandriya K.D., Kumar A., Wilkins M.R., Jones C.L., Huhnke
549 R.L. (2012) Effects of cellulose, hemicellulose and lignin on thermochemical
550 conversion characteristics of the selected biomass. *Bioresource Technology*, **114**,
551 663-669.

552 Phillips J.M., Hayman D.S. (1970) Improved procedures for clearing roots and staining
553 parasitic and vesicular-arbuscular mycorrhizal fungi for rapid assessment of
554 infection. *Transactions of the British Mycological Society*, **55**, 158-161.

555 Sallam S.M.A.H., da Silva Bueno I.C., Barbosa de Godoy P., Nozella E.F., Schmidt
556 Vitti D.M.S., Abdalla A.L. (2010) Ruminal fermentation and tannins bioactivity of
557 some browses using a semi-automated gas production technique. *Tropical and*
558 *Subtropical Agroecosystems*, **12**, 1-10.

559 Samac D.A., Jung H.-J.G., Lamb J.F.S. (2006) Development of alfalfa (*Medicago sativa*
560 L.) as a feedstock for production of ethanol and other bioproducts. *Alcoholic Fuels*,
561 **112**, 79-98.

562 Sánchez-Díaz M., Pardo M., Antolín M.C., Peña J., Aguirreolea J. (1990) Effect of
563 water stress on photosynthetic activity in the *Medicago-Rhizobium-Glomus*
564 symbiosis. *Plant Science*, **71**, 215-221.

565 Sanz-Sáez A., Erice G., Aguirreolea J., Muñoz F., Sánchez-Díaz M., Irigoyen J.J.
566 (2012) Alfalfa forage digestibility, quality and yield under future climate change

scenarios vary with *Sinorhizobium meliloti* strain. *Journal of Plant Physiology*, **169**, 782-788.

Simmons B.A., Loque D., Ralph J. (2010) Advances in modifying lignin for enhanced biofuel production. *Current Opinion in Plant Biology*, **13**, 313-320.

Toussaint J.P., Smith F.A., Smith S.E. (2007) Arbuscular mycorrhizal fungi can induce the production of phytochemicals in sweet basil irrespective of phosphorus nutrition. *Mycorrhiza*, **17**, 291-297.

Van Soest P.J., Robertson J.B., Lewis B.A. (1991) Methods for dietary fiber, neutral detergent fiber, and non-starch polysaccharides in relation to animal nutrition. *Journal of Dairy Science*, **74**, 3583-3597.

Vargas J., Cárdenas E., Pabón M., Carulla J. (2012) Enteric methane emission by grazing ruminants. *Archivos de Zootecnia*, **61**, 51-66.

Wang C., Wang S., Zhou H., Glindemann T. (2007) Effects of forage composition and growing season on methane emission from sheep in the Inner Mongolia steppe of China. *Ecological Research*, **22**, 41-48.

Figure Legends

Figure 1 (A) Nodule biomass (g plant^{-1}) in non-mycorrhizal (NM) or mycorrhizal (M) alfalfa grown either at ambient (A CO_2) or elevated (E CO_2) CO_2 in the atmosphere. Values are means ($n = 6$) $\pm$ SE. (B) Intensity of mycorrhizal colonization (%) and percentage (%) of mycorrhizal roots showing arbuscules. Values are means ($n = 6$, $n = 45$ root fragments per pot) $\pm$ SE. ANOVA (graph A): ns = not significant; *, ** and *** = significant at $P \leq 0.05$, $P \leq 0.01$ and $P \leq 0.001$, respectively.

Figure 2 (A) Leaf dry matter (LDM) (g plant^{-1}), (B) stem dry matter (St DM) (g plant^{-1}) and (C) root dry matter (RDM) (g plant^{-1}) in non-mycorrhizal (NM) or mycorrhizal (M) alfalfa grown either at ambient (A CO_2) or elevated (E CO_2) CO_2 in the atmosphere. Values are means ($n = 6$) $\pm$ SE. ANOVA: ns = not significant; *, ** and *** = significant at $P \leq 0.05$, $P \leq 0.01$ and $P \leq 0.001$, respectively.

Table 1 Concentrations (mg g⁻¹ DM) of soluble carbohydrates and total soluble sugars (TSS) in leaves and stems of alfalfa plants non-inoculated (NM) or inoculated (M) with arbuscular mycorrhizal fungi (AMF) and grown either at ambient (ACO₂) or under elevated (ECO₂) CO₂. Values are means (n = 6) ± SE. ANOVA: ns = not significant; *, ** and *** = significant at $P \leq 0.05$, $P \leq 0.01$ and $P \leq 0.001$, respectively. DM = Dry matter.

			Stachyose	Raffinose	Sucrose	Galactinol	Glucose	Xylose	Fructose	Sorbitol	TSS
Leaves											
ACO₂	NM		0.99 ± 0.18	2.64 ± 0.41	37.10 ± 8.90	6.68 ± 0.28	5.90 ± 0.24	30.29 ± 0.85	5.62 ± 0.82	0.17 ± 0.02	89.7 ± 8.5
	M		0.96 ± 0.26	1.50 ± 0.11	38.15 ± 4.22	5.29 ± 0.56	4.34 ± 0.50	24.38 ± 4.78	4.02 ± 0.56	0.17 ± 0.01	80.4 ± 4.7
ECO₂	NM		1.39 ± 0.05	1.66 ± 0.09	26.20 ± 1.20	7.20 ± 0.77	5.00 ± 0.12	28.22 ± 3.94	5.31 ± 0.18	0.20 ± 0.00	75.4 ± 4.9
	M		1.52 ± 0.11	2.88 ± 0.46	40.22 ± 1.43	6.85 ± 0.13	6.36 ± 0.32	26.85 ± 0.75	5.47 ± 0.94	0.18 ± 0.01	90.4 ± 2.1
AMF			ns	*	**	***	ns	**	ns	ns	ns
CO ₂			**	ns	ns	***	ns	ns	ns	ns	ns
CO ₂ x AMF			ns	***	ns	**	**	*	ns	ns	ns
Stems											
ACO₂	NM		0.75 ± 0.08	1.45 ± 0.14	33.21 ± 3.38	3.60 ± 1.43	4.61 ± 0.30	23.39 ± 0.99	3.89 ± 0.24	0.16 ± 0.01	74.7 ± 3.6
	M		0.75 ± 0.06	0.36 ± 0.08	40.41 ± 3.17	5.61 ± 0.75	4.04 ± 0.17	21.63 ± 0.25	3.60 ± 0.33	0.17 ± 0.00	77.6 ± 3.2
ECO₂	NM		0.68 ± 0.04	1.18 ± 0.28	29.49 ± 5.19	6.02 ± 0.37	4.29 ± 0.31	18.85 ± 1.55	3.85 ± 0.58	0.14 ± 0.02	64.9 ± 6.6
	M		0.80 ± 0.04	1.40 ± 0.13	39.95 ± 2.49	6.99 ± 0.36	6.94 ± 0.88	21.08 ± 1.16	5.82 ± 0.95	0.12 ± 0.02	83.1 ± 2.9
AMF			ns	ns	***	ns	*	ns	*	ns	*
CO ₂			ns	ns	ns	ns	**	*	*	***	ns
CO ₂ x AMF			ns	ns	ns	ns	***	ns	**	*	ns

Table 2 Cell wall components in leaves and stems of alfalfa plants non-inoculated (NM) or inoculated (M) with arbuscular mycorrhizal fungi (AMF) and grown either at ambient (ACO₂) or under elevated (ECO₂) CO₂. Values are means (n = 6) ± SE. ANOVA: ns = not significant; *, ** and *** = significant at $P \leq 0.05$, $P \leq 0.01$ and $P \leq 0.001$, respectively. CW = cell wall; DM = dry matter.

		CW (g kg ⁻¹ DM)	Cellulose (g kg ⁻¹ CW DM)	Hemicellulose (g kg ⁻¹ CW DM)	Lignin (g kg ⁻¹ CW DM)
Leaves					
ACO₂	NM	250.4 ± 4.9	273.1 ± 16.1	607.6 ± 9.7	68.3 ± 0.1
	M	267.4 ± 9.5	292.6 ± 18.2	630.9 ± 4.9	83.5 ± 8.4
ECO₂	NM	246.1 ± 12.7	261.5 ± 1.9	676.7 ± 11.5	69.5 ± 1.9
	M	296.0 ± 15.5	233.3 ± 12.7	736.7 ± 15.2	30.0 ± 3.4
AMF		*	ns	**	*
CO ₂		ns	*	***	***
CO ₂ x AMF		ns	ns	**	***
Stems					
ACO₂	NM	479.2 ± 6.1	553.9 ± 1.9	304.1 ± 4.4	122.2 ± 11.6
	M	497.0 ± 10.0	574.5 ± 5.6	305.9 ± 0.9	120.5 ± 1.6
ECO₂	NM	514.4 ± 17.1	574.8 ± 1.1	301.8 ± 4.5	124.6 ± 9.0
	M	510.8 ± 5.2	560.8 ± 1.6	312.7 ± 1.8	126.5 ± 3.4
AMF		ns	ns	ns	ns
CO ₂		ns	ns	ns	ns
CO ₂ x AMF		ns	***	ns	ns

625 **Table 3** Crude protein (CP) (mg g⁻¹ DM) and methane (CH₄) production (g kg⁻¹ DM digested) in leaves and stems of non-
626 mycorrhizal (NM) or mycorrhizal (M) alfalfa grown either at ambient (A CO₂) or under elevated (ECO₂) CO₂. Values are means
627 (n = 6) ± SE. ANOVA: ns = not significant; *, ** and *** = significant at $P \leq 0.05$, $P \leq 0.01$ and $P \leq 0.001$, respectively.

628

				629	
		CP	CH ₄		
		(mg g ⁻¹ DM)	(g kg ⁻¹ DM digest)	630	
Leaves					
	A	NM	216.2 ± 10.4	2.46 ± 0.31	631
		M	281.6 ± 2.8	3.80 ± 0.33	
	E	NM	190.5 ± 6.7	4.74 ± 0.64	632
		M	239.9 ± 10.1	4.56 ± 0.71	
	AMF	***	ns	633	
	CO ₂	**	*		
	CO ₂ x AMF	ns	ns	634	
Stems					
	A	NM	121.1 ± 4.5	3.90 ± 0.19	635
		M	122.4 ± 6.8	5.87 ± 0.47	
	E	NM	110.6 ± 2.9	4.50 ± 0.31	636
		M	112.4 ± 2.3	5.61 ± 0.30	
	AMF	ns	**	637	
	CO ₂	*	ns		
	CO ₂ x AMF	ns	ns	638	

639

Figure 1