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

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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 N$_2$, 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 (CH$_4$), which acts as an important greenhouse gas (GHG). The loss of CH$_4$ from ruminant livestock is a problem for efficient nutrition of animals and for emissions of GHG, being the amount of CH$_4$ 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-Garcia 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 N$_2$ 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 CO$_2$ 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 CO$_2$ and enhanced root biomass in plants exposed to elevated CO$_2$. 
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 *Rhizohagus 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 μmol m⁻² 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 μmol mol⁻¹ (measured experiment mean 710 ± 22 μmol 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 μmol mol⁻¹, measured experiment mean 395 ± 20 μmol 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 × 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 × 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 (μmol} \ 10^{-2} \) + 168.7 \text{ (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 (χ²) 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 ≤ 0.001 for raffinose; P ≤ 0.01 for galactinol and glucose) as well as the content of glucose in stems (AMF × CO₂, P ≤ 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 ≤ 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 CO$_2$ 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 CO$_2$ compared with that found in M plants at ambient CO$_2$ (AMF × CO$_2$, $P \leq 0.001$). Amount of lignin and hemicellulose was similar in stems of all plants independent of mycorrhizal symbiosis and atmospheric CO$_2$ concentration.

Crude protein (CP) content

When plants were cultivated at ambient CO$_2$, 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 CO$_2$ 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 CO$_2$ increased the potentiality of alfalfa leaves for producing methane (CH$_4$), regardless plants were or not associated with AMF (Table 3). When grown at ambient CO$_2$, the production of CH$_4$ was slightly higher from leaves (Table 3) and significantly higher form 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 CH$_4$ 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 (Antolin 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 CO$_2$ 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 CO$_2$. However, when leaves and stems were separately analysed, we observed that only in leaves CO$_2$ 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 CO$_2$ on the amounts of hemicellulose and lignin. At ambient CO$_2$, 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 CO$_2$, which supports the idea that, under elevated CO$_2$, 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 CO$_2$ 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 CH$_4$ production. It is possible that the increased amount of hemicellulose in leaves of M plants under elevated CO$_2$ may have enhanced the production of CH$_4$ in these organs (Monlau et al., 2012).

In summary, AMF inoculated alfalfa plants exposed to elevated CO$_2$ 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 CO$_2$ concentration. In addition, the potential of stems for producing CH$_4$ reinforces their suitability for the conversion of biomass into bioethanol.

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based on the compositional and structural features of lignocellulosic materials.

*Environmental Science & Technology, 46*, 12217-12225.


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) ± SE. (B) Intensity of mycorrhizal colonization (%) and percentage (%) of mycorrhizal roots showing arbuscules. Values are means (n = 6, n = 45 root fragments per pot) ± 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) ± 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 ≤ 0.05, P ≤ 0.01 and P ≤ 0.001, respectively. DM = Dry matter.

<table>
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<tr>
<th></th>
<th>Stachyose</th>
<th>Raffinose</th>
<th>Sucrose</th>
<th>Galactitol</th>
<th>Glucose</th>
<th>Xylose</th>
<th>Fructose</th>
<th>Sorbitol</th>
<th>TSS</th>
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<tr>
<td>ACO₂</td>
<td>NM</td>
<td>0.99 ± 0.18</td>
<td>2.64 ± 0.41</td>
<td>37.10 ± 8.90</td>
<td>6.68 ± 0.28</td>
<td>5.90 ± 0.24</td>
<td>30.29 ± 0.85</td>
<td>5.62 ± 0.82</td>
<td>0.17 ± 0.02</td>
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<td></td>
<td>M</td>
<td>0.96 ± 0.26</td>
<td>1.50 ± 0.11</td>
<td>38.15 ± 4.22</td>
<td>5.29 ± 0.56</td>
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<td>24.38 ± 4.78</td>
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<td>ECO₂</td>
<td>NM</td>
<td>1.39 ± 0.05</td>
<td>1.66 ± 0.09</td>
<td>26.20 ± 1.20</td>
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<td>5.00 ± 0.12</td>
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<td>1.52 ± 0.11</td>
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<tr>
<td>ACO₂</td>
<td>NM</td>
<td>0.75 ± 0.08</td>
<td>1.45 ± 0.14</td>
<td>33.21 ± 3.38</td>
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<td>0.75 ± 0.06</td>
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<td>NM</td>
<td>0.68 ± 0.04</td>
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<td>4.29 ± 0.31</td>
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<td>0.80 ± 0.04</td>
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<td>39.95 ± 2.49</td>
<td>6.99 ± 0.36</td>
<td>6.94 ± 0.88</td>
<td>21.08 ± 1.16</td>
<td>5.82 ± 0.95</td>
<td>0.12 ± 0.02</td>
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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 (ACO$_2$) or under elevated (ECO$_2$) CO$_2$. 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.

<table>
<thead>
<tr>
<th></th>
<th>CW (g kg$^{-1}$ DM)</th>
<th>Cellulose (g kg$^{-1}$ CW DM)</th>
<th>Hemicellulose (g kg$^{-1}$ CW DM)</th>
<th>Lignin (g kg$^{-1}$ CW DM)</th>
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<td><strong>Leaves</strong></td>
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<tr>
<td>ACO$_2$</td>
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</tr>
<tr>
<td>NM</td>
<td>250.4 ± 4.9</td>
<td>273.1 ± 16.1</td>
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<td>68.3 ± 0.4</td>
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<tr>
<td>M</td>
<td>267.4 ± 9.5</td>
<td>292.6 ± 18.2</td>
<td>630.9 ± 4.9</td>
<td>83.5 ± 8.4</td>
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<tr>
<td>NM</td>
<td>246.1 ± 12.7</td>
<td>261.5 ± 19.1</td>
<td>676.7 ± 11.5</td>
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<td>M</td>
<td>296.0 ± 15.5</td>
<td>233.3 ± 12.7</td>
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<tr>
<td><strong>AMF</strong></td>
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<tr>
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<tr>
<td>NM</td>
<td>479.2 ± 6.1</td>
<td>553.9 ± 1.9</td>
<td>304.1 ± 4.4</td>
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<tr>
<td>M</td>
<td>497.0 ± 10.0</td>
<td>574.5 ± 5.6</td>
<td>305.9 ± 0.9</td>
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<tr>
<td>NM</td>
<td>514.4 ± 17.1</td>
<td>574.8 ± 1.1</td>
<td>301.8 ± 4.5</td>
<td>124.6 ± 9.0</td>
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<tr>
<td>M</td>
<td>510.8 ± 5.2</td>
<td>560.8 ± 1.6</td>
<td>312.7 ± 1.8</td>
<td>126.5 ± 3.4</td>
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<td><strong>CO$_2$ x AMF</strong></td>
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</table>
Table 3 Crude protein (CP) (mg g⁻¹ DM) and methane (CH₄) production (g kg⁻¹ DM digested) in leaves and stems of non-mycorrhizal (NM) or mycorrhizal (M) alfalfa grown either at ambient (A CO₂) or under elevated (ECO₂) CO₂. Values are means (n = 6) ± SE. ANOVA: ns = not significant; *, ** and *** = significant at P ≤ 0.05, P ≤ 0.01 and P ≤ 0.001, respectively.

<table>
<thead>
<tr>
<th></th>
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<td>CH₄</td>
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<tr>
<td></td>
<td>(mg g⁻¹ DM)</td>
<td>(g kg⁻¹ DM digested)</td>
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<tr>
<td></td>
<td>ACO₂ NM</td>
<td>216.2 ± 10.4</td>
<td>2.46 ± 0.31</td>
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<td>281.6 ± 2.8</td>
<td>3.80 ± 0.33</td>
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<tr>
<td></td>
<td>ECO₂ NM</td>
<td>190.5 ± 6.7</td>
<td>4.74 ± 0.64</td>
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<td>239.9 ± 10.1</td>
<td>4.56 ± 0.71</td>
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<tr>
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<td>Stems</td>
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<tr>
<td></td>
<td>ACO₂ NM</td>
<td>121.1 ± 4.5</td>
<td>3.90 ± 0.19</td>
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<td></td>
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<td>122.4 ± 6.8</td>
<td>5.87 ± 0.47</td>
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<td>ECO₂ NM</td>
<td>110.6 ± 2.9</td>
<td>4.50 ± 0.31</td>
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<td>112.4 ± 2.3</td>
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<tr>
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</table>
Figure 1

![Figure 1](image1.png)

**ANOVA**

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<tr>
<th>Treatment</th>
<th>Nodule biomass (g plant$^{-1}$)</th>
<th>Mycorrhization (%)</th>
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<td>![Bar Chart ACO$_2$]</td>
<td>![Bar Chart ACO$_2$]</td>
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<tr>
<td>ECO$_2$</td>
<td>![Bar Chart ECO$_2$]</td>
<td>![Bar Chart ECO$_2$]</td>
</tr>
</tbody>
</table>

- **ANOVA** Table:
  - AMF: **
  - CO$_2$: **
  - AMF x CO$_2$: ns

- **Intensity of AMF colonization**
  - AMF x CO$_2$: ns

- **% Roots with arbuscules**
  - AMF x CO$_2$: ns