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# Elevated $CO_2$ and water-availability effect on gas exchange and nodule development in $N_2$-fixing alfalfa plants

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ABSTRACT

$N_2$-fixing alfalfa plants were grown in controlled conditions at different $CO_2$ levels (350 μmol mol$^{-1}$ *versus* 700 μmol mol$^{-1}$) and water-availability conditions (WW, watered at maximum pot water capacity *versus* WD, watered at 50% of control treatments) in order to determine the $CO_2$ effect (and applied at two water regimes) on plant growth and nodule activity in alfalfa plants. The $CO_2$ stimulatory effect (26% enhancement) on plant growth was limited to WW plants, whereas no $CO_2$ effect was observed in WD plants. Exposure to elevated $CO_2$ decreased Rubisco carboxylation capacity of plants, caused by a specific reduction in Rubisco (EC 4.1.1.39) concentration (11% in WW and 43% in WD) probably explained by an increase in the leaf carbohydrate levels. Plants grown at 700 μmol mol$^{-1}$ $CO_2$ maintained control photosynthetic rates (at growth conditions) by diminishing Rubisco content and by increasing nitrogen use efficiency. Interestingly, our data also suggest that reduction in shoot N demand (reflected by the TSP and especially Rubisco depletion) affected negatively nodule activity (malate dehydrogenase, EC 1.1.1.37, and glutamate-oxaloacetate transaminase, EC 2.6.1.1, activities) particularly in water-limited conditions. Furthermore, nodule DM and TSS data revealed that those nodules were not capable to overcome C sink strength limitations.



## 1. Introduction

For the 1000 years prior to the Industrial Revolution, atmospheric $CO_2$ concentration ($C_a$) was stable at about 270 μmol mol$^{-1}$. Human activities have been continuously increasing the concentration of atmospheric $CO_2$ from about 280 μmol mol$^{-1}$ at the beginning of the 19th century to its current value of 372 μmol mol$^{-1}$ which represents an increase of 38%. By the middle of this century, atmospheric $CO_2$ is predicted to reach 550 μmol mol$^{-1}$ and to surpass 700 μmol mol$^{-1}$ by the end of the century (Alley et al., 2007).

Because photosynthesis and biomass production of plants with $C_3$ photosynthetic metabolism are currently limited by $C_a$, both $CO_2$ fixation rates and plant growth increase as $C_a$ rises (Daepp et al., 2000; Aranjuelo et al., 2005b). An increase in $CO_2$ concentration from 300–350 to 680 μmol mol$^{-1}$ has been described as enhancing plant growth by 7% to 25% (Hadley et al., 1995; Daepp et al., 2000; Aranjuelo et al., 2006; Erice et al., 2006). Analyses of the $CO_2$ effect and its interaction with other environmental conditions is of great relevancy, since the responsiveness of plants to enhanced $CO_2$ has been shown to differ with $H_2O$ availability (Serraj et al., 1998), temperature (Aranjuelo et al., 2005a; Erice et al., 2006) and humidity (De Luis et al., 2002). Furthermore, as described by Valladares and Pearcey (1997), stress growth conditions might have different effects on plant growth that cannot be predicted if they are analysed separately, because of synergistic and antagonistic phenomena.

Although the initial stimulation of net photosynthesis associated with elevated $CO_2$ is sometimes retained during long-term exposure (Gunderson and Wullschleger, 1994), it is often partially reversed in an acclimation process, often referred as "down-regulation" (Long et al., 2004; Aranjuelo et al., 2005b; Ainsworth and Rogers, 2007). Down-regulation is usually accompanied by alterations in the gas-exchange characteristics that are indicative of a decreased carboxylation capacity (Ainsworth and Long, 2005;

*Abbreviations:* *A*, net photosynthetic rate; $A_{350}$, *A* measured at 350 μmol mol$^{-1}$ $CO_2$; $A_{700}$, *A* measured at 700 μmol mol$^{-1}$ $CO_2$; Ci, intercellular $CO_2$ concentration; $Ci_{350}$, Ci measured at 350 μmol mol$^{-1}$ $CO_2$; $Ci_{700}$, Ci measured at 700 μmol mol$^{-1}$ $CO_2$; DM, dry mass; *g*, conductance; $g_{350}$, *g* measured at 350 μmol mol$^{-1}$ $CO_2$; $g_{700}$, *g* measured at 700 μmol mol$^{-1}$ $CO_2$; GOT, glutamate–oxaloacetate transaminase; MDH, malate dehydrogenase; PPFD, photosynthetic photon flux density; TSP, total soluble protein; TSS, total soluble sugars; VPD, vapour pressure deficit; WW, plants watered at maximum soil volumetric water content; WD, partially watered plants; $\theta_v$, soil volumetric water content.

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Ainsworth and Rogers, 2007). Long-term studies also revealed that photosynthesis and the growth response to elevated $CO_2$ depend on a plant's ability to develop new sinks, expand storage capacity or the growth rate of existing sinks (Wolfe et al., 1998; Erice et al., 2006). According to Thomas and Strain (1991), when the carbohydrate production in plants exposed to elevated $CO_2$ exceeds the capacity to produce new sinks, the plants decrease their photosynthetic rate so as to balance source activity and sink capacity. Experiments conducted under controlled conditions (Stitt and Krapp, 1999; Poorter and Perez-Soba, 2002) and field conditions under free air $CO_2$ enrichment (FACE, Ainsworth and Long, 2005) have highlighted how sink development is restricted under N limiting conditions. In addition, it has been observed that the carbohydrate-mediated repression of photosynthetic genes is more severe in N-deficient plants (Stitt and Krapp, 1999; Reich et al., 2006; Ainsworth and Rogers, 2007). Several mechanisms have been proposed to explain the N limitation on growth, including limitations due to faster growth (Farage et al., 1998), increased microbial immobilization of N, or N sequestration in plant biomass (Luo et al., 2004).

Several authors (Serraj et al., 1998; Lüscher et al., 2000; Rogers et al., 2006) have postulated that legumes, since they are capable of fixing atmospheric $N_2$, will have an advantage in plant growth over non-$N_2$-fixing plants. It has been described that $N_2$-fixing species show a larger stimulation of growth and photosynthetic rate to elevated $CO_2$ than non-fixing species (Ainsworth and Rogers, 2007). Such studies suggest that $N_2$-fixing grown at elevated $CO_2$ conditions have a smaller tendency toward photosynthetic acclimation. Since legumes form a symbiotic association with $N_2$-fixing bacteria, those plants have an extra sink for additional C for exchange with the bacterial symbiont to enhance N fixation (Udvardi and Day, 1997). However, although evidence of plant growth and $N_2$ fixation stimulation in legumes has been analyzed (Zanetti et al., 1996; Lee et al., 2003), it has been described that responsiveness of legumes to predicted $CO_2$ enhancement is not clear and that it is dependent in other environmental conditions (water availability, temperature, etc.) (Serraj et al., 1998; West et al., 2005). The greater photosynthetic rate in legumes grown under high $CO_2$ conditions would imply that there is a larger supply of organic C to nodules and this is used in turn by the bacteroid nitrogenase enzyme inside the nodules as a source of energy and reducing power to fix $N_2$ (Arrese-Igor et al., 1999; Cabrerizo et al., 2001). Nodulated root can require up to 60% of photoassimilates produced over 12 h photoperiod (Gordon et al., 1987). This coupling is regulated by photosynthesis (C supply), nitrogen availability (N source strength), and N demand (N sink strength). Photoassimilates provided by the plant are hydrolysed in the nodule to obtain phosphoenolpyruvate through the glycolytic pathway. The oxaloacetate is converted to malate by malate dehydrogenase (MDH). MDH produces malate that can either be used as a source of C and energy for bacteroid consumption, enter the mitochondrial and be oxidised in the tricarboxilic acid cycle, or contribute to ammonia assimilation in the glutamine synthetase/glutamate synthase (GS/GOGAT) cycle (Cabrerizo et al., 2001). MDH also forms a complex with glutamate oxaloacetate transaminase (GOT) enzyme. The ability to fix atmospheric $N_2$ should enable these plants to adjust their enhanced N needs in elevated $CO_2$ environments. Interestingly, other authors (Serraj et al., 1998) have observed that $N_2$ fixation decreased, even though soybean plants exposed to elevated $CO_2$ and low water-availability conditions had larger nodule carbohydrate availability.

The aim of this paper was to determine the $CO_2$ (under water-limited conditions) and N availability effect in $N_2$-fixing alfalfa plant's growth. Further, most of these studies have been conducted in non-$N_2$-fixing plants. In the experiments where $N_2$-fixing plants have been analysed, the plants were usually grown under field conditions (where soil N was not totally controlled), with little attention being paid to the effect of $CO_2$ on nodule development. Since the responsiveness of the whole plant to the predicted $CO_2$ increase and low water-availability periods will depend strongly on the adaptation of nodule performance, this study was designed with a focus on the role of nodule. To achieve this, nodulated alfalfa plants were grown with 700 $\mu$mol mol$^{-1}$ $CO_2$ and different water-availability conditions over a 1-month period in controlled conditions. The experiment was conducted with an inert substrate and the plants were watered by N-free nutrient solution to ensure that the only source of N for the plant was fixed in the nodule. In order to study the role of nodule activity during photosynthetic acclimation, we have developed an experimental design that enabled us to harvest and analyse the key enzymes that are involved in C and N metabolism in the nodule.

## 2. Materials and methods

### 2.1. Plant material and experimental design

Seedlings of alfalfa (*Medicago sativa* L. cv. Aragón) were transferred into 2-l pots (5 plants per pot) containing a mixture of inert perlite and vermiculite (2/1, v/v). During the first month, plants were inoculated three times with *Sinorhizobium meliloti*, strain 102F78 (The Nitragin Co., Milwaukee, WI, USA) and grown in a greenhouse at 25/15 °C (day/night) with a photoperiod of 14 h under natural daylight, supplemented with fluorescent lamps (Sylvania DECOR 183, Professional-58W, Germany) providing a photosynthetic photon flux density (PPFD) of about 300–400 $\mu$mol m$^{-2}$ s$^{-1}$. Plants were watered twice a week with Evans N-free nutrient solution (Evans, 1974), and with tap water once a week to avoid salt accumulation in the pots.

When 30 days old, plants were transferred to controlled environment chambers (Conviron PGV 36, Winnipeg, Canada) and randomly assigned to 4 treatments (8 pots per treatment) corresponding to the applied $CO_2$ concentration (ambient, around 350 $\mu$mol mol$^{-1}$ *versus* elevated, at 700 $\mu$mol mol$^{-1}$) and soil water content (WW, watered at maximum soil volumetric water content, around 600 mm$^3$ mm$^{-3}$ *versus* WD, partially watered, 50% maximum soil volumetric water content, around 300 mm$^3$ mm$^{-3}$) during the following 30 days (i.e. 60-day-old plants). Plants were grown at 20/10 °C (day/night) and 45% RH (corresponding to 1.7 kPa vapour pressure deficit, VPD) with 14 h photoperiod and 600 $\mu$mol m$^{-2}$ s$^{-1}$ PPFD. Pots were rotated every week from one chamber to the other in order to avoid chamber effects. During this period in the growth chamber, the plants produced the 98% of final DM.

When analysing interaction between $CO_2$ and water availability, it should be remembered that elevated $CO_2$ grown plants deplete soil water at a lower rate than ambient $CO_2$ grown plants (due to lower stomatal conductance and lower transpiration rates), which means that in many experiments, elevated $CO_2$ increased the time to reach a particular water stress (De Luis et al., 1999). Therefore, the tolerance to low water-availability – meaning the ability to maintain plant productivity under given soil water stress (Jones, 1992) – induced by elevated $CO_2$ remains incompletely elucidated. The only way to test such a question was to design an experiment in which all treatments are subjected to the same soil water content, as we did in this study. Well watered (WW) plants were irrigated until they reached a maximum soil volumetric water content ($\theta_v$), whereas partially irrigated plants (WD) were watered at 50% $\theta_v$ of well-watered plants. Such $\theta_v$ levels were maintained throughout the experiment by daily measuring of transpired water (calculated by weighing the pots) and replenishing the lost water. In order to reduce soil evaporation, pots were covered with a plas-

tic sheet perforated with very small holes to allow stems to pass through. Reduced water levels were reached around 11 days (for plants grown at 350 μmol mol$^{-1}$ $CO_2$) and 13 days (plants grown at 700 μmol mol$^{-1}$ $CO_2$) after the beginning of treatment, when plants were 41 and 43 days old, respectively. WW plants were alternatively watered with Evans N-free nutrient solution (Evans, 1974) and distilled water, whereas WD plants were always watered with complete Evans solution in order to supply all treatments with the same amount of nutrients. All the determinations listed below where conducted at the end of the experiment, when the plants were 60 days old. The experiment was repeated in two consecutive years to confirm the response obtained during the first year.

### 2.2. Growth parameters and water relations

Plant production was estimated by weighing separately leaves, stem, root and nodules corresponding to 10 plants (i.e. dry mass, DM) harvested at the end of the experiment (when plants were 60 days old). Their DM was obtained after drying in an oven at 80 °C over 48 h. Leaf area was measured directly using an automatic leaf area meter (Li-3000, LiCor, NE, USA).

Plant water status was evaluated by measuring the leaf relative water content (RWC, Weatherley, 1950) of healthy and fully expanded apical leaves. Soil volumetric water content was calculated by weighing pots every day at the beginning of the photoperiod.

### 2.3. Gas exchange and chlorophyll fluorescence measurements

Fully expanded apical leaves from 60-day-old plants were enclosed in a gas-exchange leaf chamber (1010-M, Waltz, Effeltrich, Germany), and the gas-exchange rate was measured with a portable photosynthesis system (HCM-1000, Waltz). The gas-exchange response to $CO_2$ was measured at both $CO_2$ concentrations (400 μmol mol$^{-1}$ $CO_2$ and 700 μmol mol$^{-1}$ $CO_2$). For each year, four-plants-per-treatment combinations were analysed. Photosynthetic assimilation (A) was estimated at a PPFD of 1200 μmol m$^{-2}$ s$^{-1}$ using equations developed by von Caemmerer and Farquhar (1982). The leaf internal $CO_2$ concentration (Ci) was estimated as described by Farquhar and Sharkey (1982).

The maximal quantum yield of photosystem II was measured using a portable fluorometer (MINI-PAM, Walz, Effeltrich, Germany). Measurements were done simultaneously with gas-exchange determinations. Measuring conditions were the same as growth conditions, but in this case, leaves were dark-adapted during 30 min before determination.

### 2.4. Rubisco protein activity and concentration (EC 4.1.1.39)

For the analyses of Rubisco activity, leaves were harvested mid-morning, and immediately plunged into liquid nitrogen. The samples were stored at −80 °C before analysis. The leaf tissue was powdered in liquid N and homogenised in a cold mortar with an extraction buffer containing 100 mM Bicine–NaOH (pH 7.8), 10 mM $MgCl_2$, 10 mM 2-mercaptoethanol, 2% PVPP (w/v), 1% BSA (w/v) and 1% Triton X-100 (v/v). An aliquot of the extract was used to determine the chlorophyll content (Arnon, 1949). Another aliquot was clarified, by centrifugation at 13,000 × *g*, and used to determine enzyme activity by measuring the oxidation of NADH at 340 nm (Sharkey et al., 1991). The time period between extraction and the measurement of initial activity was less than 2.5 min. The activation state was calculated by considering initial activity as a percentage of total activity.

Rubisco protein content was determined through the extraction of frozen leaf subsamples, ground in a fine powder in 50 mM Tricine buffer (pH 8.0), 1 mM EDTA, 5 mM of 6-aminocaproic acid, 2 mM benzamidine, 8 mM β-mercaptoethanol and 100 mM PMSF—for 20 min on ice. This was followed by centrifugation at 12,000 × *g* at a temperature of 4 °C for 25 min. The protein concentration was measured in the decanted supernatant (Bradford, 1976), and five volumes of cold acetone were added to an aliquot containing 300 μg of protein, which was subsequently left overnight in the freezer. The sample was then centrifuged at 12,000 × *g* at a temperature of 4 °C for 15 min. The acetone was allowed to evaporate. The precipitate was dissolved in 65 mM Tris–HCl (pH 6.8), 25% glycerol (v/v), 0.6 M β-mercaptoethanol, 2.5% SDS (w/v) and 0.01% bromophenol blue at a temperature of 96 °C for 7 min. The samples were cooled to room temperature and loaded onto a 13% SDS-PAGE gel (Martín del Molino et al., 1995). The solubilised proteins were separated by a discontinuous SDS-PAGE system (Laemmli, 1970) using a 0.75-mm thick gel (13% separating, 4% stacking). Electrophoresis was carried out at room temperature and at a constant current of 200 V. Aliquots of the SDS dissociated extracts containing 9 μg of protein were applied to each well. The gels were stained in 0.1% (w/v) Coomassie blue dissolved in 5/5/2 (v/v/v) water/methanol/acetic acid overnight and subsequently destained in 12.5% (v/v) isopropanol and 10% (v/v) acetic acid. Finally, the gels were scanned with a Molecular Dynamics (CA, USA) densitometer. The percentage of Rubisco (small and large subunits) relative to TSP was calculated by taking the value of TSP as 100%.

### 2.5. Total soluble proteins (TSP) and sugars (TSS)

Leaf total soluble proteins (TSP) and total soluble sugars (TSS) were quantified by grinding and filtering 100 mg of fresh weight frozen leaf tissue in a cold mortar using an extraction buffer containing 50 mM K-phosphate (pH 7.5). The extract was filtered and centrifuged at 28,710 × *g* for 15 min at 4 °C. The supernatant was used for TSP and TSS quantification. TSP was measured by the protein dye-binding method of Bradford (1976). TSS was determined according to Yemm and Willis (1954).

### 2.6. Nitrogen content

Leaf samples, previously dried at 60 °C for 48 h, were ground in a mill with titanium blades and stored in vials into desiccators over silica gel. The N concentration was determined by means of sulphuric acid digestion in a Büchi K-424 (Büchi, Switzerland). Samples of 100 mg were digested by adding 20 ml $H_2SO_4$ and a Kjeldhal Cu–Se catalytic pill. The digestion process was left to run (i.e. for 1 h at 250 °C) until the samples were clarified. The samples were then diluted to 50 ml with distilled water. For determining the N content, 5 ml of ionic strength adjuster (ISA, Ref. 951211, Orion, NY, USA) were added to 5 ml of measuring solution. Measurements were done with an ammonia selective electrode (Orion 95-12BN, NY, USA) using 0.1 mM ammonium chloride as a standard.

### 2.7. Nodule enzymatic determinations

Five hundred mg of freshly harvested nodules were crushed in 10 ml of 50 mM K-phosphate buffer (pH 7.8), with 0.2% (v/v), 2-mercaptoethanol, 0.1 mM $Na_2$-EDTA and 10% (w/w) PVPP in a cold mortar. Malate dehydrogenase (MDH, EC 1.1.1.37) and glutamate–oxaloacetate transaminase (GOT, EC 2.6.1.1) activity in the nodule plant fraction were assayed spectrophotometrically by NADH oxidation at 340 nm. The reaction medium and assay conditions were based on those of Vance and Stade (1984). TSP and TSS content were determined as previously described.

**Table 1**
The interactive effect of $CO_2$ (ambient 350 μmol mol$^{-1}$ *versus* elevated 700 μmol mol$^{-1}$) and water availability (well watered, WW *versus* water deficit, WD) on total (g plant$^{-1}$), leaf (g plant$^{-1}$), stem (g plant$^{-1}$), root (g plant$^{-1}$), nodule (mg plant$^{-1}$) dry mass (DM) production and total leaf area (cm$^2$ plant$^{-1}$) in nodulated alfalfa

| | Total DM | Leaf DM | Stem DM | Root DM | Nodule DM | Total leaf area |
|---|---|---|---|---|---|---|
| WW-350 | 1.12 ± 0.08 b | 0.46 ± 0.04 b | 0.41 ± 0.04 a | 0.33 ± 0.02 b | 25 ± 1.4 a | 53.16 ± 3.54 b |
| WW-700 | 1.53 ± 0.10 a | 0.57 ± 0.03 a | 0.44 ± 0.03 a | 0.51 ± 0.04 a | 27 ± 0.2 a | 60.96 ± 2.71 a |
| WD-350 | 0.61 ± 0.05 c | 0.13 ± 0.01 c | 0.09 ± 0.01 b | 0.37 ± 0.03 b | 12 ± 0.8 b | 27.68 ± 4.28 c |
| WD-700 | 0.69 ± 0.05 c | 0.20 ± 0.014 c | 0.11 ± 0.01 b | 0.38 ± 0.03 b | 11 ± 0.7 b | 32.98 ± 4.55 c |

The measurements were carried out when the plants were 60 days old. Each value represents the mean ± S.E. ($n$ = 8). The different letters indicate significant differences ($P$ < 0.05) between the treatment as determined by Tukey-b test.

### 2.8. Statistical analysis

As previously mentioned, the experiment was carried out over 2 consecutive years, and under the same growth conditions. No statistical differences were observed between years in the analyses corresponding to the same treatment combination, which means that no year effect was observed consequently all the data were merged. Values presented in this paper correspond to mean values of data collected during the 2 consecutive years. Two factor analyses of variance (ANOVA; Sokal and Rholf, 1986) at 0.5% and 0.1% levels were performed to partition the variance into the main effects and the interaction between the two factors ($CO_2$ and water availability). When the $F$-ratio was significant, least significant differences were evaluated by the Tukey-b test ($P$ < 0.05).

## 3. Results

$CO_2$ effect on plant growth was mediated by water availability ($P$ = 0.03) (Table 1). Exposure to elevated $CO_2$ conditions increased total DM production (27%) in WW plants, whereas in WD conditions no statistical differences were observed associated with $CO_2$ enhancement (Table 1). The separated analyses of tissue DM revealed that the larger DM production observed in fully watered plants exposed to elevated $CO_2$ conditions were caused by the increase in leaf and root production of those plants (Table 1). Water limitation decreased DM production (Table 1). Table 1 also revealed that although under fully water-availability conditions elevated $CO_2$ enhanced total leaf area, under water deficit conditions, no $CO_2$ effect was observed.

The analyses of leaf relative water content (RWC; Fig. 1A) revealed that no differences associated with applied $CO_2$ and water regime level were observed between treatments. Fig. 1B shows that, regardless water regimen, specific transpiration decreased by 40% high $CO_2$ treatments ($P$ = 0.020).

Interestingly, gas-exchange data showed that, measured at growth conditions, elevated $CO_2$ conditions ($P$ = 0.51) did not affect the photosynthesis of WW and WD plants (Table 2). No differences associated with the water availability on the level of photosynthesis were detected either. Table 2 also showed that when $CO_2$ fixation rates were determined at 700 and 350 μmol mol$^{-1}$ atmospheric $CO_2$, elevated $CO_2$ plants had lower (28% depletion and 34% depletion) photosynthetic rates ($P$ = 0.019). Leaf stomatal conductance

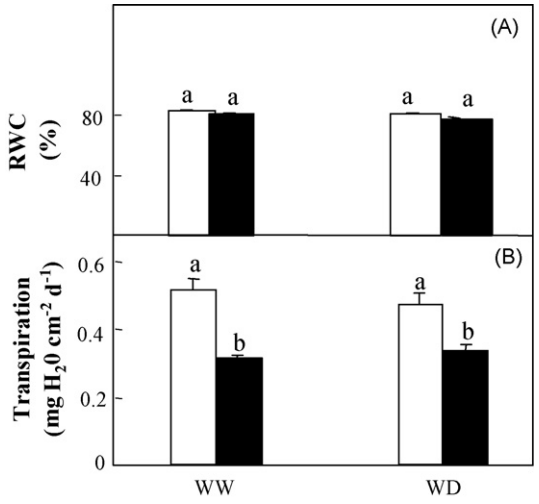


**Fig. 1.** The interactive effect of $CO_2$ (350 μmol mol$^{-1}$, unshaded bars *versus* 700 μmol mol$^{-1}$, shaded bars) and water availability (well watered *versus* water deficit) on (A) leaf relative water content (RWC) and (B) transpiration in nodulated alfalfa plants. Each RWC represents the mean ± S.E. of eight leaves. The different letters indicate significant differences ($P$ < 0.05) among treatments as determined by Tukey-b test.

($g$) data, measured at growth condition, showed that elevated $CO_2$ treatments had lower $g$ values. However, when $g$ was determined at 700 and 350 μmol mol$^{-1}$ $CO_2$, no statistical differences were observed between treatments (Table 2). The quantification of intercellular $CO_2$ concentration (Ci) showed that elevated $CO_2$ increased the intercellular $CO_2$ concentration. Water limitations had no effect on Ci concentration. The chlorophyll fluorescence study did not detect differences in $F_v/F_m$ between treatments associated with the $CO_2$ concentration and soil water content (Fig. 2).

TSS analyses revealed that plants grown at 700 μmol mol$^{-1}$ $CO_2$ had higher TSS values (Table 3) ($P$ = 0.001). There were not statistical differences on leaf N content (Table 3) associated with elevated $CO_2$ ($P$ = 0.551).

Enhanced $CO_2$ depleted (by 18%) the TSP concentration in WD ($P$ = 0.028) plants whereas no statistical differences were observed in WW (Table 3). Interestingly, the analysis of the Rubisco activity showed that both initial and total Rubisco activities decreased

**Table 2**
The interactive effect of $CO_2$ (ambient 350 μmol mol$^{-1}$ *versus* elevated 700 μmol mol$^{-1}$) and water availability (well watered, WW *versus* water deficit, WD) on leaf photosynthesis ($A$, μmol m$^{-2}$ s$^{-1}$), conductance ($g$, mmol m$^{-2}$ s$^{-1}$) and internal $CO_2$ concentration (Ci, μmol mol$^{-1}$) in nodulated alfalfa, measured either at 350 μmol mol$^{-1}$ $CO_2$ ($A_{350}$, $g_{350}$ and $Ci_{350}$) or 700 μmol mol$^{-1}$ $CO_2$ ($A_{700}$, $g_{700}$ and $Ci_{700}$)

| | $A_{700}$ | $A_{350}$ | $g_{700}$ | $g_{350}$ | $Ci_{700}$ | $Ci_{350}$ |
|---|---|---|---|---|---|---|
| WW-350 | 19.6 ± 0.6 a | 12.1 ± 0.48 a | 73.4 ± 8.7 a | 91.8 ± 8.6 a | 273.9 ± 28.3 c | 123.1 ± 36.9 b |
| WW-700 | 14.1 ± 0.7 b | 7.7 ± 0.3 b | 65.2 ± 2.7 a | 79.9 ± 3.6 a | 381.0 ± 17.6 ab | 246.4 ± 44 b |
| WD-350 | 19.1 ± .4 a | 12.2 ± 0.9 a | 76.9 ± 9.4 a | 102.9 ± 13.4 a | 304.2 ± 22.0 bc | 158.2 ± 15.1 a |
| WD-700 | 13.3 ± 1.1 b | 8.2 ± 0.5 b | 70.2 ± 7.9 a | 86.5 ± 7.0 a | 441.9 ± 21.5 a | 305.7 ± 60.1 a |

The measurements were carried out when the plants were 60 days old. Each value represents the mean ± S.E. ($n$ = 8). The different letters indicate significant differences ($P$ < 0.05) between the treatment as determined by Tukey-b test.

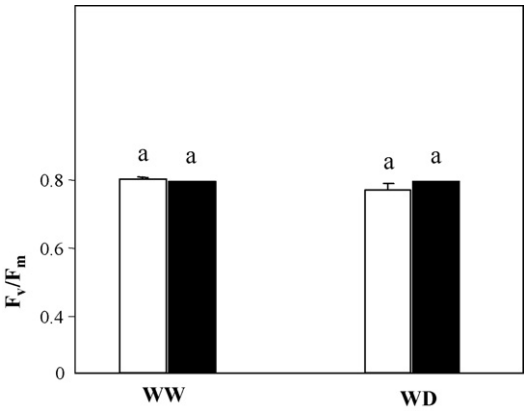


**Fig. 2.** The interactive effect of $CO_2$ (350 μmol mol$^{-1}$, unshaded bars *versus* 700 μmol mol$^{-1}$, shaded bars) and water availability (well watered *versus* water deficit) on maximal quantum yield of PSII ($F_v/F_m$) in nodulated alfalfa plants. Each value represents the mean ± S.E. of eight leaves. Otherwise, as for Fig. 1.

**Table 3**
The interactive effect of $CO_2$ (ambient 350 μmol mol$^{-1}$ *versus* elevated 700 μmol mol$^{-1}$) and water availability (well watered, WW *versus* water deficit, WD) on the leaf total soluble sugar (TSS, g m$^{-2}$), leaf N content ($N_{leaf}$, g N m$^{-2}$), and the total soluble protein (TSP, g m$^{-2}$) content in nodulated alfalfa plants

| | TSS | $N_{leaf}$ | TSP |
|---|---|---|---|
| WW-350 | 0.59 ± 0.004 d | 1.36 ± 0.04 a | 7.45 ± 0.18 a |
| WW-700 | 1.54 ± 0.002 a | 1.44 ± 0.07 a | 7.88 ± 0.15 a |
| WD-350 | 0.98 ± 0.001 c | 1.01 ± 0.02 b | 4.97 ± 0.03 b |
| WD-700 | 1.24 ± 0.016 b | 1.14 ± 0.04 b | 4.07 ± 0.04 c |

The measurements were carried out when the plants were 60 days old. Each value represents the mean ± S.E. ($n$ = 8). The different letters indicate significant differences ($P$ < 0.05) between the treatment as determined by Tukey-b test.

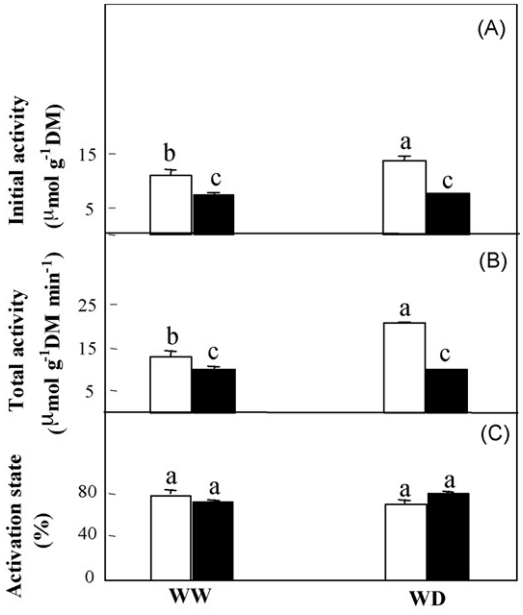


**Fig. 3.** The interactive effect of $CO_2$ (350 μmol mol$^{-1}$, unshaded bars *versus* 700 μmol mol$^{-1}$, shaded bars) and water availability (well watered *versus* water deficit) on Rubisco (A) initial and (B) total activities and (C) activation state in nodulated alfalfa plants. Each column represents the mean ± S.E. of eight samples. Otherwise, as for Fig. 1.

in WW and especially in WD treatments grown under elevated $CO_2$ conditions (Fig. 3A and B). However, no differences ($P$ = 0.482) were detected between treatments for the Rubisco activation state (Fig. 3C). The quantification of Rubisco concentration showed that the Rubisco large and small subunit, and consequently the total Rubisco concentration, diminished in WW (11%) and WD (43%) plants grown at 700 μmol mol$^{-1}$ $CO_2$ ($P$ = 0.025) (Table 4). Water limitation diminished Rubisco protein concentration. Calculation of the percentage of Rubisco in the TSP content indicated that compared with ambient $CO_2$ plants, plants exposed to elevated $CO_2$, suffered a specific decrease in the Rubisco content (Table 4).

The analyses of nodule enzymatic activity showed that the inhibitory effect of elevated $CO_2$ on malate dehydrogenase (MDH) was limited to WD plants were its activity was 35% lower ($P$ = 0.033) (Fig. 4A). No differences were detected in the MDH specific activity (Fig. 4B). Plants grown under elevated $CO_2$ conditions ($P$ = 0.036) had lower glutamate–oxaloacetate transaminase (GOT) activity in both WW (22%) and WD (29%) plants (Fig. 4C). Elevated $CO_2$ did not modify GOT specific activity (Fig. 4D). Nodule TSP ($P$ = 0.038) concentration decreased in WW (19%) and WS (17%) plants exposed to elevated $CO_2$ conditions (Fig. 5A). The analyses of nodule TSS concentration showed that plants exposed to 700 μmol mol$^{-1}$ $CO_2$, had higher TSS, in WW treatments, whereas under water limitation not induce statistical differences (Fig. 5B) were observed.

## 4. Discussion

Short-term studies have described that elevated $CO_2$ results in a rise in photosynthetic rates (Drake and González-Meler, 1997) and therefore an increase in DM production. However, as it has been previously described, the $CO_2$ effect on plants might vary depending on exposure time, soil water and nutrient availability, ambient relative humidity, temperature, radiation, and other factors (De Luis et al., 1999; Aranjuelo et al., 2005a,b, 2006; Erice et al., 2006, 2007). Our study revealed that 98% of final DM was produced during the second month of experiment, in the growth chambers. The effect of $CO_2$ on plant growth was strongly conditioned by water availability. The analyses of total DM production showed that the stimulatory effect associated with elevated $CO_2$ on production was only detected in fully watered plants (Table 1) where DM increased up to 26% (as compared to the respective ambient $CO_2$ treatment). The further analyses of tissue production highlighted the fact that the larger DM production of plants grown at 700 μmol mol$^{-1}$ was explained by the fact that in WW plants, elevated $CO_2$ increased leaf and root production, however no statistical differences were found

**Table 4**
The interactive effect of $CO_2$ (ambient 350 μmol mol$^{-1}$ *versus* elevated 700 μmol mol$^{-1}$) and water availability (well watered, WW *versus* water deficit, WD) on Rubisco total, large and small subunit contents (g m$^{-2}$) and Rubisco relative to the total soluble protein content (%) in nodulated alfalfa plants

| | Rubisco total | Large subunit | Small subunit | Rubisco relative to TSP |
|---|---|---|---|---|
| WW-350 | 2.65 ± 0.07 a | 2.03 ± 0.06 a | 0.59 ± 0.01 a | 35.57 ± 2.01 a |
| WW-700 | 2.36 ± 0.02 b | 1.72 ± 0.01 b | 0.61 ± 0.00 a | 29.95 ± 2.68 b |
| WD-350 | 1.82 ± 0.04 c | 1.40 ± 0.06 c | 0.39 ± 0.01 b | 36.62 ± 4.33 a |
| WD-700 | 1.03 ± 0.01 d | 0.80 ± 0.01 d | 0.25 ± 0.00 c | 25.31 ± 1.34 c |

The measurements were carried out when the plants were 60 days old. Each value represents the mean ± S.E. ($n$ = 8). The different letters indicate significant differences ($P$ < 0.05) between the treatment as determined by Tukey-b test.

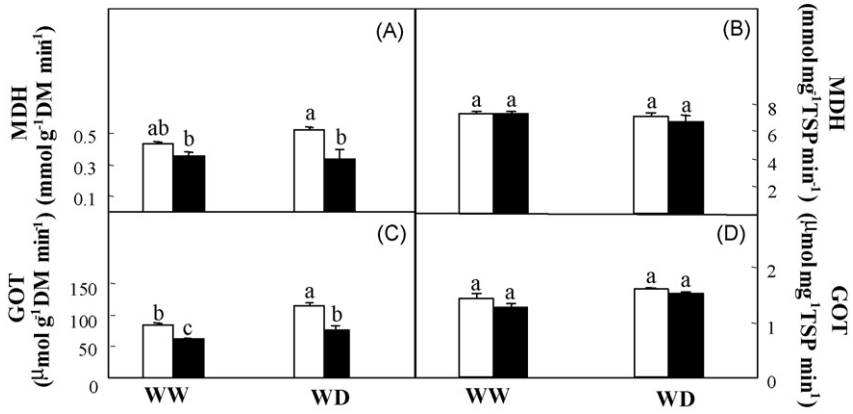


**Fig. 4.** The interactive effect of $CO_2$ (350 μmol mol$^{-1}$, unshaded bars *versus* 700 μmol mol$^{-1}$, shaded bars) and water availability (well watered *versus* water deficit) on (A) total and (B) specific malate dehydrogenase (MDH) activity and glutamate–oxalacetate transaminase (GOT) (C) total and (D) specific activity in the nodules of nodulated alfalfa plants. Each column represents the mean ± S.E. of eight samples. Otherwise, as for Fig. 1.

on stem and nodule production. Lack of statistical differences in the photosynthetic rate between $CO_2$ treatments (Table 2) revealed that the larger DM production of fully watered plants exposed to elevated $CO_2$ could be explained by their larger leaf area (Table 1), and this in turn implies a higher $CO_2$ fixation rate at the whole plant level. Similarly, the absence of a water-availability effect on the $CO_2$ fixation rate of plants exposed to ambient and elevated $CO_2$ suggests that the inhibitory effect of low water availability on plant growth was targeted to leaf area. Indeed, the limitation of water availability decreased leaf area (Table 1) and, consequently, total C assimilation per plant.

The fact that there were no statistical differences in leaf relative water content (RWC, Fig. 1A) showed that differences in dry mass were not caused by a different water status. It is also noteworthy that there were not differences in RWC associated with soil water content. Furthermore, no differences were observed in leaf transpiration rates between WW and WD (Fig. 1B). According to the description by Azcón-Bieto et al. (2004), reduction in leaf area is one of the main strategies developed by plants to diminish water loss during drought periods. Diminished leaf area expansion in WD plants (Table 1) would be due to reduced cell division and elongation (Boyer et al., 1985; Legg et al., 1975), both processes being extremely dependent on water availability. Absence of statistical differences in the photosynthetic, leaf conductance and intercellular $CO_2$ concentration rates (Table 2) confirmed that WD plants adapted their growth to available soil water content without suffering stressful growth conditions. Similar responses have been described in alfalfa (Aranjuelo et al., 2005b; Erice et al., 2006) and in wheat (Cabrera-Bosquet et al., 2007) exposed to long-term water limiting regimes.

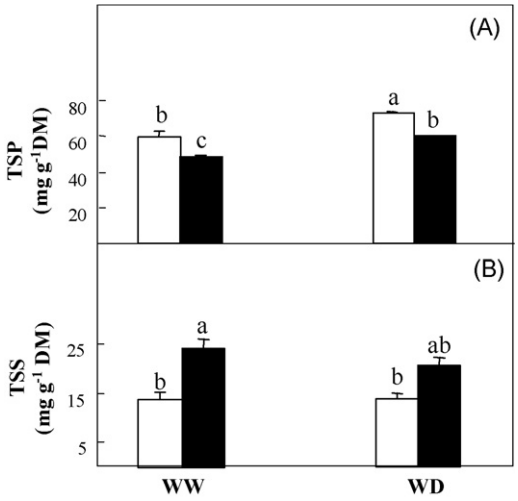


**Fig. 5.** The interactive effect of $CO_2$ (350 μmol mol$^{-1}$, unshaded bars *versus* 700 μmol mol$^{-1}$, shaded bars) and water availability (well watered *versus* water deficit) on (A) total soluble proteins (TSP) and (B) total soluble sugars (TSS) in the nodules of nodulated alfalfa plants. Each column represents the mean ± S.E. of eight samples. Otherwise, as for Fig. 1.

As previously discussed, gas-exchange data showed that, when measured at the corresponding growth conditions (350 μmol mol$^{-1}$ $CO_2$ for ambient $CO_2$ treatment and 700 μmol mol$^{-1}$ $CO_2$ for elevated $CO_2$ treatment), neither the water regime nor elevated $CO_2$ exposure significantly affected photosynthesis rates (Table 2). However when photosynthesis measurements were conducted at the same $CO_2$ concentration (i.e. 700 μmol mol$^{-1}$ and especially at 350 μmol mol$^{-1}$), $CO_2$ fixation rates in plants grown at 700 μmol mol$^{-1}$ $CO_2$ were generally lower than those in plants grown in ambient $CO_2$ indicating a photosynthesis down-regulation or acclimation induced by the elevated $CO_2$ (Stitt and Krapp, 1999; Aranjuelo et al., 2006). Reduction in Rubisco carboxylation capacity could be caused by stomatal and/or non-stomatal limitations of photosynthesis. Gas-exchange measurements showed that leaf conductance ($g_s$) was diminished by elevated $CO_2$. Such reduction explained the fact that plants exposed to elevated $CO_2$ conditions required two more days to reach desired $\theta_v$ because of their lower evapotranspiration values. In other hand, the intercellular $CO_2$ concentration (Ci) of plants grown under elevated $CO_2$ conditions was larger (Table 2). This implies that plants grown in elevated $CO_2$ environments had a higher intercellular $CO_2$ concentration. These results suggest that non-stomatal limitations (e.g. PSII activity and/or carboxylation efficiency) are the main cause of the decreased carboxylation efficiency observed in elevated $CO_2$ treatments.

Chlorophyll fluorescence was measured in order to test whether photosynthetic acclimation was a result of reduced PSII activity (Fig. 2). The chlorophyll fluorescence data indicated that photosynthetic acclimation was not caused by a decreased maximal photochemical efficiency ($F_v/F_m$). Photosynthetic limitation might also be caused by a decrease in RuBP regeneration. According to some authors (Wolfe et al., 1998; Jifon and Wolfe, 2002), reduction in photosynthetic activity is caused by a C source/sink imbalance. Plant growth data (Table 1) suggest that a decrease in photosynthetic capacity was caused by a reduced ability (fully watered

treatments increased dry mass production by 26%) or an inability (water-limited treatments) of plants exposed to elevated $CO_2$ to develop greater/new sinks. The inability to increase sink strength suggests that the demand for carbohydrates was insufficient to balance the enhanced carbohydrate supply under enhanced $CO_2$ conditions, with the consequent end-product inhibition of photosynthetic capacity (Strain and Thomas, 1995; Rogers et al., 2006). TSS data (Table 3) confirmed that plants exposed to elevated $CO_2$, especially in WD plants, had a massive production and build-up of carbohydrates. Carbon sink/source imbalance induced photosynthetic acclimation caused by depletions in the expression of Calvin cycle enzymes (Moore et al., 1999; Stitt and Krapp, 1999; Aranjuelo et al., 2005b; Reich et al., 2006; Rogers et al., 2006). However, it should also be noted that all the enzymes involved in the photosynthetic processes may not be equally affected by enhanced $CO_2$ (Moore et al., 1999). Nie et al. (1995) observed that, although transcripts for Rubisco subunits and phosphoglycerate kinase are particularly sensitive to moderate increases in $CO_2$, sedoheptulose-1,7-bisphosphatase (SBP) and phosphoribulosekinase (PRK) mRNAs are not.

As it has been previously described, a decrease in Rubisco content is often correlated with a decline in photosynthesis after exposure to elevated $CO_2$ (Urban, 2003). Determination of the initial and total activities of Rubisco (Fig. 3A and B) confirmed that the decreased photosynthetic rates that were observed in fully and partially watered plants grown at 700 $\mu$mol mol$^{-1}$ $CO_2$ were caused by inhibition of Rubisco activity (Fig. 3A and B). Since there was no change in the enzyme activation state, our data revealed that the decreased initial and total Rubisco activity was the result of a depleted Rubisco protein content (Moore et al., 1999). The fact that WD plants exposed to elevated $CO_2$ had depleted Rubisco and TSP contents, and a larger TSS value suggests that those plants had problems to increase sink strength (reflected by the lack of $CO_2$ effect on DM production). Such limitation induced a more severe photosynthetic down-regulation of WD plants when compared with WW plants. According to Stitt and Krapp (1999), increased growth rates in elevated $CO_2$ may either lead to plants becoming N limited or may exacerbate an existing N limitation. However, in our case and similarly to what described by Ainsworth and Rogers (2007) lack of statistical differences on leaf N concentration (Table 3) highlighted that alfalfa plants exposed to elevated $CO_2$ conditions were capable to reach control N values. Such results imply that reduction in Rubisco content was not caused by a lower leaf N content. Similarly to what described by other authors (Drake and González-Meler, 1997; Ainsworht and Rogers, 2007) our data suggest that the large C accumulation observed in plants grown at 700 $\mu$mol mol$^{-1}$ $CO_2$, induced the reduction of Rubisco activity and, consequently, the diminishment of excess capacity for carboxylation. The fact that % of Rubisco relative to TSP (Table 4) of plants exposed to 700 $\mu$mol mol$^{-1}$ $CO_2$ was lower highlighted that those plants suffered a specific decrease in Rubisco content. This kind of regulation of the amount of Rubisco might serve to optimise $CO_2$ acquisition with the utilisation of fixed carbon (Woodrow, 1994). In a recent review, Ainsworth and Rogers (2007) observed that since legumes grown at elevated $CO_2$ concentration have an excess of Rubisco, and less Rubisco is required, redistribution of the excess N invested in this protein could increase nitrogen use efficiency without impacting potential C acquisition. These authors also observed that legumes are preferentially reducing their carboxylation capacity in order to optimise their resources.

There has been a considerable debate (Serraj et al., 1998; Arrese-Igor et al., 1999) about $N_2$ fixation in elevated $CO_2$ conditions and its relationship with photosynthetic C supply and nodule C sink strength. Our data showed that even if plants exposed to elevated $CO_2$ conditions had a larger C availability (at whole plant level), such enhancement was not reflected in a larger nodule DM. However, our results (Fig. 5) revealed that in WW plants exposed to elevated $CO_2$, TSS availability was larger, whereas in WD plants no statistical differences were observed. In the other hand, the study of the activity of enzymes involved in C (i.e. MDH) and N (i.e. GOT) (Fig. 4) metabolisms in the cytosolic nodule fraction, indicating a lower flux of carbon and nitrogen in the nodule, probably associated with a lower nodules fixing activity. Clearly, our results showed that the $CO_2$ effect on MDH activity varied depending on the water availability. The inhibitory effect associated with the $CO_2$ increase was limited to WD plants, whereas in WW plants, no effect was observed. As malate is synthesised through the MDH, inhibition of MDH total activity in WD plants grown at 700 $\mu$mol mol$^{-1}$ $CO_2$ was reflected in diminished malate (considered as the main C supply for $N_2$ fixation) production. Malate depletion implies that less C and energy were available for bacteroid consumption, and also that less C was redirected to mitochondria for ammonia assimilation. On the other hand, GOT, an enzyme mainly associated with the assimilation of fixed $N_2$, also showed that elevated $CO_2$ diminished its activity in both WW and WD plants. The inhibitory effect of elevated $CO_2$ on GOT might have contributed to a lower N assimilation capacity of the nodule plant cells, which may difficult the ammonia assimilation and therefore may induce ammonia accumulation and nitrogenase inhibition. Unchanged MDH and GOT specific activity revealed that decreases in total enzymatic activities were a consequence of depleted TSP content, which is a symptom of lower nodule activity as observed in several stresses as drought (Aranjuelo et al., 2007).

Similar results were obtained by Serraj et al. (1998), under elevated $CO_2$ and drought conditions, suggesting that nodulation and nodule activity were not regulated by carbohydrate availability but by a feedback mechanism mediated by the plant's demand for nitrogen as we observed in alfalfa nodules (Fig. 5). According to Serraj et al. (1999), when the shoot N demand decreases, the concentration of N-transporting solutes declines with a consequent accumulation of products associated with the $N_2$ fixation (ureides) in the nodules that leads to inhibition of nitrogenase activity in the bacteroids. The fact that leaves of plants exposed to elevated $CO_2$ had lower TSP and specially Rubisco protein content suggests that shoot N demand of those plants was lower, with the consequent inhibition of nodule activity, as also described by Serraj et al. (1998).

In summary, our study revealed that the $CO_2$ effect on plant growth (i.e. 26% increase) was limited to fully watered plants, whereas under conditions of restricted water availability, no $CO_2$ effect on plant DM production was observed. Exposure to elevated $CO_2$ conditions induced photosynthetic acclimation, and this was probably explained by the inability of these plants to increase carbon sink activity, especially in WD plants. The absence of differences in water status and chlorophyll fluorescence data suggested that the photosynthetic down-regulation (particularly in WD plants) was caused by a depleted Rubisco protein content, which was a result of carbohydrate build-up. Lack of $CO_2$ effect on leaf N concentration values of WW and WD suggest that those plants maintained control C acquisition values by diminishing, specifically, Rubisco content and by increasing nitrogen use efficiency. The analyses of nodule DM and nodule TSS data revealed that nodules of plants grown at 700 $\mu$mol mol$^{-1}$ were not capable to increase C sink strength in a significant degree. Interestingly, our data also suggest that reduction in shoot N demand (reflected by the TSP and especially Rubisco depletion) affected negatively nodule activity (MDH and GOT) particularly in water-limited conditions. This diminishment of nodule activity was reflected by TSP depletion. The fact that nodules from plants grown in an elevated $CO_2$ environment possessed the same (WD) or larger (WW treatments) TSS availability as plants grown at ambient $CO_2$ implies that

such different levels of carbohydrate availability could not explain the obtained results. Instead, nodule activity depletion could have been caused by the reduction in shoot N demand in those plants and perhaps by an increase of the end product of $N_2$ fixation activity (ammonia) which may inhibit nitrogenase.

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