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**EFFECTS OF CLIMATE CHANGE ON NODULATED ALFALFA
PLANTS: PHYSIOLOGY, BIOCHEMICAL AND MOLECULAR
ANALYSES**

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Resumen

El aumento del dióxido de carbono (CO₂) atmosférico es el mayor responsable del incremento del efecto invernadero, y por lo tanto el mayor impulsor del cambio climático. El Grupo Intergubernamental de Expertos que estudian el cambio climático (IPCC), prevé que el aumento de la concentración de CO₂ en la atmósfera se incrementará desde 375 actuales hasta alcanzar 700 $\mu\text{mol mol}^{-1}$ de CO₂ en los próximos 100 años, lo que también conducirá a un aumento promedio de 4 °C en la temperatura media de la atmósfera (IPCC, 2007). Las actividades humanas no sólo afectan a la concentración de CO₂ en la atmósfera, sino que también alteran el ciclo global del nitrógeno (N) al aumentar las concentraciones de nitrógeno disponible, debido al uso intensivo de fertilizantes químicos (Vitousek *et al.*, 1997).

Se ha observado que un aumento en la concentración de CO₂ en la atmósfera afecta a diversos procesos fisiológicos en las plantas, destacando el aumento de la fotosíntesis neta en las plantas C₃ (Drake *et al.*, 1997), lo cual incrementa el rendimiento vegetal en exposiciones a corto plazo (Oliveira *et al.*, 2010). Sin embargo, a largo plazo la aclimatación fotosintética al CO₂ elevado reduce el potencial de crecimiento de la planta (Erice *et al.*, 2006a; Ainsworth y Rogers, 2007; Aranjuelo *et al.*, 2009). El fenómeno conocido como "aclimatación de la fotosíntesis (ó regulación a la baja)" se debe a una limitación metabólica, que habitualmente se atribuye a una disminución de la actividad carboxilatoria de la Rubisco y/o a una disminución de la cantidad de Rubisco (Ribulosa-1, 5-bisfosfato carboxilasa/oxigenasa) en CO₂ elevado (Urban, 2003; Aranjuelo *et al.*, 2005b).

El nitrógeno (N) puede limitar la respuesta de las plantas al CO₂ elevado, y por lo tanto el factor "disponibilidad de N" debe siempre tenerse en cuenta en todos los estudios sobre los efectos del CO₂ en las plantas (Sanz-Sáez *et al.*, 2010). El crecimiento acelerado de la alfalfa en condiciones de CO₂ elevado (Erice *et al.*, 2007) aumenta la demanda de nutrientes, y en un suelo con baja disponibilidad de N como los de las regiones Mediterráneas, el N sería un nutriente limitante del crecimiento (Lüscher *et al.*, 1998; Lüscher *et al.*, 2000). Este hecho podría reducir la fotosíntesis y por lo tanto disminuir el rendimiento vegetal (Luo *et al.*, 2004; Peterson *et al.*, 1999).

La alfalfa (*Medicago sativa* L.) es una de las leguminosas forrajeras más cultivadas en el mundo, debido a razones tanto ecológicas (mejora la fertilidad del suelo y reduce la contaminación del suelo y del agua al requerir menor uso de fertilizantes) como económicas (alta calidad del forraje y menos uso de fertilizantes). La alfalfa puede establecer simbiosis con bacterias fijadoras de N₂ del género *Sinorhizobium meliloti*, lo que le permite cubrir sus requerimientos de N, a través de la fijación del N₂ de la atmósfera en los nódulos.

El objetivo del proyecto era estudiar la respuesta de plantas noduladas de alfalfa (*Medicago sativa* L.) de las variedades Aragón y Europa al aumento de CO₂ atmosférico (CO₂ ambiente, alrededor de 400 µmol mol⁻¹, y CO₂ elevado, 700 µmol mol⁻¹) y fertilizadas con 0 mM (0N) ó 15 mM (15N) de NH₄⁺NO₃.

Plantas de alfalfa inoculadas con *S. meliloti* cepa 102F78 de un mes de edad, a las que se les aplicó 0 ó 15 mM de NH₄⁺NO₃, crecieron en invernaderos a CO₂ ambiente (400 µmol mol⁻¹ aproximadamente) o elevado (700 µmol mol⁻¹) durante un mes. A lo largo del mes creciendo en estas condiciones, se realizaron tres cosechas a los 37, 52 y 58 días de edad de las plantas. Se analizaron el crecimiento, el intercambio gaseoso foliar (fotosíntesis y parámetros asociados), la actividad nodular (actividad aparente de la nitrogenasa, ANA), y análisis bioquímicos en hojas y nódulos de las plantas. Dado que las medidas de intercambio gaseoso no eran destructivas, se realizaron medidas adicionales a las mencionadas de las tres cosechas, a los 36, 40, 45, 47 y 54 días de edad de las plantas. El experimento se realizó dos veces: una en los meses de octubre a noviembre de 2011, y por segunda vez los meses de febrero a marzo de 2012. Los resultados en ambos experimentos fueron muy similares, por lo que se decidió presentar los resultados del primero de los experimentos.

Con los parámetros medidos se han elaborado dos capítulos de la tesis. El primero hace referencia a los parámetros de crecimiento, intercambio gaseoso y diversos análisis bioquímicos realizados en las hojas. El segundo de los capítulos se centró en la actividad nodular y en el análisis bioquímico de las fracciones vegetal y bacteriana de los nódulos.

El aumento de CO₂ estimuló la producción de biomasa (materia seca total) en la variedad Aragón y de forma especial en el tratamiento de 15 mM de NH₄⁺NO₃. La variedad Europa no mostró un efecto significativo de los tratamientos aplicados en la producción de biomasa total. Las hojas fueron los órganos más sensibles al aumento del CO₂, de tal forma que el efecto se observó en ambas variedades de alfalfa. El resto de los parámetros de crecimiento medidos, no mostraron respuestas muy significativas. Las medidas de intercambio gaseoso realizadas a CO₂ de crecimiento (los tratamientos de ambiente a 400 μmol mol⁻¹, y las de CO₂ elevado a 700 μmol mol⁻¹) mostraron que en ambas variedades se producía un descenso de las tasas de fotosíntesis con la edad. También se intuyó un proceso de aclimatación fotosintética, especialmente a partir de los 47 días de edad. Sin embargo, ninguno de los parámetros bioquímicos medidos en las hojas, actividad extractable de la Rubisco *in vitro*, concentración de subunidades grande (RbcLs) o pequeña de la Rubisco (RbcS), concentraciones de azúcares y proteínas solubles totales, así como la relación C/N, corroboraron dicha aclimatación fotosintética. Tan sólo la acumulación de almidón y el aumento de la relación C/N inducida por el CO₂ elevado apoyó la posible aclimatación sobre todo en la variedad de alfalfa Aragón. El CO₂ elevado redujo la conductancia estomática y las tasas de transpiración específica. Sin embargo, y a pesar de las menores conductancias estomáticas inducidas por el CO₂ elevado, las concentraciones intercelulares de CO₂ en las hojas fueron muy superiores en las plantas crecidas en CO₂ elevado (valores medios de 500 μmol mol⁻¹ frente a valores menores a 300 μmol mol⁻¹ en los tratamientos de CO₂ ambiente).

En lo que respecta a la actividad nodular, señalar la fuerte inhibición del desarrollo nodular inducido por el tratamiento de 15 mM en plantas noduladas de alfalfa de ambas variedades. Por ello, las medidas de la actividad nitrogenasa aparente (ANA) y las actividades enzimáticas de las fracciones vegetal y bacteriana sólo se realizaron en las plantas de los tratamientos de 0 mM de NH₄⁺NO₃. Las actividades ANA fueron muy superiores en la variedad Aragón que en Europa, y el aumento de CO₂ no tuvo efecto en ninguna de las dos variedades. Los azúcares y las proteínas solubles totales y el almidón de la fracción vegetal del nódulo no se vieron muy afectados por los tratamientos. La concentración de almidón de los nódulos de la variedad Aragón fue

significativamente mayor que el de la variedad Europa. Las actividades enzimáticas malato deshidrogenasa (MDH), isocitrato deshidrogenasa (ICDH), fosfoenolpiruvato carboxilasa (PEPC) y glutamato-oxaloacetado transaminasa (GOT), no se vieron afectadas por el CO₂ elevado. En la fracción bacteriana del nódulo, las proteínas solubles y la actividad malato deshidrogenasa (MDH) no se vieron afectadas por el tratamiento de CO₂ elevado. No así la actividad isocitrato deshidrogenasa que aumento en Aragón y disminuyó en Europa. Las menores tasas de actividad aparente de la nitrogenasa (ANA) medidas en Europa se asociaron con niveles de almidón en la fracción vegetal y de proteínas solubles en la fracción bacteriana significativamente menores.

Del estudio se concluye que la respuesta de la producción de biomasa total de las dos variedades de alfalfa (Aragón y Europa) al aumento de CO₂ no fue igual. La variedad Aragón fue más sensible al aumento de CO₂ atmosférico en los dos tratamientos de nitrógeno aplicados (0 y 15 mM), si bien el efecto estimulador de la producción de biomasa fue mayor en 15 mM de NH₄⁺NO₃. Por el contrario, la variedad Europa se mostró insensible al aumento de CO₂ en ambas condiciones de nitrógeno. Si bien las tasas fotosintéticas en ambas variedades dejaron intuir una aclimatación fotosintética al CO₂ elevado, los distintos parámetros bioquímicos medidos en las hojas no lo apoyaron. El tratamiento de 15 mM de NH₄⁺NO₃ inhibió la formación de nuevos nódulos en las dos variedades. Los nódulos no mostraron una respuesta muy destacada al aumento del CO₂ en ninguno de los parámetros analizados. Se observó que los nódulos de las plantas de Aragón fueron más eficientes fijando N₂ que las plantas de Europa, lo que podría estar asociado a los mayores niveles de almidón en la fracción vegetal y de proteínas solubles en la fracción bacteriana de los nódulos de esta variedad.

Palabras claves: aclimatación, cambio climático, dióxido de carbono (CO₂ elevado), fijación de nitrógeno, fotosíntesis, *Medicago sativa* (alfalfa), metabolismo de nódulo, nitrógeno, *Sinorhizobium meliloti*.

Abstract

Increased atmospheric CO₂ is a major contributor to the greenhouse effect and is one of the main inducers of climate change. According to Intergovernmental Panel on Climate Change (IPCC) predictions, the atmospheric CO₂ will be enhanced from 375 to 700 $\mu\text{mol mol}^{-1}$ CO₂ in the next 100 years, leading also to an increase of 4 °C in atmospheric mean temperature (IPCC, 2007). Human activities do not only affect the CO₂ concentration, they also alter the global nitrogen (N) cycle by increasing the inputs of N fixed forms, mainly because of the extensive use of chemical fertilizers (Vitousek *et al.*, 1997).

Elevated CO₂ concentration in the atmosphere is also shown to affect a variety of physiological processes in plants, including the increase of the potential net photosynthesis in C₃ plants (Drake *et al.*, 1997), so enhancing yield over short-term exposures (Oliveira *et al.*, 2010). However, photosynthesis acclimation to elevated CO₂ over long-term experiments, can cause a reduction in potential growth (Erice *et al.*, 2006a; Ainsworth and Rogers, 2007; Aranjuelo *et al.*, 2009). This phenomenon often known as “photosynthetic acclimation (down-regulation)” is due to a metabolic limitation usually attributable to reduced carboxylation activity and/or a reduced amount of Rubisco (Ribulose-1,5-bisphosphate carboxylase/oxygenase) at elevated CO₂ (Urban, 2003; Aranjuelo *et al.*, 2005b).

Nitrogen (N) can limit the responses to elevated CO₂ in plants, and therefore “N availability factor” should always be considered in all the studies about effects of the elevated CO₂ on plants (Sanz-Sáez *et al.*, 2010). Accelerated alfalfa plant growth under elevated CO₂ (Erice *et al.*, 2007) increases the demand for nutrients, and in a soil with low N availability as in the Mediterranean region; the N will usually be a growth-limiting nutrient (Lüscher *et al.*, 1998; Lüscher *et al.*, 2000). This fact may lead to lower photosynthesis and so yield reduction (Luo *et al.*, 2004; Peterson *et al.*, 1999).

Alfalfa plant (*Medicago sativa* L.) is one of the most used legume forage crops for ecological (improvement of soil fertility and decreases the risk of soil and water pollution due to the lower requirement of fertilizers) and economical reasons (high quality forage and lower need of fertilizers) in the world. Alfalfa plants can establish symbiosis with N₂-fixing bacteria

Sinorhizobium meliloti allowing them to obtain their N requirements from atmospheric N₂ fixation in nodules.

The aim of the project was to study the response of two varieties (Aragón and Europe) of nodulated alfalfa (*Medicago sativa* L.) to the increase in atmospheric CO₂ concentration (ambient CO₂, around 400 µmol mol⁻¹ and elevated CO₂, 700 µmol mol⁻¹) and fertilized with 0 mM (0N) or 15 mM (15N) NH₄⁺NO₃.

One-month-old alfalfa plants inoculated with *S. meliloti* strain 102F78, were fertilized (15N) or not (0N) with 15 mM NH₄⁺NO₃ and exposed to ambient (400 µmol mol⁻¹) or elevated CO₂ (700 µmol mol⁻¹) during 1 month in the greenhouses. During the month growing at different CO₂ concentrations, three harvests were performed at 37, 52 and 58 days old plants. Growth measurements, leaf gas exchange (photosynthesis and associated parameters), nodule activity (apparent nitrogenase activity, ANA) and leaf and nodule biochemical analyses were performed. Non destructive measurements of gas exchange were additionally taken, when plants were 36, 40, 45, 47 and 54 days old. The experiment has been repeated two times: the first one from october to november 2011, and the second one from february to march 2012. Since the results in both experiments were almost the same, in this thesis work we decided to present the results of the first experiment.

With the data collected in the experiment we prepared the two chapters of the master thesis. The first chapter includes plant growth parameters, leaf gas exchange measurements and finally several leaf biochemical analyses. The second chapter is dedicated to the nodule activity, including biochemical analyses of the nodule plant and bacterial fractions.

The increase of CO₂ stimulated the production of biomass (total dry matter) in the variety Aragón and especially in the treatment of 15 mM of NH₄⁺NO₃. The variety Europe did not show a significant effect of the applied treatments on the production of total biomass. The leaves were the organs most sensitive to the effect of the increase of CO₂, in such a way that the effect of the high CO₂ was observed in both varieties of alfalfa. The rest of the growth parameters recorded did not show a noticeable response to the elevated CO₂. Leaf gas exchange parameters measured at CO₂ growth conditions (ambient treatments at 400 µmol mol⁻¹ and elevated CO₂ ones at 700 µmol mol⁻¹), shown

a plant age decline tendency in net photosynthetic rates in both varieties (Aragón and Europe). According to the net photosynthetic data it can be suggested a photosynthetic acclimation tendency to the elevated CO₂, especially for the 47 days old-plant measurements. However, any of the several biochemical parameters measured in the leaves, as Rubisco *in vitro* activity, concentrations of large (RbcLs) and small (RbcSs) subunits of Rubisco, total soluble sugars and proteins, and C/N ratio, confirmed the presence of photosynthetic acclimation. Only leaf starch accumulation and high C/N ratio induced by the elevated CO₂ may support the acclimation phenomenon especially in Aragón variety. High CO₂ reduced stomatal conductance and transpiration rates. However, even if the stomatal conductance was lower in elevated CO₂ conditions, the leaf intercellular concentration of CO₂ in these plants was much higher than in ambient CO₂ treatments (500 µmol mol⁻¹ of CO₂ in elevated CO₂ treated plants *versus* values lower than 300 µmol mol⁻¹).

With respect to nodule activity, it should be noted the high inhibition of the nodule development induced by 15 mM nitrogen treatment in nodulated alfalfa plants in both varieties. Therefore, the apparent nitrogenase activity (ANA) measurements, as well as the enzyme activities in the nodule and bacterial fractions were only assayed in 0 mM NH₄⁺NO₃ treatments. The ANA values were higher in Aragón than in Europe, but not effect of the elevated CO₂ was observed in any of both varieties. Total soluble sugars and proteins in the nodule plant fractions were not affected by treatments. The nodule starch concentrations in Aragón were always higher than in Europe. Enzymes activities as malate dehydrogenase (MDH), isocitrate deshydrogenase (ICDH), phosphoenolpyruvate carboxylase (PEPC) and glutamate-oxaloacetate transaminase (GOT), were not affected by the elevated CO₂. In the nodule bacterial fraction, the soluble proteins and malate dehydrogenase activity were not affected by the elevated CO₂ treatment. By the contrary, the isocitrate dehydrogenase (ICDH) in the nodule bacterial fraction increased in Aragón and decreased in Europe. The lower ANA rates measured in Europe variety can be associated with the significantly lower plant fraction starch and total soluble protein levels in bacterial fraction of its nodules.

From the study we can conclude that the response of the production of total biomass to the high atmospheric CO₂ was different in both varieties of

alfalfa (Aragon and Europe). The variety Aragon was more sensitive to the increase in CO₂ in both treatments of nitrogen applied (0 and 15 mM), although the stimulatory effect in the production of biomass was significantly higher in 15 mM of NH₄⁺NO₃. On the contrary, the variety Europe proved to be more insensitive to the increase of CO₂ in both nitrogen conditions. Even if the analysis of the photosynthetic rates suggest an acclimation process (down regulation) induced by the elevated CO₂, however all the biochemical parameters measured in leaves did not ratify the acclimation. The 15 mM NH₄⁺NO₃ treatment inhibits the development of new nodules. Any of the parameters measured in nodules did not show a noticeable response to the elevated CO₂ treatment. Alfalfa nodules of Aragón variety showed more efficiency fixing N₂ than those of Europe, which may be associated with the higher levels of starch in nodule plant fraction and total soluble proteins in the bacterial fraction of Aragón nodules.

Keywords: acclimation, climate change, carbon dioxide (elevated CO₂), photosynthesis, N₂-fixation, *Medicago sativa* (alfalfa), nodule metabolism, nitrogen, *Sinorhizobium meliloti*.

Résumé

L'augmentation du taux du dioxyde de carbone (CO₂) atmosphérique est un contributeur majeur à l'effet de serre. Ainsi, il est l'un des inducteurs principaux du changement climatique. Le groupe d'experts intergouvernemental étudiant le changement climatique (IPCC), prévoit que l'augmentation de la concentration du CO₂ dans l'atmosphère sera renforcée de 375 à 700 $\mu\text{mol mol}^{-1}$ CO₂ dans les 100 prochaines années, conduisant également à une augmentation moyenne de 4°C de la température atmosphérique (IPCC, 2007). Les activités humaines ne touchent pas seulement la concentration du CO₂, elles modifient aussi le cycle global de l'azote (N) en augmentant les apports des formes fixes d'azote, à cause de l'utilisation intensive d'engrais chimiques (Vitousek *et al.*, 1997).

Il est connu qu'une concentration élevée du CO₂ dans l'atmosphère affecte différents processus physiologiques chez les plantes, et provoque une augmentation du potentiel net de la photosynthèse dans les plantes C₃ (Drake *et al.*, 1997), augmentant ainsi le rendement au cours de des expositions à court terme (Oliveira *et al.*, 2010). Toutefois, des expériences montrent que la photosynthèse caractérisée par des concentrations élevées en CO₂ provoque à long terme une réduction du potentiel de la croissance (Erice *et al.*, 2006a; Ainsworth et Rogers, 2007; Aranjuelo *et al.*, 2009). Ce phénomène souvent appelé « acclimatation photosynthétique (régulation à la baisse) » est dû à une limitation du métabolisme, qui s'attribue généralement à une réduction de l'activité de carboxylation et / ou une quantité réduite de Rubisco (Ribulose-1, 5-diphosphate carboxylase/oxygénase) à des taux élevés de CO₂ (Urban, 2003; Aranjuelo *et al.*, 2005 b).

L'azote (N) peut limiter la réponse des plantes au CO₂ élevé, et par conséquent "la disponibilité de N" doit être toujours considéré dans toutes les études des effets du CO₂ aux plantes (Sanz-Sáez *et al.*, 2010). La croissance accélérée de la luzerne dans des conditions de CO₂ élevé fait que la demande de nutriments augmente, et dans un sol avec une basse disponibilité de N comme dans la région Méditerranéenne, le N serait un nutriment limitant de la croissance (Lüscher *et al.*, 1998 ; Lüscher *et al.*, 2000). Ce fait pourrait réduire la photosynthèse et par conséquent diminuer le rendement végétal (Luo *et al.*, 2004; Peterson *et al.*, 1999).

La luzerne (*Medicago sativa* L.) est une des plantes légumineuses fourragères les plus cultivées, pour des raisons écologiques (amélioration de la fertilité du sol et une moindre contamination du sol et d'eau grâce à une moindre nécessité d'engrais) comme économiques (une haute qualité du fourrage et un moindre usage d'engrais) dans le monde. La luzerne peut établir une symbiose avec des bactéries fixatrices du N₂ -*Sinorhizobium meliloti*-, ça leur permet d'obtenir leurs besoins en azote de la fixation d'azote dans les nodules.

L'objectif du projet était d'étudier la réponse de plantes nodulées de luzerne (*Medicago sativa* L.) des variétés Aragon et Europe à l'augmentation du CO₂ atmosphérique (ambient CO₂, environ 400 µmol mol⁻¹, au élevé CO₂ de 700 µmol mol⁻¹) et fertilisé avec 0 mM (0N) ou 15 mM (15N) de NH₄⁺NO₃.

Plantes de luzerne nodulées, âgées d'un mois, et inoculées avec la souche *S. meliloti* 102F78, supplémenté (15N) ou non (0N) avec une solution 15 mM de NH₄⁺NO₃, ils ont crû en présence des taux de CO₂: ambient (environ 400 µmol mol⁻¹) ou élevé (700 µmol mol⁻¹) pendant un mois dans des serres. Pendant le mois en croissance dans ces conditions, trois récoltes ont été réalisées quand les plantes avaient 37, 52 et 58 jours d'âge. La croissance, l'échange gazeux dans les feuilles (photosynthèse et paramètres associés), l'activité nodulaire (l'activité apparente de la nitrogénase, ANA), et des analyses biochimiques dans les feuilles et dans les nodules des plantes ont été réalisées dans chaque récolte. Étant donné que les mesures d'échange gazeux n'étaient pas destructives, des mesures additionnelles aux trois récoltes mentionnées ont été réalisées, aux 36, 40, 45, 47 et 54 jours d'âge des plantes. L'expérience a été réalisée deux fois: l'une entre le mois d'octobre et novembre 2011, et la seconde fois entre février et mars 2012. Les résultats dans les deux expériences ont été très similaires, par ce que l'on a décidé présenter les résultats du premier des expériences.

Avec les paramètres mesurés on a pu élaborer deux chapitres de la thèse. Le premier fait référence aux paramètres de croissance de la plante, à l'échange gazeux et les diverses analyses biochimiques réalisées dans les feuilles. Le deuxième chapitre se concentre sur l'activité nodulaire et sur les analyses biochimiques des fractions végétale et bactérienne des nodules.

L'augmentation de CO₂ a stimulé la production de biomasse (la matière sèche totale) dans la variété l'Aragon et d'une forme spéciale dans le traitement de 15 mM de NH₄⁺NO₃. La variété Europe n'a pas montré d'effet significatif des traitements appliqués dans la production de biomasse totale. Les feuilles ont été les organes les plus sensibles à l'effet de l'augmentation de CO₂, tellement que l'effet du CO₂ élevé a été observé dans les deux variétés de luzerne. Le reste de paramètres de croissance mesurés, ils n'ont pas montré de réponses très significatives. Les mesures d'échange gazeux réalisées à CO₂ de croissance (les traitements a CO₂ ambiant à 400 µmol mol⁻¹, et ceux de CO₂ élevé à 700 µmol mol⁻¹) ils ont montré que dans les deux variétés se produisait une descente des taxes de photosynthèse avec l'âge. On pourrait aussi deviner une acclimatation photosynthétique, spécialement à partir de 47 jours d'âge. Cependant, aucun des paramètres biochimiques mesurés dans les feuilles, ni l'activité extractable de la Rubisco *in vitro*, ni les concentrations des sous-unités majeure et petite de la Rubisco (RbcL et RbcS), ni les niveaux solubles de sucres ou des protéines, ainsi que la relation C/N, ils ont fortifié la dite acclimatation photosynthétique. Seulement l'accumulation d'amidon et de la relation C/N induite par le CO₂ élevé a appuyé la possible acclimatation dans la variété Aragón. Le CO₂ élevé a réduit la conductance stomatique et les taxes de transpiration spécifique. Cependant, et malgré les moindres conductances stomatique induites par le CO₂ élevé, dans les feuilles les concentrations intercellulaires de CO₂ ont été très supérieures aux plantes élevé dans de haute concentration de CO₂ (des valeurs moyennes de 500 µmol mol⁻¹ en face de valeurs inférieur à 300 µmol mol⁻¹ dans les traitements de CO₂ ambiant).

En ce qui concerne l'activité nodulaire, remarquer la forte inhibition du développement nodulaire induit par le traitement de 15 mM aux plantes nodulées de luzerne des deux variétés. Par cela, les mesures de l'activité apparente de la nitrogénase (ANA) et les activités enzymatiques des fractions végétal et bactérienne ont été seulement réalisées aux plantes des traitements de 0 mM de NH₄⁺NO₃. Les activités ANA ont été très supérieures dans la variété Aragon que dans Europe, et l'augmentation de CO₂ n'a pas eu d'effet dans aucune de deux variétés. Les sucres et les protéines solubles totales et l'amidon de la fraction végétale du nodule ne se sont pas trouvés très affectés par les

traitements. La concentration d'amidon des nodules de la variété Aragon a été significativement supérieures que celle de la variété Europe. Les activités enzymatiques malate déshydrogénase (MDH), isocitrate déshydrogénase (ICDH), phosphoénolpyruvate carboxylase (PEPC) et glutamate-oxaloacétate-transaminase (GOT), ne se sont pas trouvés affectés par le CO₂ élevé. De la même façon, la fraction bactérienne du nodule, les protéines solubles et l'activité malate déshydrogénase (MDH) ne se sont pas trouvées affectées par le traitement de CO₂ élevé. Par contre l'activité isocitrate déshydrogénase a augmenté à Aragon et a diminué à Europe. Les taxes inférieurs d'activité apparente de la nitrogénase (ANA), mesurées dans la variété Europe, se pourraient associées aux niveaux significativement plus bas de amidon dans la fraction végétale et des protéines solubles dans la fraction bactérienne de leur nodules.

De l'étude on peu conclure que la réponse de la production de biomasse totale des deux variétés de luzerne (l'Aragon et Europe) à l'augmentation de CO₂ a été différent. La variété Aragon a été plus sensible à l'augmentation de CO₂ atmosphérique dans les deux traitements d'azoté appliqués (0 et 15 mM), bien que l'effet stimulant de la production de biomasse a été plus évidant dans 15 mM de NH₄⁺NO₃. Au contraire, la variété Europe s'est montrée plus insensible à l'augmentation de CO₂ dans les deux conditions d'azote. Bien que les taxes photosynthétiques laissent deviner une acclimatation photosynthétique au CO₂ élevé, cependant les paramètres biochimiques mesurés dans des feuilles ne l'ont pas appuyé. Le traitement de 15 mM de NH₄⁺NO₃ il a empêché la formation de nouveaux nodules. Les nodules n'ont pas montré des réponses très remarquables à l'augmentation de CO₂ dans les paramètres analysés. On a observé que les nodules des plantes de Aragon ont été plus efficaces en fixant N₂ que les plantes de Europe, ce qui pourrait être associé aux plus grands niveaux de amidon dans la fraction végétale et des protéines solubles totales dans la fraction bactérienne des nodules des plantes de Aragón.

Mots clés : acclimatation, changement climatique, dioxyde de carbone (élevé de CO₂), photosynthèse, fixation d'azote, *Medicago sativa* (la luzerne), métabolisme de nodule, azote, *Sinorhizobium meliloti*.

Abbreviations

AA0N: Nodulated alfalfa variety Aragon grown with 0 mM NH_4^+NO_3 at ambient CO_2

AA15N: Nodulated alfalfa variety Aragon grown with 15 mM NH_4^+NO_3 at ambient CO_2

AE0N: Nodulated alfalfa variety Aragon grown with 0 mM NH_4^+NO_3 at elevated CO_2

AE15N: Nodulated alfalfa variety Aragon grown with 15 mM NH_4^+NO_3 at elevated CO_2

AM: arbuscular mycorrhiza

ANA: apparent nitrogenase activity

ANOVA: analysis of variance

A_{growth}: net photosynthesis measured at growth CO_2 concentration

A₄₀₀: net photosynthesis measured at 400 $\mu\text{mol mol}^{-1}$ CO_2 concentration

A₇₀₀: net photosynthesis measured at 700 $\mu\text{mol mol}^{-1}$ CO_2 concentration

BSA: Bovine serum albumin

$\delta^{13}\text{C}$: C isotope composition

C_i: intercellular CO_2 concentration or mesophyll CO_2 concentration

CO_2A : Plants grown at ambient CO_2 concentration (around 400 $\mu\text{mol mol}^{-1}$)

CO_2E : Plants grown at elevated CO_2 concentration (700 $\mu\text{mol mol}^{-1}$)

C/N: Carbon to nitrogen ratio

DM: dry matter or dry mass

E: specific transpiration

EA0N: Nodulated alfalfa variety Europe grown with 0 mM NH_4^+NO_3 at ambient CO_2

EA15N: Nodulated alfalfa variety Europe grown with 15 mM NH_4^+NO_3 at ambient CO_2

EE0N: Nodulated alfalfa variety Europe grown with 0 mM NH_4^+NO_3 at elevated CO_2

EE15N: Nodulated alfalfa variety Europe n grown with 15 mM NH_4^+NO_3 at elevated CO_2

FACE: free air CO_2 enrichment

FM: fresh mass

g_s: stomatal conductance

GS/GOGAT: glutamine synthetase/glutamate synthase

GOT: glutamate oxaloacetate transaminase

ICDH: isocitrate dehydrogenase

IPCC: intergovernmental panel on climate change

IUCN: international union for conservation of nature

INSPV: Instituto Nacional de Semillas y Plantas de Vivero

MDH: malate dehydrogenase

NADH: reduced form of nicotinamide adenine dinucleotide (NAD^+)

NADPH: reduced form of nicotinamide adenine dinucleotide phosphate (NADP^+)

NH_4^+NO_3 : ammonium nitrate

PBM: peribacteroidal membranes

PCM: parallel climate model

PEPC: Phosphoenolpyruvate carboxylase

PPFD: photosynthetic photon flux density

PVPP: polyvinyl polypyrrolidone
RbcL: Rubisco large subunit
RbcS: Rubisco small subunit
Rubisco: Ribulose-1, 5-bisphosphate carboxylase/oxygenase
SDS-PAGE: sodium dodecyl sulfate polyacrylamide gel electrophoresis
SLA: specific leaf area
S/L: steam/leaf ratio
TOM: total organic matter
USGCRP: United States global change research program
VSP: vegetative storage proteins
* ***P*<0.05:** significant difference at 5%
* ***P*<0.01:** significant difference at 1%
* ***P*<0.001:** significant difference at 0.1%



1. General Introduction

1.1. General Aspects of Climate Change

Our climate is changing due to the observed increases in human activities, particularly burning fossil fuel, that have increased atmospheric carbon dioxide (CO₂) concentrations over the 20th century. At the same time that CO₂ concentration in the atmosphere increased, the average global air and ocean temperature increased too, and as a consequence a widespread melting of snows and ice had raised the global sea levels.

Continued CO₂ emissions are expected to induce global climate change. According to the Intergovernmental Panel on Climate Change (IPCC), the concentration of CO₂ has been increasing since 1750, when it was 280 $\mu\text{mol mol}^{-1}$ and nowadays, it has already reached 392 $\mu\text{mol mol}^{-1}$, and continues to grow up at a rate of 1.9 $\mu\text{mol mol}^{-1}$ per year (IPCC, 2007). According to some models, land ecosystems can sequester carbon fast enough to help to counteract CO₂ emissions. Models featured in the IPCC suggest that increasing atmospheric CO₂ alone could cause 350 to 890 Pg of carbon (1 Pg = 1015 g) to accumulate in the terrestrial biosphere by 2100 (Figure 1).

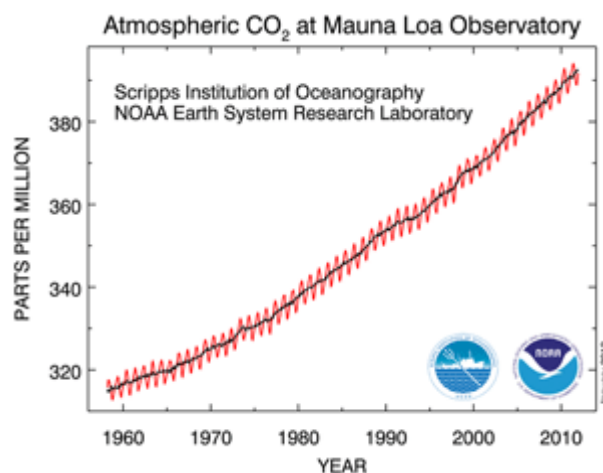


Figure 1. Evolution of the atmospheric concentration of CO₂ in the last 50 years. Measurements done in Mauna Loa's observatory. **Source:** NOAA (2012).

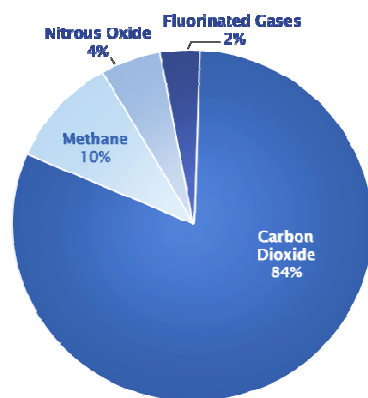


Figure 2. Emissions of main greenhouse gases and their concentrations in the atmosphere.
Source: U.S Greenhouse Gas Emissions (2010).

The Iberian Peninsula is not an exception for this impact of climate change, and the increase of temperature (1.2-1.5°C) and the rainfall decrease (Spanish National Institute of Meteorology) confirm the mentioned changes that targets with especial impact vegetation. Some of these impacts on vegetation are: changes in species distribution, tree vulnerability and mortality, and effects in crops. Therefore, the climate change associated with CO₂ and temperature increases, is one of the major ecological, social and economic problems of our planet (EU, 2006), and an important challenge to the society.

Carbon dioxide is considered the greenhouse gas that has more effect on the atmosphere temperature increase, through the phenomenon known as global warming. CO₂ is along with other greenhouse gases such as methane (CH₄), nitrous oxide (N₂O), hydrochlorofluorocarbon-22 (HCFC-22), fluorinated gases, etc. (Figure 2 and Figure 3), the gases that could cause a 4°C rise in the average global temperature by the end of this century (IPCC, 2007).

Each of these gases can remain in the atmosphere for different amounts of time, at different time scales ranging from years to millennia. For CH₄, N₂O, and other trace gases such as HCFC-22 (a refrigerant fluid) the lifetimes can be reasonably determined. For example, CH₄ lifetime is about 12 years, N₂O about 110 years and HCFC-22 about 12 years, but a lifetime for CO₂ cannot be defined. Besides, because of slow removal processes, atmospheric CO₂ will continue to increase in the long term even if its emission is substantially

reduced from present levels. So, greenhouse gases are slowing or preventing the loss of heat to space. In this way, they are acting like a blanket and making Earth warmer than it would otherwise be. This process is commonly known as the “greenhouse effect”.

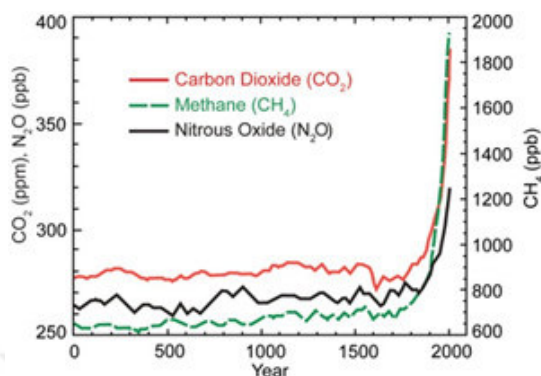


Figure 3. The increase in greenhouse gases concentrations in the atmosphere over the last 2,000 years. Concentration units are parts per million (ppm) or parts per billion (ppb), indicating the number of molecules of the greenhouse gas per million or billion molecules of air. **Source:** USGCRP (2009).

Many factors can cause changes in Earth’s energy balance *via* alteration in the greenhouse effect. When the sun’s energy is reflected back into space, Earth avoids warming. If we consider only the effects of the natural processes, it might not explain the current and past global warming. It is also needed to account for the greenhouse gases emitted by humans, in order to explain this warming (U.S Greenhouse Gas Emissions, 2010).

Besides natural causes, human activities release large amounts of carbon to the atmosphere, causing CO₂ concentrations in the atmosphere to rise. Looking for the evolution of the atmospheric CO₂ concentration and air temperature we can found a very close relationship (Figure 4). Considering the rise of atmospheric CO₂ year by year according to Figure 4, it appears to be able to predict changing CO₂ and temperature for next 100 years (Figure 5).

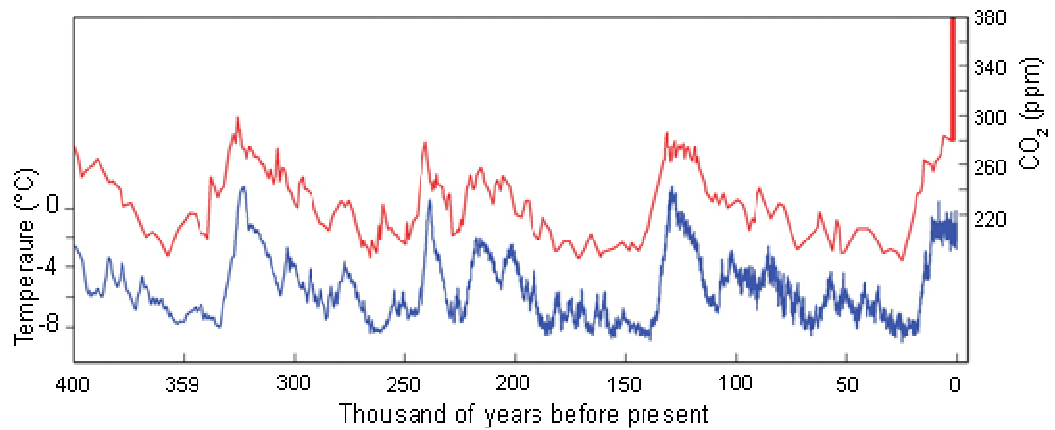


Figure 4. Relationship between atmospheric CO₂ (upper line) and temperature (lower line) during years. *Source: IPCC (2001).*

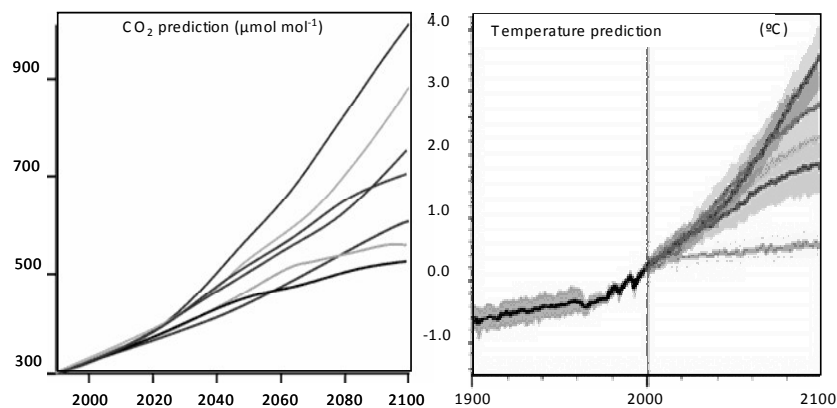


Figure 5. Prediction of atmospheric CO₂ and temperature for next 100 years. *Source: IPCC (2001).*

1.2. Climate Change and the Effects on Plants

Solely due the impact of climate change on leguminous plants in Madagascar Island, experts predicts that by the year 2100, 83% of plant species will decline in their present range, 19-35% of species will be considered threatened according to IUCN Red List categories, and 2-3% of species are predicted to become extinct (Rivers *et al.*, 2010).

Plants interact with the environment directly by exchanging water and energy and indirectly by temperature and moisture, etc. The most important role of plants is the production of oxygen and the absorption of carbon dioxide from the atmosphere during the process of photosynthesis, which is the basic process for plant life. Environmental conditions, such as light intensity, temperature, water availability and wind strength, etc., affect plant growth. It is expected that all the changes associated with climate change (increase in atmospheric CO₂ concentration, increase in temperature and changes in rainfall pattern, etc.) will affect plants grow.

Higher temperatures cause heat stress in plants. Plants grow less and produce fewer yields. Increased temperature may be beneficial in areas which are very cold at present.

Water availability is another environmental factor that directly and indirectly affects plant growth. Climate change may also results in flooding as a consequence of excessive rainfall. Waterlogged soil causes plant roots to rot and heavy rainfall damages tender young plants. However, more water availability without flooding can be beneficial in very dry areas, allowing to obtain higher yields and to increase the number of crops that can be cultivated in the area. By the contrary, as expected in many areas of the world affected by climate change, the reduced rainfall will negatively affect crop production. Drought is one of the worst factors limiting plant cultivation and production in the world.

Changes in our climate are happening more quickly now than they have ever done before in the geological past. Plants have to adapt to new climate conditions more rapidly than they have ever had to do so before (ESPERE, 2012).

Global impacts are positive or beneficial under conditions of the Parallel Climate Model (PCM) which comes from the National Center Atmospheric Research (Washington *et al.*, 2000), because it is a very mild climate change scenario (Mendelsohn *et al.*, 2006). Some repots demonstrates some positive

impacts of increased CO₂ in both agriculture and environment. In agriculture impacts such as increased growing season in Greenland (Nyegaard, 2007) and in productivity of sour orange trees (Kimball *et al.*, 2007) are expected. Other effects associated to climate change are the increase in vegetation activity in high northern latitudes (Zhou *et al.*, 2001), in plankton biomass in the North Pacific Subtropical Gyre (Corno *et al.*, 2005), in forest growth (McMahon *et al.*, 2010) and in Arctic tundra plant reproduction (Klady *et al.*, 2011).

However, there are several researches about direct or indirect negative impact of increased CO₂ such as decreased water supply (McCabe and Wolock., 2007; Cai and Cowan., 2008; Solomon *et al.*, 2009), or warmer night time minimum temperature declining rice yields (Peng *et al.*, 2004; Tao *et al.*, 2008). Also negative effect are proposed on global phytoplankton (Boyce *et al.*, 2010) and global net primary production - the amount of carbon absorbed by plants (Zhao *et al.*, 2010), or increased Western United States wildfire activity associated with higher temperatures and earlier spring snowmelt (Westerling *et al.*, 2006) and higher mortality rates of healthy trees in Western U.S. forest (Pennisi *et al.*, 2009). Other negative effect includes encroachment of shrubs into grasslands, rendering rangeland unsuitable for domestic livestock grazing (Morgan *et al.*, 2007) and release of CO₂ from rainforests as these regions become drier (Saleska *et al.*, 2009).

Nevertheless, there are more specific researches on plants under controlled environments to measure real sole effect of CO₂ or CO₂ interactions with other environmental factors such as temperature, drought, UV radiation, nutrition, etc., than researches under natural conditions. The continuous increase of atmospheric CO₂ concentration has led to extensive research over last two decades, during which more than 1,400 scientific papers describing impacts of elevated CO₂ on photosynthesis have been published. However, the degree of response is very variable, depending on species, growing conditions, mineral nutrition and duration of CO₂ enrichment (Urban, 2003). In the other hand, most experiments analyzing the climate change effect on plant growth have considered the effect of CO₂ or CO₂ × temperature under optimal water

conditions (Aranjuelo, 2004), and less interest has been pay to the scarce water conditions.

1.3. Alfalfa (*Medicago sativa*) a Model to Study the Climate Change Effects on Plants

Alfalfa (*Medicago sativa*) is one of the most important cropping legume forage crops in the world. That is why called as the “Queen of Forages” in all over the world. It originated around east of Turkey, west of Iran, around Hazar Sea and north of Kyrgyzstan (Tarman, 1954). There are 83 wild *Medicago* taxa and 6 *Medicago sativa* subspecies in the world. Thirty of them are found in Turkey (Başbağ *et al.*, 2009). It is an autotetraploid, allogamous, heterozygous species, perennial, important forage crop for ecological and economical reasons, it establishes a symbiotic relationship with a N₂-fixing bacterium (*Sinorhizobium meliloti*) providing an extra source of N for the plant.

Alfalfa as the major cultivated legume forage crop in the all over the world has a very important mission for animal nutrition. For this reason, alfalfa is cultivated on approximately 15.2 million ha in the world and, it has an average world forage yield of 28,664 kg ha⁻¹ (Başbağ *et al.*, 2009). Alfalfa production has also a very important economical impact for both Spain and Turkey countries. During the years 2002-2005, Turkey produced on average 1905 tonnes of legumes on 4,874 ha and the main legume was alfalfa (EU, Screening Repot Turkey, 2006).

Alfalfa is the third cultivated and first harvestable forage producing crop in Spain. The main producing area of alfalfa is in north-eastern Spain in a zone called Valle del Ebro. Alfalfa is also an essential pillar of forage secondary industry in the cultivated area. This industry dehydrated the production of alfalfa, approximately 173,000 ha in Spain. The seventy six percent of this industry is located in Valle del Ebro, although in recent years it has become an important production in Castilla-La Mancha, and more recently it has spread throughout Castille and Leon. Spain is currently the leader producing

dehydrated alfalfa with two million tonnes per year in the European Union (Delgado *et al.*, 2005).

Nitrogen is an essential plant nutrient that limits plant growth. In order to obtain sufficient nitrogen, phosphorus and water, etc. many plants form a symbiotic interaction with arbuscular mycorrhizal fungi (AM) (Bonfante and Anca, 2009). Many legumes are, in addition to arbuscular mycorrhizal symbiosis, capable of establishing symbiosis with rhizobial bacteria to obtain plant's nitrogen requirement. This is the case of alfalfa; in fact frequently alfalfa plants grown in the fields have double symbiosis in their roots, especially when the inputs of fertilizers are not high. Alfalfa plants are frequently used in rotation with other crops (wheat, corn, etc.) as a way to improve the soil fertility.

Medicago sativa L. is also one of the most popular model plant for investigations on the symbiosis with nitrogen fixing rhizobia (Rhizobia is a generic term for all bacteria that are able to form symbiotic interactions with legumes and they are classified in four genera: *Rhizobium*, *Bradyrhizobium*, *Azorhizobium*, and *Sinorhizobium*.), which infect roots, modifying root morphology into a nodular structure where N₂ fixation takes place (Estaún *et al.*, 2007).

Alfalfa is also a very interesting plant to study the relationship between C and N cycle interactions. The ability of the plant to fix simultaneously CO₂ in the leaves and N₂ in the roots, give to the alfalfa symbiosis an especial interest. In fact, several studies of the Plant Biology Department of the University of Navarra, where the present master thesis has been developed, have investigated the effect of environmental constraints as water stress, salinity, atmospheric humidity, etc., on single (*Medicago sativa*-*Sinorhizobium* or *Medicago sativa*-Mycorrhiza fungi) and double symbiotic alfalfa plant (*Medicago sativa*-*Sinorhizobium*-Mycorrhiza fungi). Most of the studies have been focused on growth and the response of photosynthesis and nodule metabolisms (see Web site <http://www.unav.es/departamento/bvegetal/publis>).

As reported in the first two sections of this general introduction (General Aspects of Climate Change and Climate Change and the Effects on Plants), climate change is real and important subject in our days. The effects of the climate change and the response of the plants to the modification of the climatic conditions (precipitation, precipitation distribution, drought episodes, temperature increase, etc.) are aspects of high interest to be investigated.

As also reported previously in this introduction, when plants grow under elevated CO₂, it is expected that photosynthesis rates will be enhanced, because photosynthetic rates, at least in C₃ plants, are limited at the actual atmospheric CO₂ concentration (around 400 $\mu\text{mol mol}^{-1}$). Therefore, it is expected that an increase in the CO₂ concentration will enhance photosynthesis and probably growth. However, the interaction with other environmental factors such as temperature, drought, etc., may change the expected response.

In fact, the Department of Plant Biology of the University of Navarra, has developed since 1995 until nowadays an intense research work to investigate the response of nodulated alfalfa to climate change scenarios. The most relevant results of this research are summarized in the next paragraphs.

The experiments have been done in growth chambers to investigate the individual climate change factors effects (CO₂, temperature, drought, humidity, etc.), as well as in greenhouses and temperature gradient greenhouses to study the interaction between these factors (CO₂-Temperature, CO₂-Drought, Temperature-Drought, and CO₂-Temperature-Drought) in semi natural conditions. At present, the more realistic studies about climate change, should be carried out in FACEs (Free Air CO₂ Enrichment). However, one of the most important and determining factor altering the plant response to the CO₂ enrichment is the temperature, and this interaction (CO₂-Temperature) can be properly investigated only in temperature gradient greenhouses.

Several studies in the department have demonstrated in different plant growth facilities (growth chambers, greenhouses and in temperature gradient

greenhouse) that elevated CO₂ enhances photosynthesis and growth (hay production) in nodulated alfalfa. However, photosynthesis and growth enhancements are not related with a plant water status improvement (De Luis, 2000).

Experimental results, showed that nodulated alfalfa is negatively affected by drought imposed in different ways and intensities (moderate, severe, short, progressive, maintained, periodic, etc.). Photosynthesis and, especially, N₂ fixation activity were seriously affected. However, when elevated CO₂ was applied, the plants suffered less. Therefore, it was concluded that elevated CO₂ associated with climate change help the nodulated alfalfa plants to tolerate drought better. Once again, the beneficial effect of the CO₂ was not associated with an improvement in plants water status (De Luis *et al.*, 1999 ; Aranjuelo *et al.*, 2005a).

Always, the response of nodulated alfalfa plants to an increase in atmospheric CO₂ is affected by the interaction with other environmental factors. One of the factors especially affecting the plant response to the CO₂ enrichment was temperature. High temperature enhances the positive effect of elevated CO₂ on plant biomass accumulation. This effect of temperature has been seen in most of the studies done with alfalfa during the first and the second cutting cycle (alfalfa regrowth) (Aranjuelo *et al.*, 2005a, 2005b, 2006; Erice, 2005; Erice *et al.*, 2006a, 2006b, 2007).

An additional result, also relevant to the climate change effect on nodulated alfalfa, is that elevated CO₂ and temperature compromise the quality of the alfalfa forage. Decline in protein content and simultaneous increase in fibres, reduced the digestibility of the alfalfa hay produced under these conditions (Sanz-Sáez *et al.*, 2012b). Photosynthesis acclimation has been frequently described in the elevated CO₂ experiments with nodulated alfalfa plants (Sanz-Sáez *et al.*, 2010; Erice *et al.*, 2006a; Aranjuelo *et al.*, 2005b). Photosynthesis acclimation or photosynthesis “down regulation”, limited or even inhibited the stimulatory effects of the CO₂ enrichment on biomass accumulation in many experiments. According to Sanz-Sáez *et al.*, (2010),

photosynthesis acclimation in nodulated alfalfa is partly due to the insufficient nitrogen supply from the nodules to the plants. When nodulated alfalfa was fertilized with NH_4^+NO_3 application (especially with 15 mM), photosynthesis acclimation was avoided, and therefore, the beneficial effect of elevated CO_2 was enhanced significantly. Efficiency of the *S. meliloti* strains used to inoculate alfalfa plants is also another factor altering the response of nodulated alfalfa to elevated CO_2 (Sanz-S  ez *et al.*, 2012a).

At present, one of the aspects that we would like to investigate is, if the variety or cultivar of alfalfa used in the study may affect the response of nodulated alfalfa to the proposed climate change scenarios. To study the subject, two commercial alfalfa varieties were chosen. One of the varieties will be Aragon, variety in which most of the already presented results were obtained. Aragon is an alfalfa variety mostly cultivated in continental climates (Continental variety). The other variety will be Europe, more typical from more humid climates (Oceanic variety). It will be interesting to investigate if morphology, physiology, etc., characteristics of these two varieties that made them more adapted or able to grow in their respective climates, can affect the response of alfalfa to a climate change scenario (CO_2 enriched atmosphere).

The study aims to answer the question about how the two mentioned varieties of alfalfa, adapted to grow in different climate conditions, respond to the increase in atmospheric CO_2 in the atmosphere. An additional treatment with plants supplied with inorganic N will be included. This additional treatment, will help us to know if the nitrogen supply by the nodule activity is limiting alfalfa growth under elevated CO_2 due to a photosynthesis acclimation, which may limit biomass production. The work also might evidence those characteristics of the plants that are more crucial to better respond to the increase in CO_2 concentration. Those data might allow to explore new varieties of alfalfa or to improve alfalfa with genetic tools, in order to ameliorate the alfalfa adaptation to climate change. Therefore, the objective of the proposed work, adjusts largely to the aims of the master, in which the student (Miss Tefide Kizildeniz) will present and defend the thesis entitle “*EFFECTS OF*

*CLIMATE CHANGE ON NODULATED ALFALFA PLANTS: PHYSIOLOGY,
BIOCHEMICAL AND MOLECULAR ANALYSES’.*



General Objectives

General objective of this project was to investigate the response of two varieties (Aragón and Europe) of nodulated alfalfa (*Medicago sativa* L.) to the increase in atmospheric CO₂ concentration (ambient CO₂, around 400 µmol mol⁻¹ and elevated CO₂, 700 µmol mol⁻¹) and fertilized with 0 mM (0N) or 15 mM (15N) NH₄⁺NO₃. In order to facilitate the presentation and the discussion of the master thesis manuscript, the general objective, has been divided into two subaims:

1. The response of growth, gas exchange and biochemical parameters measured in leaves of two varieties (Aragón and Europe) of nodulated alfalfa (*Medicago sativa* L.) grown under ambient or elevated CO₂ and fertilized with 0 mM (0N) or 15 mM (15N) NH₄⁺NO₃, corresponding with the content of the **Chapter I**.
2. The response of nodule dry mass, apparent nitrogenase activity (ANA) and biochemical parameters measured in nodules of two varieties (Aragón and Europe) of nodulated alfalfa (*Medicago sativa* L.) grown under ambient or elevated CO₂ and fertilized with 0 mM (0N) or 15 mM (15N) NH₄⁺NO₃, corresponding with the content of the **Chapter II**.



Chapter I

The response of growth, gas exchange and biochemical parameters measured in leaves of two varieties (Aragón and Europe) of nodulated alfalfa (*Medicago sativa* L.) grown under ambient or elevated CO₂ and fertilized with
0 mM or 15 mM NH₄⁺NO₃

Introduction

Broadly supported by experimental data is that, fertilized crops and pasture species have a greater photoassimilate availability at elevated CO₂ (Rogers and Ainsworth, 2006). Soybean (*Glycine max*) and clover (*Trifolium repens*) FACE experiments demonstrate stimulation of photosynthetic C uptake and leaf carbohydrate content at elevated CO₂ (Ainsworth *et al.*, 2003).

An increase in the relative contribution of symbiotically fixed N to total plant N at elevated CO₂ has been reported in a number of experiments (e.g. Schortemeyer *et al.*, 2002; Lee *et al.*, 2003). In clover, the relative contribution of symbiotically fixed N₂ at elevated CO₂ was critical factor in maintaining the C to N ratio in the ecosystem as a whole (Zanetti *et al.*, 1996). These experiments may provide evidence enough showing that legumes increase N₂ fixation at elevated CO₂ (Rogers *et al.*, 2009). On the other hand, N₂ fixation in fertilized agricultural environments, in contrast to N₂ fixation in natural ecosystems, is not consistently stimulated by elevated CO₂ (Van Groenigen *et al.*, 2006). Some studies reveal that stimulation of N₂ fixation by elevated CO₂ may be species specific (West *et al.*, 2005) or driven by the effects of elevated CO₂ on competition and abundance of legumes in the ecosystem (Reich *et al.*, 2006).

Alfalfa (*Medicago sativa* L.) is an important forage crop for ecological and economical reasons. It establishes a symbiotic relationship with N₂-fixing bacterium (*S. meliloti*) that reduces atmospheric nitrogen into ammonia that can be used by plant for providing an extra source of N. As mentioned above, N₂-fixing legumes often show a larger stimulation of growth and photosynthetic rates at elevated atmospheric CO₂ than species without this capability.

Alfalfa is also the main forage legume in the crop livestock systems of North America and many European countries. It produces the greatest amount of forage protein per unit area among the legumes. Moreover, alfalfa is an extremely adaptable plant that grows under a wide range of climate conditions from the equator to regions close to the Arctic Circle (Michaud *et al.*, 1988).

Alfalfa is cultivated on 61% of the national Spanish field. Alfalfa Aragón variety is the major cultivated alfalfa in the irrigated Spanish forage lands. In 1993, 849,700 kg of Aragón ecotype was cultivated and represented a 74.6% of all cultivated alfalfa varieties in Spain, while Victoria 11.8%, Resto 6.3%, Estival 3.0%, Capitana 2.6% and Tierra de Campos 1.7% (INSPV, 1994).

The importance of alfalfa Aragón variety or ecotype in the forage field production in Spain is well known for its wide adaptation to irrigated land in major part of Spanish agricultural regions. This high presence of Aragón variety, is due to his good level of production, retains its characteristics both in spring and autumn for cold resistance, high persistence, good regrowth capacity and a good sanitary level. Seed production of this ecotype is performed in a wide area on the middle of Valle del Ebro including provinces as Logroño, Navarra and Zaragoza, according classification of Spanish Alfalfas by Hidalgo (1965). Aragón is a cultivar originating from Spain that is classified as Mediterranean-type alfalfa.

There are many studies investigating the stress physiology of alfalfa species (e.g. water stress, drought, elevated CO₂). Li *et al.* (2011) reported two alfalfa varieties, Long-Dong and Algonquin, the former being a Chinese native cultivar and Algonquin a higher productive and more adapted cultivar to a wider cultural practices. Under severe drought conditions, Algonquin altered root morphology, reduced biomass production, and lower water dissipation, increasing water use efficiency of biomass production. However, these adaptive changes in plants depend on stress level, species, cultivars, interaction with other environmental factors, etc.

Erice *et al.* (2011) examined the water use efficiency, transpiration and net CO₂ exchange to progressive drought and subsequent recovery in four different alfalfa varieties: “Tafilalet” variety or ecotype from Morocco, “Tierra de Campos” originating from Spain that is classified as Mediterranean-type alfalfa, “Moapa” from the USA and derived from African genotypes, and “Europe” classified as Nordic-type alfalfa and adapted to oceanic climate. The results showed that Tafilalet was the most productive cultivar, but under severe drought conditions there was no difference in dry matter accumulation among cultivars.

As reported previously, there are many varieties of alfalfa adapted to different climatic conditions. Advantages to growth in specific climatic conditions among the different varieties of alfalfa could be due to physiological, biochemical and molecular characteristics. Previous studies revealed that nodulate alfalfa response to the CO₂ enrichment is affected by several environmental conditions (temperature, water and nutrient availability, humidity, *Sinorhizobium* infecting strain, etc.), and it could be possible that intraspecific differences amount alfalfa varieties or cultivars may also affect CO₂ response. Therefore, it will be interesting to investigate the response of photosynthesis and nodule metabolism of two varieties of alfalfa, with contrasting climatic growth conditions, to the elevated CO₂, one of the factors associated to the climate change. The alfalfa varieties chosen were Aragón, a more continental variety, and Europe, representing a more oceanic alfalfa variety (Crochemore *et al.*, 1998). Growth parameters will also be measured. Inorganic nitrogen application treatment (15 mM NH₄⁺NO₃) will be included to check the possible N supply limitation from the nodules, inducing photosynthesis acclimation and depression of plant biomass accumulation.

Materials and Methods

Plant Material

Seeds of two alfalfa cultivars *Medicago sativa* L. cv. Aragón and cv. Europe, were sterilized in a solution of NaClO (10%, w/v) and germinated in Petri dishes. Aragón is an ecotype from Spain and with resistance to cold. Also Aragón is well adapted to Mediterranean climate (Marqués *et al.*, 1999). In contrast, Europe is a Nordic-type alfalfa (Crochemore *et al.*, 1998) well adapted to an oceanic climate (Marqués *et al.*, 1999). One week-old seedlings were transferred into 2 L pots (4 plants per pot) containing a mixture of inert perlite and vermiculite (2/1, v/v). During the first month, all plants were inoculated three times with *S. meliloti* 102F78 strain isolated from North Carolina (USA). The inoculum was provided by The Nitragin[®] Co., Milwaukee, WI, USA. The bacteria grew on yeast mannitol agar medium at 25 °C for around 5 days, and the cultures in the mid-to-late exponential phase of growth were resuspended in 2% (w/v) sucrose (cell density: 10^9 cell ml⁻¹). For reliable nodulation, 3 ml of bacterial suspension was added to each plant. Inoculation was performed once a week during the first month, starting from the second week. During that period, plants were irrigated three times per week with Evans N-free solution (Evans, 1974). In order to avoid salt accumulation in the pots, all plants were also irrigated with distilled water once a week. Plants were grown at ambient CO₂ concentration (around 400 $\mu\text{mol mol}^{-1}$ CO₂), with a thermoperiod of 25 /15°C day/night, 60% relative humidity and 14 h (from 7.00 to 21.00) of photoperiod under natural daylight, supplemented with metal halide lamps (Powerstar[®] HQI[®] – TS, OSRAM 400W, Germany) providing a photosynthetic photon flux density (PPFD) of 400 $\mu\text{mol m}^{-2} \text{s}^{-1}$ (Figure 7).

Experimental Design

When inoculated alfalfa plants were 30 days old, plants were transferred into two greenhouses. Half of the plants continued growing at the same conditions as reported before during initial growing period (ambient CO₂ treatment, around 400 $\mu\text{mol mol}^{-1}$), and the other half of the plants grew at elevated CO₂ (elevated CO₂ treatment, 700 $\mu\text{mol mol}^{-1}$). The rest of the growth conditions (thermoperiod, photoperiod, relative humidity, etc.), were the same as in the initial growth period. In each of the

greenhouses (ambient and elevated CO₂ treatments), half of the plants, continued being irrigated with Evans N-free solution (0 mM treatment) (Evans, 1974), but the rest of the plants (half of the plants in each treatment) received 15 mM NH₄⁺NO₃ in Evans solution (15 mM treatment). The irrigation schedule was as follow: Monday and Friday with Evans nutrient solution (0 or 15 mM according to the treatment), and Wednesday with distilled water to avoid salt accumulation in the pots. The experiment was repeated twice, from october to november 2011, and from february to march 2012. Since the results in both replications were almost the same, the results and data presented in this thesis work correspond to the first experiment (october to november 2011).

Plants of the four treatments (A0N: Nodulated alfalfa grown with 0 mM NH₄⁺NO₃ at ambient CO₂; A15N: Nodulated alfalfa grown with 15 mM NH₄⁺NO₃ at ambient CO₂; E0N: Nodulated alfalfa grown with 0 mM NH₄⁺NO₃ at elevated CO₂; E15N: Nodulated alfalfa grown with 15 mM NH₄⁺NO₃ at elevated CO₂) for each variety (A: Aragón and E: Europe). They continue growing during an additional month until they become 60 days-old. During this treatment period in the greenhouses, three harvests were performed (when plants were 37th, 52nd and 58th days-old) (Figure 6). Therefore, there were 8 treatments, 3 repetitions, 3 harvests and 34 extra ones which gave a total of 106 plants (Table 1).

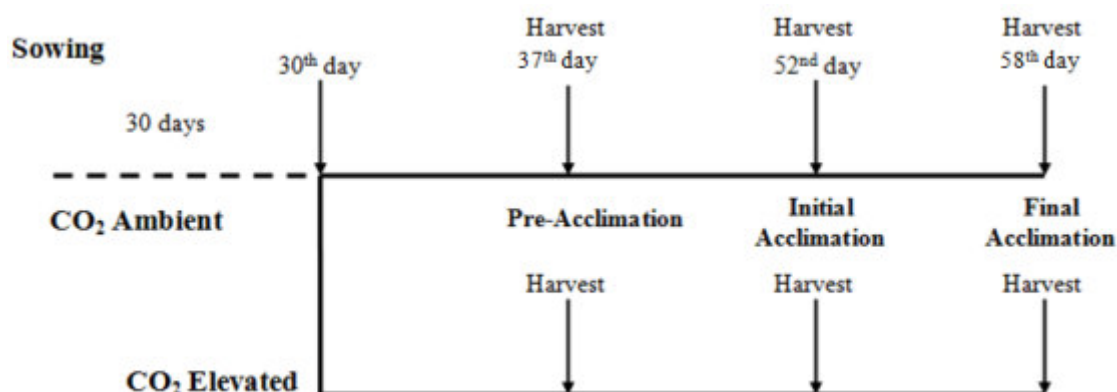


Figure 6. Scheme of experimental design

Ambient CO ₂ ~ 400 µmol mol ⁻¹				Elevated CO ₂ 700 µmol mol ⁻¹			
Variety	0 mM	15 mM	Total No. of Plants	Variety	0 mM	15 mM	Total No. of Plants
Europe	13	13	26	Europe	13	13	26
Aragon	13	13	26	Aragon	13	13	26
Total No. of Plants	26	26	52	Total No. of Plants	26	26	52

8 treatments x 3 repetition x 3 harvest = 72 + 34 extra = 106 plants

Table 1. Experimental treatments

Plant Growth Parameters

In each harvest (37th, 52nd and 58th days) plants were separated into leaves, stems, roots and nodules and all plant organs were weighted for obtaining fresh mass (FM). Dry mass (DM) of each organ was obtained after drying in oven at 80 °C for 48h. Leaf area was measured using an automatic leaf area meter (Li-3000, LICOR[®], NE, U.S.A.) (Figure 7). The specific leaf area (SLA) was calculated as leaf area/leaf dry matter. Shoot to leaf ratio (Shoot/Leaf Ratio), as alfalfa forage quality parameter, was calculated dividing the dry matter of the stem by the leaf dry matter.



Figure 7. Automatic leaf area meter (Li-3000, LICOR[®], NE, U.S.A.)



Figure 8. Aspect of the two varieties (Aragón and Europe) of nodulated alfalfa plants (*Medicago sativa* L.) grown under ambient CO₂ (Amb, around 400 $\mu\text{mol mol}^{-1}$) or elevated CO₂ (Elev, around 700 $\mu\text{mol mol}^{-1}$) and fertilized with 0 mM or 15 mM NH_4^+NO_3 .

Gas Exchange Parameters

Gas exchange parameters were measured in fully expanded young leaves corresponding to 33, 36, 40, 45, 47, 52, and 54 days old plants, using a portable photosynthesis system GFS-3000 (Walz, Effeltrich[®], Germany) (Figure 9). Net photosynthesis was measured at CO₂ growth conditions (A_{growth}). Gas exchange in ambient CO₂ plants was measured at 400 $\mu\text{mol mol}^{-1}$ of CO₂, and in elevated CO₂ grown plants at 700 $\mu\text{mol mol}^{-1}$. Leaf conductance (g), intercellular CO₂ concentration (C_i) and transpiration (E) were calculated as described by Long and Hallgreen (1985).



Figure 9. Portable photosynthesis system GFS-3000 (Walz, Effeltrich[®], Germany).

Leaf Biochemical Measurements: Total Soluble Proteins, Sugars and Starch

Total soluble proteins, total soluble sugars and starch concentration were quantified in leaf extracts by grinding and filtering 0.5 g of fresh leaf mass weight, in cold mortar using an extraction buffer containing 0.1 M K-phosphate (pH 7). The extract was filtered and centrifuged at 18,000 g (13,000 rpm) for 15 minutes at 4 °C. After the centrifugation the pellet was stored in a freezer at -80 °C until later starch analysis. Total soluble protein and sugar quantifications were performed in the centrifuged supernatant. Starch was measured in the pellet following the procedure described by Jarvis & Walker (1993). Total soluble protein levels were measured using the method of Bradford (1976), while the total soluble sugar determinations followed the procedure of Yemm and Willis (1954).

Rubisco In vitro Activity Assay (EC 4.1.1.39)

The same leaves in which the gas exchange measurements were performed, were harvested, immediately frozen in liquid nitrogen and stored in a freezer at -80 ° C, awaiting analysis. The leaf tissue was powdered in liquid nitrogen and homogenized in a cold mortar with an extraction buffer containing 100 mM Bicine-NaOH (pH 7.8), 10 mM MgCl₂, 10 mM 2-mercaptoethanol, 2% PVPP (polyvinyl polypyrrolidone) (w/v), 1% BSA (w/v) and 1% Triton X-100 (v/v). The supernatant was clarified, by centrifugation at 13,000 g, and used to determine Rubisco *in vitro* enzyme activity by measuring the oxidation of NADH at 340 nm in coupled enzyme assay (Sharkey *et al.*, 1991).

Rubisco Protein Concentration

The same supernatant used for Rubisco *in vitro* activity measurement, was used to measure Rubisco subunit (RbcL, Rubisco large subunit; RbcS, Rubisco small subunit) concentrations. Leaf extracts used for Rubisco activity analysis were frozen in liquid nitrogen and stored at -80 ° C until the concentrations of Rubisco subunits were determined. For protein quantification, the leaf extracts were precipitated using the sodium deoxycholate-trichloroacetic acid protocol described by Peterson (1983). The resulting pellet was air dried and resuspended in Laemmli lysis buffer (Laemmli 1970) and boiled for 10 min to denature proteins. For SDS-PAGE (BIO-RAD equipment) (Figure 10), 4 µg of soluble proteins was prepared and performed using a 150 g L⁻¹ acrylamide separation gel and stained with silver nitrate (Blum *et al.*, 1987). Gel images were scanned and analyzed using the ImageQuant TL software (GE Amersham Biosciences, UK). The concentration of Rubisco protein large (RbcL) and small (RbcS) subunits was referred to a Rubisco standard protein (provided by Agrisera AB; Vännäs, Sweden) and was calculated per gram of leaf dry matter (mg g⁻¹ leaf DM).

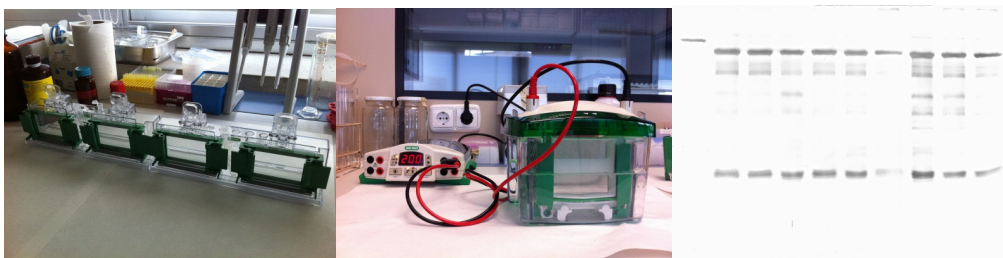


Figure 10. SDS-PAGE system and one gel aspect.

C/N Ratio

Leaf samples were collected at the end of the experiment (58 days), dried at 60 °C for 48 h, and analyzed for the C/N ratio. One milligram of ground sample was used for each determination, and three replicates were analyzed for each treatment. Determinations were conducted at the Serveis Científico-Tècnics of the University of Barcelona using an elemental analyser (EA1108, Series 1, Carbo Erba Instrumentazione, Milan, Italy) coupled to an isotope ratio mass spectrometer (Delta C, Finnigan, Mat., Bremen, Germany) operating in continuous flow mode.

Statistical Analysis

Statistical analysis of the data was made by three factor analyses (CO₂: ambient or elevated; nitrogen supply: 0 or 15 mM; and alfalfa variety: Aragón or Europe) of variance (ANOVA). Means $\pm$ standard errors (S.E.) and significant differences between factors and interactions were calculated at 5% level of significance. When differences between treatments were significant according to the ANOVA analysis, least significant differences were evaluated by a Tukey's *t*-test, as found in the Statistical Package for Social Science (SPSS) (SPSS Inc., Chicago, USA) version 15.0 programs for Windows XP.

Results

Plant Dry Matter

Effect of CO₂ (ambient or elevated) and nitrogen supply (0 or 15 mM NH₄⁺NO₃) on plant total dry matter (DM), including above and below ground organs (leaves, stem, roots and nodules) of nodulated alfalfa during growing period (30 days) are represented in figure 11. Dry matter increase during this period was more progressive in Europe than in Aragón. Aragón variety showed higher increase in the dry matter accumulation between the second and the third harvests.

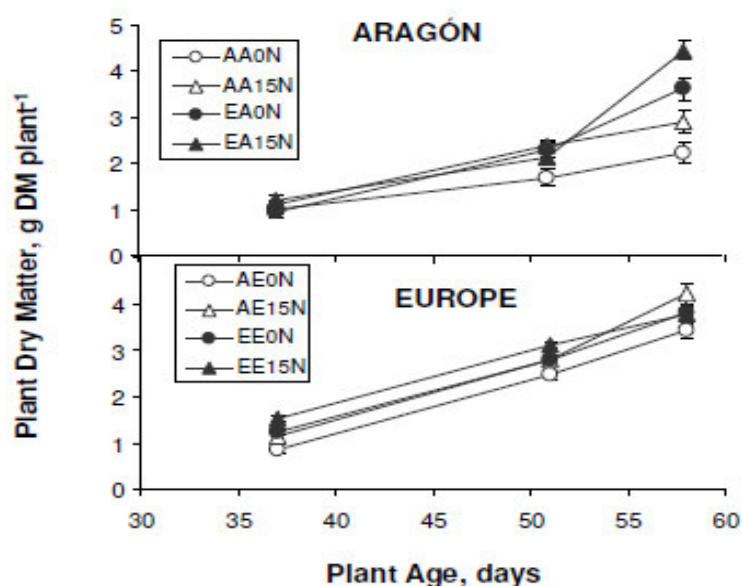


Figure 11. Effect of CO₂ (ambient, around 400 $\mu\text{mol mol}^{-1}$ or elevated 700 $\mu\text{mol mol}^{-1}$) and nitrogen (0 or 15 mM NO₃ NH₄⁺) on total plant dry matter (DM, g DM Plant⁻¹) in two varieties (Aragón and Europe) of nodulated alfalfa (*Medicago sativa* L.) grown in greenhouse conditions during one month. Three harvests were performed when plants were 37, 52 and 58 days old. Abbreviations of the treatments were: AA0N, Ambient CO₂, Aragón alfalfa variety supplied with 0 mM; AA15N, Ambient CO₂, Aragón alfalfa variety supplied with 15 mM; EA0N, Elevated CO₂, Aragón alfalfa variety supplied with 0 mM; EA15N, Ambient CO₂, Aragón alfalfa variety supplied with 15 mM; AE0N, Ambient CO₂, Europe alfalfa variety supplied with 0 mM; AE15N, Ambient CO₂, Europe alfalfa variety supplied with 15 mM; EE0N, Elevated CO₂, Europe alfalfa variety supplied with 0 mM; EE15N, Elevated CO₂, Europe alfalfa variety supplied with 15 mM. Each value is the mean of 4 plants $\pm$ SE. Data were analysed statistically by three factors analyses of variance (ANOVA) test and least significant differences were evaluated by a Tukey's *t*-test.

Almost no differences between treatments were observed in any of the harvest data in both varieties, with the exception of the third harvest (58 days old plants) in Aragón. Nodulated Aragón plants grown in elevated CO₂ and 15 mM NH₄⁺NO₃ reached the highest dry matter accumulation, followed by elevated CO₂ and 0 mM treated plants and then ambient 15 mM plants. The smaller plants were those grown in ambient CO₂ and 0 mM NH₄⁺NO₃. In the case of Europe no effect of the treatments were observed in the final harvest.

Plant Leaf Dry Matter

The effect of CO₂ (A, ambient or E, elevated) and nitrogen supply (0 or 15 mM NH₄⁺NO₃) on plant leaf dry matter accumulation at the end of the vegetative normal growth period (3rd harvest) is shown in figure 12. Only the third harvest is presented, because similar results were observed in the two previous harvests (37 and 52 days old plants) in both varieties. In contrast to that observed in total dry matter (Figure 11), leaf dry matter production responded positively to the increase in CO₂ and sometimes, as in Aragón, 15 mM nitrogen application interacted positively under elevated CO₂.

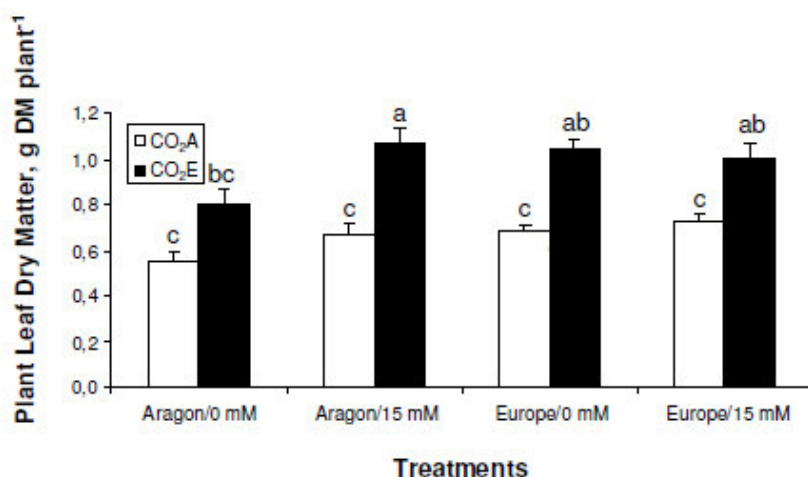


Figure 12. Effect of CO₂ (ambient, around 400 μmol mol⁻¹ or elevated 700 μmol mol⁻¹) and nitrogen (0 or 15 mM NH₄⁺NO₃) on plant leaf dry matter (g DM plant⁻¹) in two varieties (Aragón and Europe) of nodulated alfalfa (*Medicago sativa* L.) grown in greenhouse conditions during one month. Data correspond to the third harvest (58 days old plants). Otherwise as for figure 11.

Plant Stem Dry Matter

Effect of CO₂ and nitrogen supply on plant stem dry matter accumulation in nodulated alfalfa at 3rd harvest is reported in figure 13. As in plant dry matter accumulation (Figure 11), the best response of stem dry matter to both, nitrogen and CO₂ treatments, was observed in Aragón which responded positively to the increase in CO₂ and nitrogen, being the interaction between elevated CO₂ and 15 mM NH₄⁺NO₃ clearly evident. In Europe variety the response to nitrogen supply was more evident, at least at ambient CO₂ concentration.

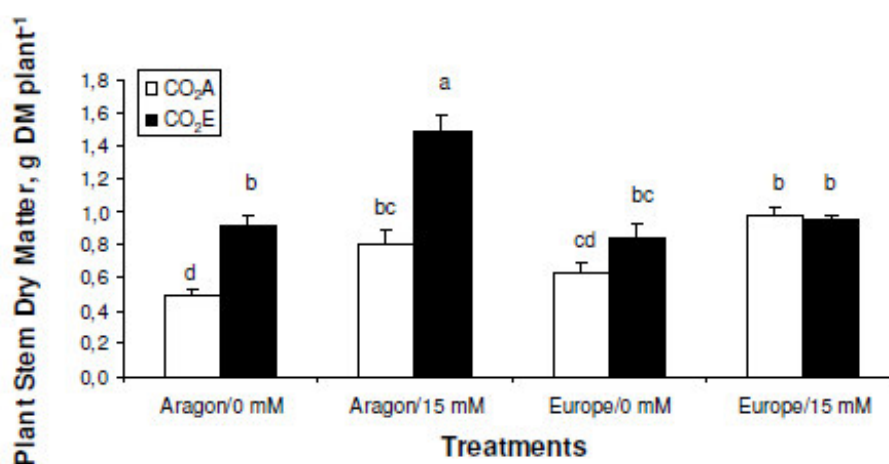


Figure 13. Effect of CO₂ (ambient, around 400 $\mu\text{mol mol}^{-1}$ or elevated 700 $\mu\text{mol mol}^{-1}$) and nitrogen (0 or 15 mM NH₄⁺NO₃) on plant stem dry matter (g DM plant⁻¹) in two varieties (Aragón and Europe) of nodulated alfalfa (*Medicago sativa* L.) grown in greenhouse conditions during one month. Data correspond to the third harvest (58 days old plants). Otherwise as for figure 11.

Plant Root Dry Matter

Effect of CO₂ and nitrogen supply on plant root dry matter accumulation in nodulated alfalfa of Aragón and Europe varieties in the 3rd harvest is represented in figure 14. Europe root dry matter is higher than Aragón in ambient CO₂. However the responses of the root dry matter to CO₂ and to nitrogen supply were not evident in any of the two varieties. An increase in root dry matter accumulation was observed in the

Aragón 0 mM NH_4^+NO_3 treatment, but it was not the case for Europe. The biggest root are observed in Europe at ambient CO_2 under 15 mM NH_4^+NO_3 fertilization

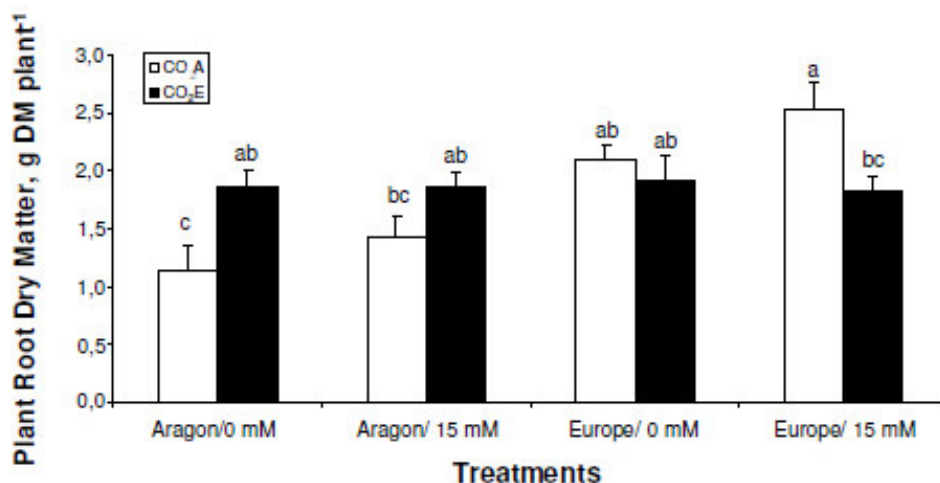


Figure 14. Effect of CO_2 (ambient, around $400 \mu\text{mol mol}^{-1}$ or elevated $700 \mu\text{mol mol}^{-1}$) and nitrogen (0 or 15 mM NH_4^+NO_3) on plant root dry matter (g DM plant⁻¹) in two varieties (Aragón and Europe) of nodulated alfalfa (*Medicago sativa* L.) grown in greenhouse conditions during one month. Data correspond to the third harvest (58 days old plants). Otherwise as for figure 11.

Stem to Leaf Dry Matter Ratio (Stem/Leaf Ratio)

Alfalfa aerial organs (leaves and stems) are very appreciated by the high quality forage to feed herbivores. However, leaves have higher nutritional values than stem due to the higher content in proteins and lower in lignin. Therefore, a measure of quality in alfalfa hay is the ratio between dry matter of stems to leaves. An increase in this ratio values means a lower quality of the forage. The figure 15 represents the effect of the CO_2 and nitrogen treatments in Aragón and Europe stem/leaf ratio during the last harvest of the experiment (58 days old plants). Not major effect of the treatments (ambient *versus* elevated CO_2 , 0 mM *versus* 15 mM NH_4^+NO_3 or the interaction between CO_2 and nitrogen) was observed, with the exception of the significant reduction in stem/leaf ratio in Europe at elevated CO_2 and 15 mM of nitrogen.

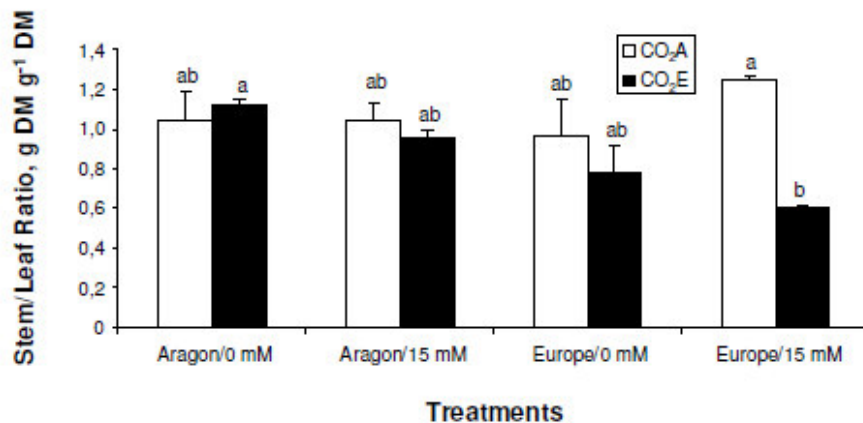


Figure 15. Effect of CO₂ (ambient, around 400 $\mu\text{mol mol}^{-1}$ or elevated 700 $\mu\text{mol mol}^{-1}$) and nitrogen (0 or 15 mM NH₄⁺NO₃) on Stem/Leaf ratio (g DM plant⁻¹) of nodulated alfalfa (*Medicago sativa* L.) Aragón and Europe varieties grown in greenhouse conditions during one month. Data correspond to the third harvest (58 days old plants). Otherwise as for figure 11.

Specific Leaf Area (SLA)

Another anatomical and adaptive parameter of the plants to the environment is the thickness of the leaves which can be estimated by the Specific Leaf Area (SLA) or relationship between leaf area and leaf dry weight. The SLA values corresponding to the third harvest (58 days old plants) of Aragón and Europe varieties are represented in figure 16. According to the data and the statistical analysis not differences between treatments and between varieties were observed.

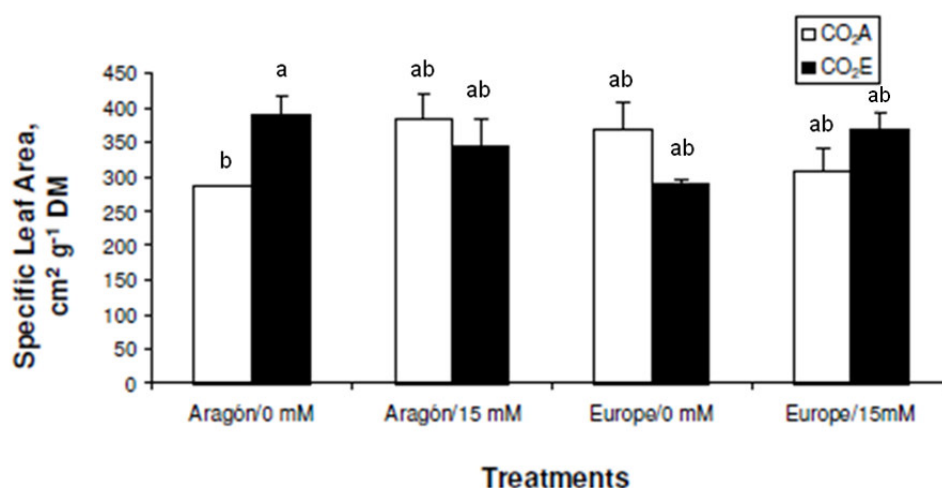


Figure 16. Effect of CO₂ (ambient, around 400 $\mu\text{mol mol}^{-1}$ or elevated 700 $\mu\text{mol mol}^{-1}$) and nitrogen (0 or 30 mM NH_4^+NO_3) on Specific Leaf Area ($\text{m}^2 \text{g}^{-1} \text{DM}$) in two varieties (Aragón and Europe) of nodulated alfalfa (*Medicago sativa* L.) grown in greenhouse conditions during one month. Data correspond to the third harvest (58 days old plants). Otherwise as for figure 11.

Plant Leaf Area

The response of the total leaf area of nodulated alfalfa to the treatments (CO₂ and nitrogen) in both varieties in the third harvest is shown in figure 17. Some tendencies of elevated CO₂ to decrease leaf area, like in Aragón 0 mM, and to the high nitrogen supply to increase leaf area under elevated CO₂, like in Europe, were observed. However, statistical analysis did not ratify those appreciations.

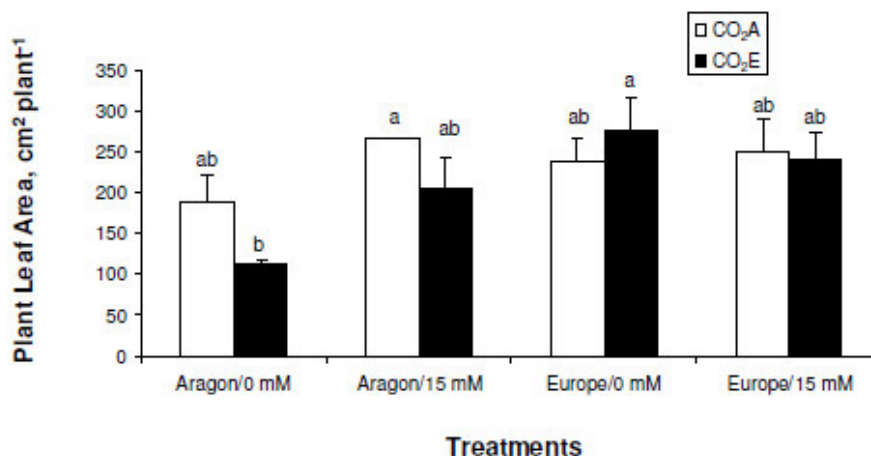


Figure 17. Effect of CO₂ (ambient, around 400 $\mu\text{mol mol}^{-1}$ or elevated 700 $\mu\text{mol mol}^{-1}$) and nitrogen (0 or 15 mM NH_4^+NO_3) on Plant Leaf Area ($\text{cm}^2 \text{ plant}^{-1}$) in two varieties (Aragón and Europe) of nodulated alfalfa (*Medicago sativa* L.) grown in greenhouse conditions during one month. Data correspond to the third harvest (58 days old plants). Otherwise as for figure 11.

Net Photosynthesis Rates (A_{growth})

To determine the presence or not of photosynthetic acclimation in the different treatments, net photosynthetic rates were measured at 400 $\mu\text{mol mol}^{-1}$ of CO₂ in the ambient treatments, and at 700 $\mu\text{mol mol}^{-1}$ in the elevated CO₂ treatments of both varieties. If net photosynthetic rates measured at 700 $\mu\text{mol mol}^{-1}$ were lower or equal than those measured at 400 $\mu\text{mol mol}^{-1}$, it can be concluded that plants grown under elevated CO₂ are probably acclimated or down regulated. This technique to detect photosynthesis acclimation is called measurements of net photosynthesis at growth conditions (A_{growth}). A_{growth} were determined in 33, 36, 40, 45, 47, 52 and 54 day's old plants.

The response of A_{growth} to the treatments (ambient and elevated CO₂, and 0 and 15 mM NH_4^+NO_3) in both varieties (Aragón and Europe) is represented in figure 18. It is noticeable to indicate that net photosynthesis showed a progressive decrease as the plants become older, even taken the precaution of measuring always the photosynthetic rates in the recent expanded leaves. The decline is observed in both varieties. The

elevated CO₂ treatments (AE and EE, represented in the figure 18 by filled symbols), almost never showed higher photosynthetic rates than ambient CO₂ treatments, and even in some cases were lower than ambient values. This may indicate an acclimation process in photosynthesis from the beginning of the experiment, independently of the variety.

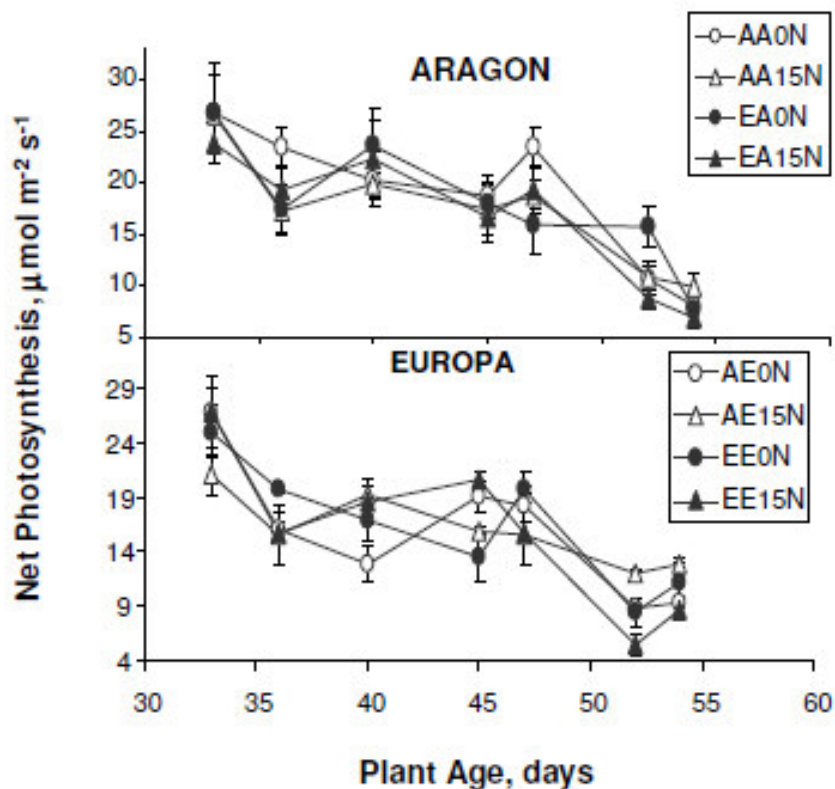


Figure 18. Effect of CO₂ (ambient, around 400 μmol mol⁻¹ or elevated 700 μmol mol⁻¹) and nitrogen (0 or 15 mM NH₄⁺NO₃) on net photosynthesis (μmol m⁻² s⁻¹) measured at CO₂ growth conditions (A_{growth}) in two varieties (Aragón and Europa) of nodulated alfalfa (*Medicago sativa* L.) greenhouse conditions during one month. Gas exchange measures were performed at ambient CO₂ (around 400 μmol mol⁻¹ CO₂) in plant grown at ambient CO₂ conditions and at 700 μmol mol⁻¹ CO₂ in plants grown at elevated CO₂. Gas exchange was measured when plants were 33, 36, 40, 45, 47, 52 and 54 days old. Each values is the mean of 4 plants ± SE. Data were analysed statistically by one factor analyses of variance (ANOVA) test and least significant differences were evaluated by a Tukey's *t*-test.

Intercellular CO₂ Concentration (C_i)

Intercellular CO₂ concentration (C_i, mesophyll CO₂ concentration) was calculated from the gas exchange parameters (net photosynthesis, stomatal conductance and external CO₂ concentration). The response of C_i to the treatments (CO₂ and nitrogen) in both varieties of nodulated alfalfa is included in figure 19. There is a clear difference in C_i between ambient and elevated CO₂ treatments. No effect of nitrogen supply was observed. The values of C_i were quite constant. In elevated CO₂ treatments, leaves of both varieties showed C_i values around 500 $\mu\text{mol mol}^{-1}$ of CO₂. In ambient CO₂ treatments C_i never reached 300 $\mu\text{mol mol}^{-1}$ remaining below about 280 $\mu\text{mol mol}^{-1}$.

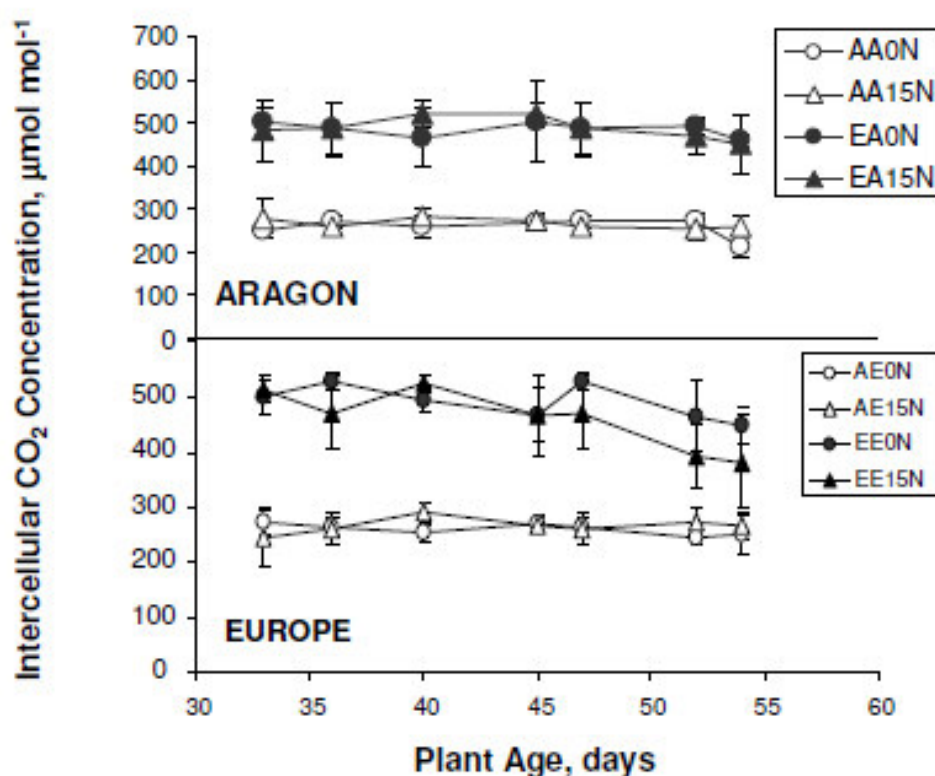


Figure 19. Effect of CO₂ (ambient, around 400 $\mu\text{mol mol}^{-1}$ or elevated 700 $\mu\text{mol mol}^{-1}$) and nitrogen (0 or 15 mM NH_4^+NO_3) on leaf Intercellular CO₂ Concentration ($\mu\text{mol mol}^{-1}$) measured at CO₂ growth conditions in two varieties (Aragón and Europe) of nodulated alfalfa (*Medicago sativa* L.) grown in greenhouse conditions during one month. Otherwise as for figure 18.

Stomatal Conductance (g_s)

Effect of CO₂ and nitrogen supply on g_s in nodulated alfalfa in both varieties (Aragón and Europe) during the growing period is shown in figure 20. It is well known that high CO₂ induces stomatal closure, and therefore decreased stomatal conductance. In the figure it can be observed, that the stomatal conductance values in the elevated CO₂ treatments in both alfalfa varieties (represented by filled symbols), were generally in the lower part of the figure, indicating a lower g_s values in those leaves. The tendency to have more closed stomata induced by elevated CO₂ was clearer in Aragón.

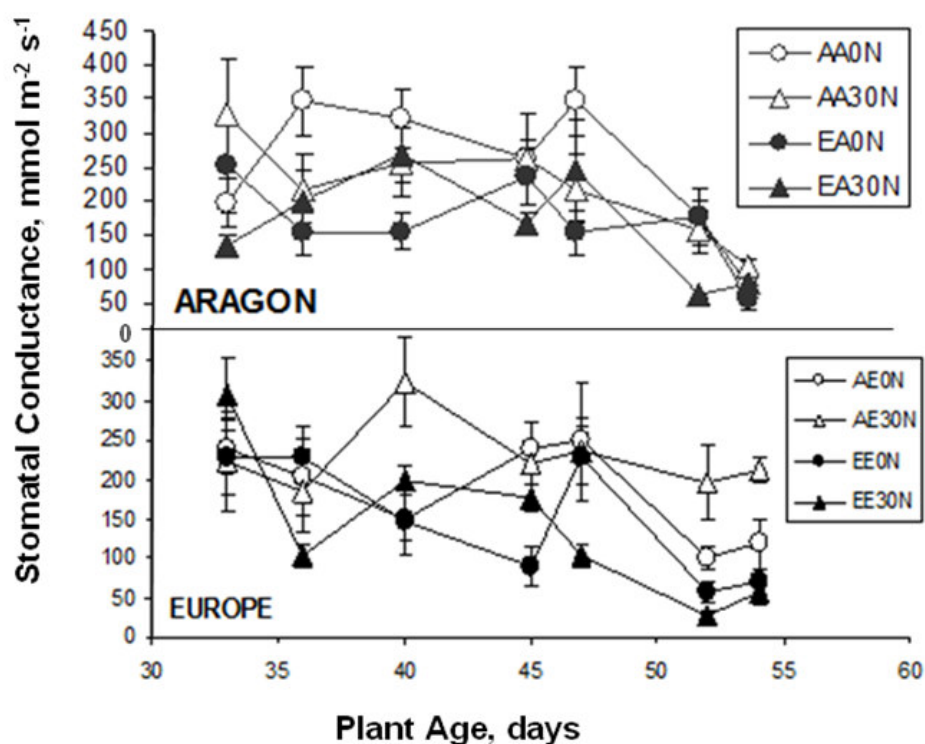


Figure 20. Effect of CO₂ (ambient, around 400 $\mu\text{mol mol}^{-1}$ or elevated 700 $\mu\text{mol mol}^{-1}$) and nitrogen (0 or 15 mM NH_4^+NO_3) on Stomatal Conductance ($\text{mmol m}^{-2} \text{s}^{-1}$) measured at CO₂ growth conditions in two varieties (Aragón and Europe) of nodulated alfalfa (*Medicago sativa* L.) grown in greenhouse conditions during one month. Otherwise as for figure 18.

Specific Transpiration (E)

Specific transpiration (E) is a gas exchange parameter, closely related to the previous analyzed stomatal conductance (g_s). Effect of CO₂ and nitrogen supply on E in nodulated alfalfa of Aragón and Europe varieties during growing period is represented in figure 21. The behavior of E values is quite similar to the described previously in stomatal conductance. The E values tend to be lower in the elevated CO₂ treatments

(filled symbols) and higher under ambient CO_2 . No specific response was observed in the N treatments in any of the varieties.

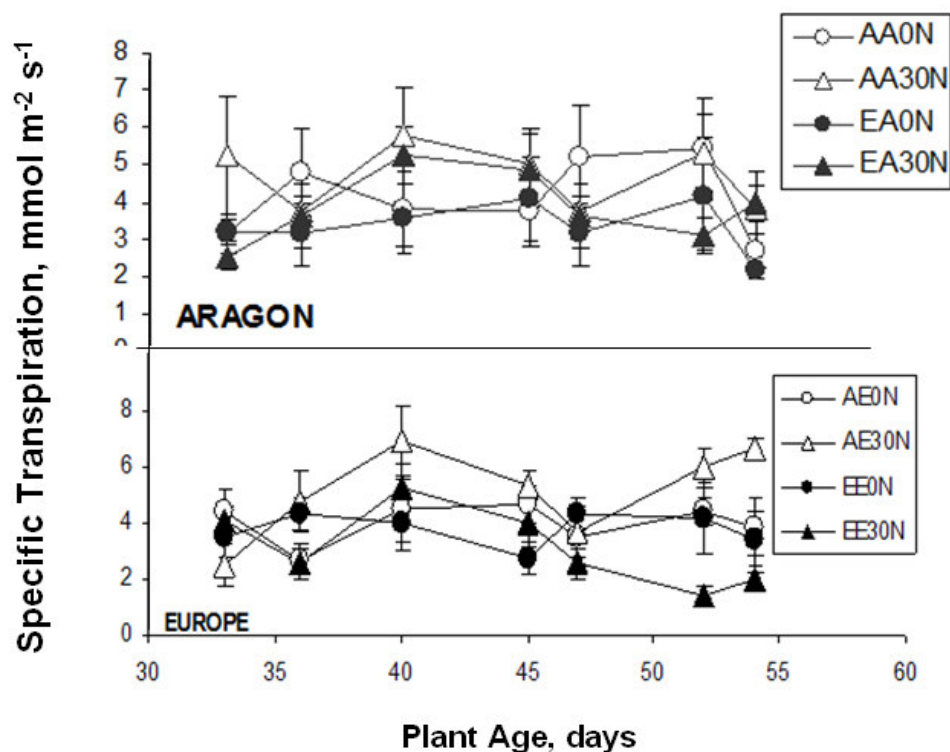


Figure 21. Effect of CO_2 (ambient, around $400 \mu\text{mol mol}^{-1}$ or elevated $700 \mu\text{mol mol}^{-1}$) and nitrogen (0 or 15 mM NH_4^+NO_3) on Specific Transpiration (E , $\text{mmol m}^{-2} \text{s}^{-1}$) measured at CO_2 growth conditions in two varieties (Aragón and Europe) of nodulated alfalfa (*Medicago sativa* L.) grown in greenhouse conditions during one month. Otherwise as for figure 18.

Leaf Starch

Starch is the main storage carbohydrate polymer in most of the plants. Effect of CO_2 and N supply on leaf starch concentrations in nodulated alfalfa during third harvest (58 days old plants) is shown in figure 22. Leaf starch concentration increased with CO_2 especially in Aragón. Nitrogen application (15 mM) has no remarkable effect, except for Europe growing at ambient CO_2 , in which an increase was observed.

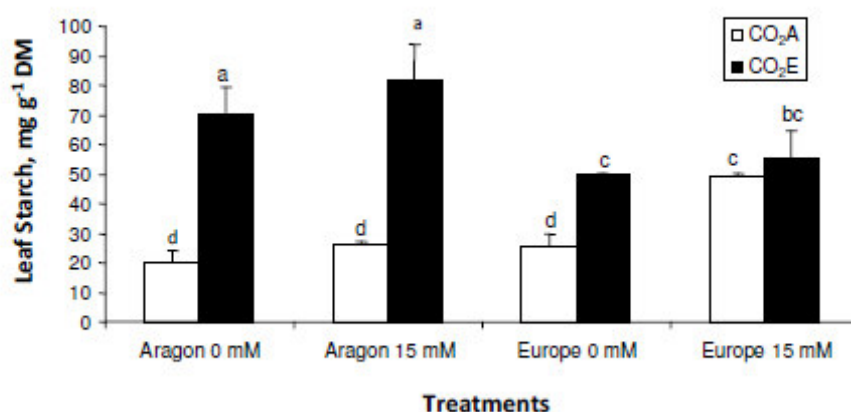


Figure 22. Effect of CO₂ (ambient, around 400 $\mu\text{mol mol}^{-1}$ or elevated 700 $\mu\text{mol mol}^{-1}$) and nitrogen (0 or 15 mM NH₄⁺NO₃) on leaf starch concentration (mg g⁻¹ DM) in two varieties (Aragón and Europe) of nodulated alfalfa (*Medicago sativa* L.) grown in greenhouse conditions during one month. Data correspond to the third harvest (58 days old plants). Otherwise as for figure 18.

Leaf Total Soluble Sugars

Effect of CO₂ and N supply on leaf total soluble sugars of nodulated alfalfa Aragón and Europe in the third harvest (58 days old) is shown in figure 23. The response of leaf total soluble sugars to treatments (CO₂ and N) seems to be more variable than that observed for leaf starch concentrations. Leaf soluble sugars increased significantly at elevated CO₂ in Aragón grown at 0 mM NH₄⁺NO₃. The rest of the treatments in Aragón and Europe showed lower values, and no remarkable differences were observed.

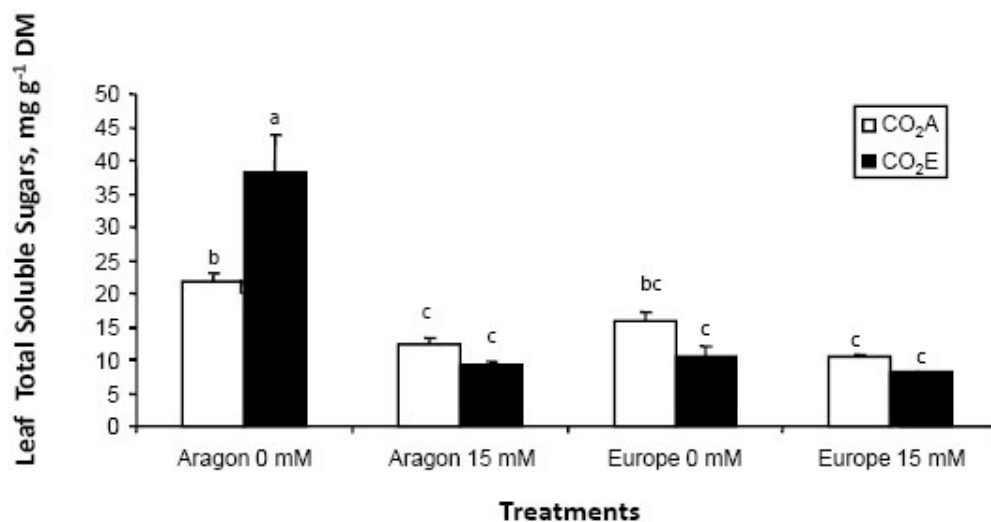


Figure 23. Effect of CO₂ (ambient, around 400 $\mu\text{mol mol}^{-1}$ or elevated 700 $\mu\text{mol mol}^{-1}$) and nitrogen (0 or 15 mM NH_4^+NO_3) on leaf total soluble sugars ($\text{mg g}^{-1}\text{DM}$) in two varieties (Aragón and Europe) of nodulated alfalfa (*Medicago sativa* L.) grown in greenhouse conditions during one month. Data correspond to the third harvest (58 days old plants). Otherwise as for figure 22.

Leaf Total Soluble Proteins

Effects of CO₂ (ambient and elevated) and nitrogen supply (0 and 15 mM) on leaf total soluble proteins in nodulated alfalfa Aragón and Europe in the third harvest (58 days old) are presented in figure 24. No effect of N or CO₂ was observed in Aragón. In Europe, only in 15 mM NH_4^+NO_3 treatment, a significant decrease in leaf soluble proteins was observed associated to the elevated CO₂.

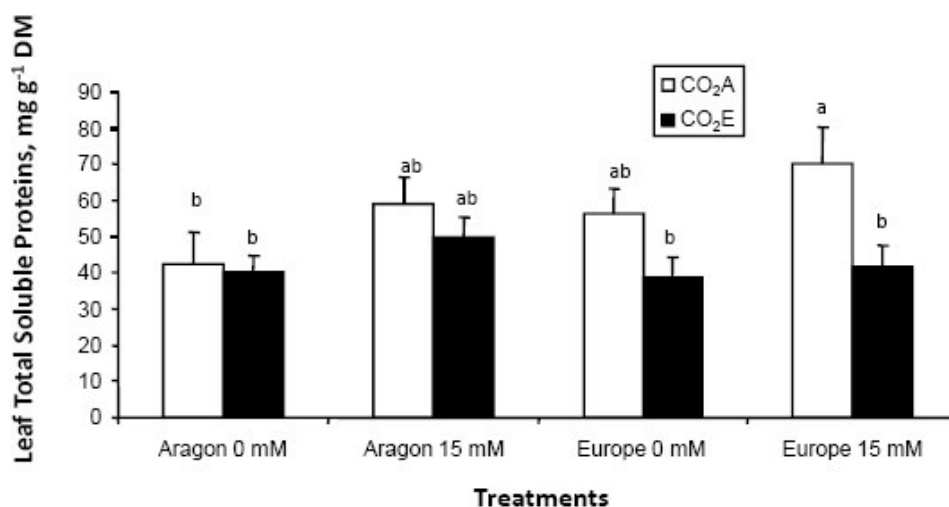


Figure 24. Effect of CO₂ (ambient, around 400 $\mu\text{mol mol}^{-1}$ or elevated 700 $\mu\text{mol mol}^{-1}$) and nitrogen (0 or 15 mM NH_4^+NO_3) on leaf total soluble proteins (mg g⁻¹ DM) in two varieties (Aragón and Europe) of nodulated alfalfa (*Medicago sativa* L.) grown in greenhouse conditions during one month. Data correspond to the third harvest (58 days old plants). Otherwise as for figure 22.

Rubisco Extractable Activity *In Vitro*

The rates of extractable Rubisco enzyme activity *in vitro* in the different CO₂ and nitrogen supply treatments in leaves of nodulated alfalfa Aragón and Europe in the third harvest (58 days old) are represented in figure 25. With the exception of 15 mM NH_4^+NO_3 in Europe, Rubisco *in vitro* activities tend to decrease in elevated CO₂. However the reductions were not statistically significant. Europe variety appears to have higher Rubisco activities; however, once again not statistical differences were found.

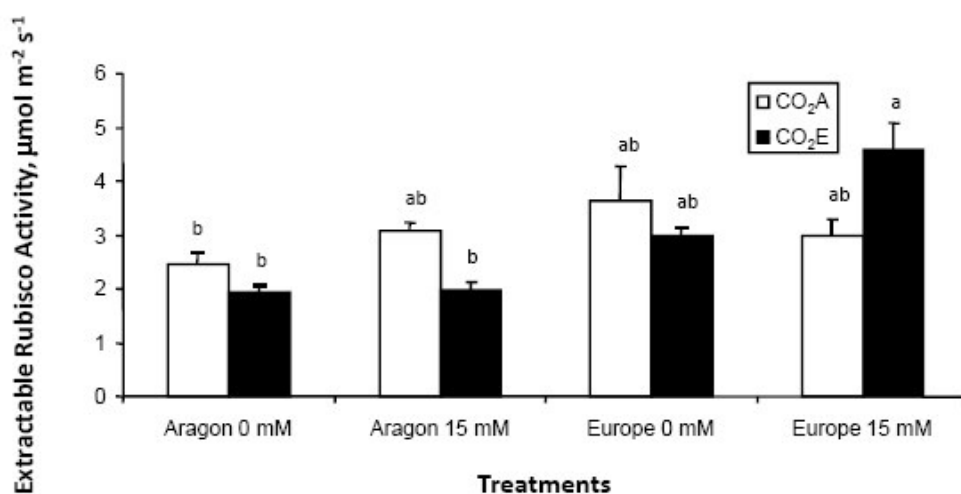


Figure 25. Effect of CO₂ (ambient, around 400 μmol mol⁻¹ or elevated 700 μmol mol⁻¹) and nitrogen (0 or 15 mM NH₄⁺NO₃) on leaf Rubisco extractable activity (μmol m⁻² s⁻¹) in two varieties (Aragón and Europe) of nodulated alfalfa (*Medicago sativa* L.) grown in greenhouse conditions during one month. Data correspond to the third harvest (58 days old plants). Otherwise as for figure 22.

Rubisco Large Subunit Concentration (RbcL)

The effect of CO₂ (ambient and elevated) and nitrogen supply (0 and 15 mM NH₄⁺NO₃) on Rubisco large subunit concentration (RbcL) in leaves of nodulated alfalfa Aragón and Europe in the third harvest (58 days old plants) is shown in figure 26. No effect of CO₂ enrichment was observed in Aragón, but in Europe a reduction was observed, despite it was only statistical difference in 15 mM NH₄⁺NO₃ treatment.

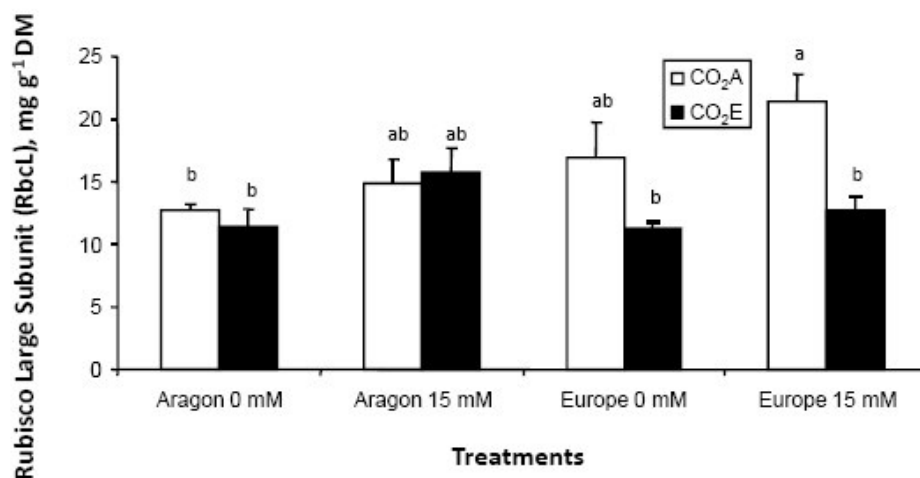


Figure 26. Effect of CO₂ (ambient, around 400 $\mu\text{mol mol}^{-1}$ or elevated 700 $\mu\text{mol mol}^{-1}$) and nitrogen (0 or 15 mM NH₄⁺NO₃) on Rubisco Large Subunit concentration in leaf tissues (RbcL, mg g⁻¹ DM) in two varieties (Aragón and Europe) of nodulated alfalfa (*Medicago sativa* L.) grown in greenhouse conditions during one month. Data correspond to the third harvest (58 days old plants). Otherwise as for figure 22.

Rubisco Small Subunit Concentration (RbcS)

Data of the concentrations of the small subunit of Rubisco (RbcS) in alfalfa Aragón and Europe leaves in the different treatments are presented in figure 27. Small subunit Rubisco (RbsS) concentrations appear to be more affected by the treatments than large Rubisco subunit (RbsL) (Figure 26); however, statistical analysis did not corroborate such appreciation. In the case of the small subunit (RbcS), Aragón variety appears to be more sensitive to the treatments. Elevated CO₂ declined RbcS levels in the leaves of Aragón, and high nitrogen supply (15 mM NH₄⁺NO₃) enhanced RbcS concentration at least in ambient CO₂ for Aragón.

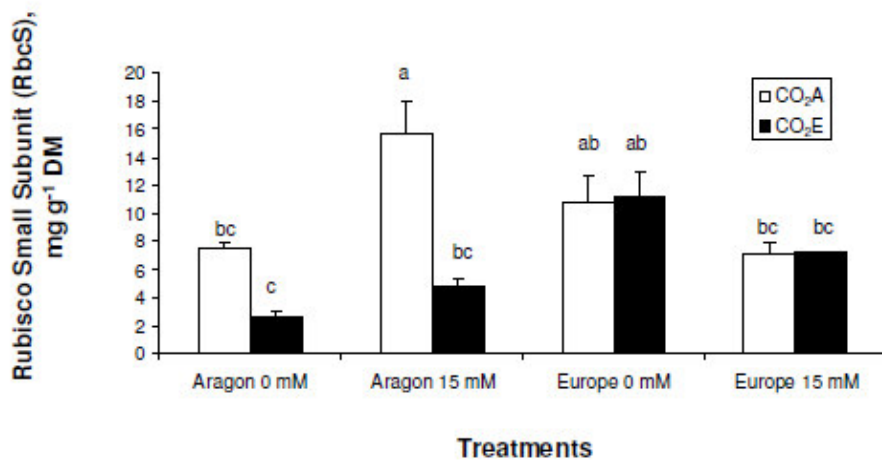


Figure 27. Effect of CO₂ (ambient, around 400 $\mu\text{mol mol}^{-1}$ or elevated 700 $\mu\text{mol mol}^{-1}$) and nitrogen (0 or 15 mM NH₄⁺NO₃) on Rubisco Small Subunit concentration in leaf tissues (RbcS, mg g⁻¹ DM) in two varieties (Aragón and Europe) of nodulated alfalfa (*Medicago sativa* L.) grown in greenhouse conditions during one month. Data correspond to the third harvest (58 days old plants). Otherwise as for figure 22.

Leaf Carbon to Nitrogen Ratio (C/N Ratio)

The response of the ratio between carbon and nitrogen (C/N Ratio) in the leaf tissues of Aragón and Europe nodulated plants to the CO₂ (ambient and elevated) and nitrogen supply (0 and 15 mM) treatments are represented in figure 28. The ratio increased significantly due to the elevated CO₂ in Aragón, in both nitrogen treatments. In Europe no noticeable variations were observed.

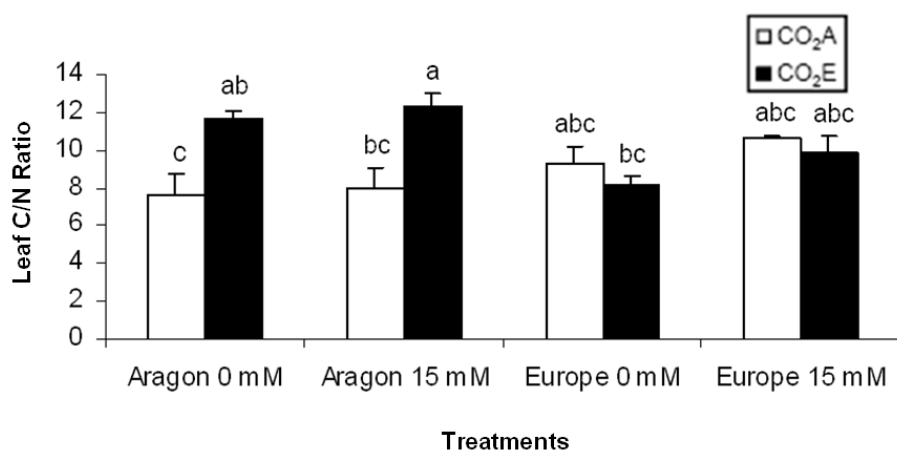


Figure 28. Effect of CO₂ (ambient, around 400 $\mu\text{mol mol}^{-1}$ or elevated 700 $\mu\text{mol mol}^{-1}$) and nitrogen (0 or 15 mM NH_4^+NO_3) on leaf carbon to nitrogen ratio (C/N Ratio) in two varieties (Aragón and Europe) of nodulated alfalfa (*Medicago sativa* L.) grown in greenhouse conditions during one month. Data correspond to the third harvest (58 days old plants). Otherwise as for figure 22.

Discussion

The expected increase in atmospheric CO₂ concentration by the end of this century (around 700 $\mu\text{mol mol}^{-1}$) may enhance photosynthesis in C₃ plants according to the Intergovernmental Panel on Climate Change (IPCC, 2007). This increase in photosynthesis by elevated CO₂ leads to the enhancement of dry matter (Drake *et al.*, 1997). In this study Aragón increased total plant dry matter (DM) (Figure 11). Also dry matter in some plant organs, especially leaf DM (Figure 12) and stem DM (Figure 13), increased significantly. The stimulatory effect of elevated CO₂ in Aragón was especially significant in 15 mM NH₄⁺NO₃, which suggests a high interaction effect between elevated CO₂ and nitrogen supply, as previously reported by Sanz-Sáez *et al.* 2010 and Sanz-Sáez, 2011. In Europe, total dry matter was not enhanced by CO₂ enrichment (Figure 11). However, leaf DM (Figure 12) of Europe plants also showed a significant stimulation due to elevated CO₂. The rest of the organs (stems, and roots) were not positively affected by the CO₂ in this variety. In previous work with nodulated alfalfa, it has been observed that the aerial part of the plant, especially leaves and sometimes stems, are more sensitive to the beneficial effect of the CO₂ enrichment than subterranean organs (root and nodules) (Erice *et al.*, 2006b; Erice *et al.*, 2007).

Growth data, especially total plant DM and organs DM (leaves, stems and roots), reveal that Aragón variety of alfalfa appears to be more sensitive than Europe to the studied environmental factors (CO₂ and NH₄⁺NO₃). Under the studied greenhouse conditions, Europe did not show remarkable differences in growth response to the CO₂ or nitrogen treatments alone or in interaction (Figure 11).

There are some morphological characteristics differentiating Aragón and Europe varieties. Aragón has slender and long leaves, whereas Europe has more swollen and short leaves. Another difference observed between the two alfalfa varieties is in the values of the allometric parameter root to shoot ratio. This ratio takes values of 1.6 in Europe and 1.1 in Aragón, and decline to values of 0.9 and 0.7 under elevated CO₂ and 15 mM NH₄⁺NO₃ treated plants, respectively. Supply of nitrogen in the form of NH₄⁺NO₃ also declines the root to shoot ratio, indicating a stimulation of the aerial parts of the plants instead of the subterranean ones (data not shown).

Alfalfa forage quality can be defined or measured according to several parameter as dry matter composition (crude protein, composition of fibres, digestibility, etc.), or by measuring several growth allometric parameters as root to shoot ratio, stem to leaf ratio, etc. In the present study no noticeable changes induced by the applied treatments (CO₂ or nitrogen supply) were detected in the alfalfa forage quality parameter defined by stem/leaf ratio (Figure 15). The only treatment that changed the ratio was the elevated CO₂ and 15 mM NH₄⁺NO₃. In this treatment, the ratio declined, indicating an increase in leaves proportion instead of stem tissues. Leaves usually have less lignin and higher proportion of proteins and then the digestibility is higher compared to that of other tissues as stems; therefore the forage of this treatment in Europe variety is the one with the highest quality. The general insensitivity of the leaves to shoot ratio to elevated CO₂, has also been observed in Aragón inoculated with the same *S. meliloti* 102F78 strain by Sanz-Sáez *et al.*, (2012b).

Another allometric growth parameter calculated in plant growth analysis is the ratio between the leaf area and the leaf dry matter, parameter named as Specific Leaf Area (SLA). This ratio informs about the thickness of the leaf (high SLA means thicker and probably with more leaf cell layers in the leaf lamina). However, another interpretation could also be the greater density of the leaf, perhaps due to the accumulation of high density polymer such as starch a sugar storage polymer (Sanz-Sáez *et al.*, 2010). Frequently, SLA declines significantly under elevated CO₂, as Sanz-Sáez *et al.*, 2010 observed in Aragón nodulated plants growth under elevated CO₂ in growth chambers, and not effect of the nitrogen application was detected. The authors observed that the increase in SLA was generally associated with starch accumulation in the leaf tissues. However, in the present study, Aragón variety increased leaf starch contents (Figure 22), despite of that SLA did not show any change (Figure 16). Therefore we can conclude, that at least in the present experimental conditions, the changes in starch accumulations in leaves did not affect the SLA in leaves of Aragón variety.

Total plant leaf area (Figure 17) is a significant parameter in many CO₂ enriched atmosphere studies. Frequently, long term elevated CO₂ treatments (weeks, months or years) induce photosynthetic acclimation, which means a reduction in photosynthetic capacity of the tissues (mainly leaves), and therefore the growth potential of the plants

is reduced. However, quite frequently leaf area of high CO₂ grown plants experience leaf area stimulation in the early periods of the CO₂ enrichment, and therefore even if the photosynthetic rates declined, plants are able to reach high growth due to the enhanced exposed area. However this is not the case in Aragón and Europe subjected to CO₂ and nitrogen supply treatments studied in this work. No stimulatory effects of elevated CO₂ on the total leaf area in any of both varieties of nodulated alfalfa was observed, as also reported Erice *et al.*, (2006b) and Sanz-Sáez *et al.*, (2010). Nitrogen supply (15 mM of NH₄⁺NO₃) enhanced plant total leaf area in Aragón grown at ambient and elevated CO₂; however the increase was not statistically different (Figure 17). Contrariwise to that observed in this study, Sanz-Sáez *et al.* (2011) described in Aragón nodulated plants supplied with 10 a 15 mM NH₄⁺NO₃, a significant enhancement of total leaf area. However the Sanz-Sáez and coworkers study was performed in growth chambers and not in greenhouse like this study, where conditions were more variable.

A central point in the analysis of the effects of elevated CO₂ in plant physiology is the study of the photosynthetic rates (Figure 18). Usually, as many studies described, photosynthetic rates increased in the first moments of CO₂ enriched atmosphere, as in this study when atmospheric CO₂ increased from 400 to 700 µmol mol⁻¹. However, after a long period (weeks or months depending of the physiology of the plants and the growth rates), photosynthetic capacity of the assimilation tissues (leaves) decreased, phenomenon known as photosynthetic acclimation or photosynthetic down regulation (Sanz-Sáez, 2011). Sanz-Sáez (2011) performed a complete and interesting comparative study about the different ways to detect photosynthesis acclimation in Aragón alfalfa grown in elevated CO₂. After a deep study including more than ten parameters (photosynthetic rates at 400 and 700 µmol mol⁻¹ measurements, V_{cmax} estimation from A/Ci curves, Rubisco activity, Rubisco protein total amount obtained from large and small subunit concentrations, C/N ratio, etc.), the author concluded that the comparison between the photosynthetic rates measured at the CO₂ concentration in which the plants grow (photosynthetic rates at growth conditions, abbreviated as A_{growth}), ambient CO₂ treatments measured at 400 and elevated CO₂ plants at 700 µmol mol⁻¹, demonstrates clearly photosynthetic acclimation to enriched CO₂ atmosphere in alfalfa. It is expected that, if the photosynthetic rates of the plants grown under elevated CO₂ (700 µmol mol⁻¹) and measured at 700 µmol mol⁻¹ CO₂ concentration are equal or lower than the

rates measured in ambient treatment plants at $400 \mu\text{mol mol}^{-1}$, plants grown at elevated CO_2 ($700 \mu\text{mol mol}^{-1}$) are probably acclimated or suffering photosynthetic down regulation. According to the data in the Figure 18, where A_{growth} of the different treatments in both varieties are represented, we can conclude, that elevated CO_2 treatments of both varieties of alfalfa independently of the nitrogen supply are acclimated.

One of the reasons to explain such equal or lower photosynthetic rates, could be CO_2 limitation induced by enriched CO_2 treatment due to stomatal closure stimulated by elevated CO_2 . In fact, the analysis of the stomatal conductance (Figures 20) and specific transpiration (Figure 21), demonstrated, even considering the high variability of the values, that the stomatal conductance and specific transpiration values of the elevated CO_2 treatments, are, in general, in the base or lower part of the figures, indicating a lower stomatal conductance (stomata more closed). However, even though CO_2 concentration in the intercellular spaces of the leaves (estimated by the C_i parameter, Figure 19), reached values of around $500 \mu\text{mol mol}^{-1}$ in elevated CO_2 treatments and $300 \mu\text{mol mol}^{-1}$ in the ambient ones. The CO_2 availability to the photosynthetic tissues (mesophyll cells) is significantly higher in elevated than in ambient treatments. In conclusion, the equal or lower rates of photosynthesis rates measured in elevated CO_2 treatments compared with ambient CO_2 rates (Figure 18), are not due to lower CO_2 availability, because the C_i values are higher (500 versus $300 \mu\text{mol mol}^{-1}$) and therefore, the limitation of photosynthetic rates in elevated CO_2 treatments should be associated to non stomatal limitations as decrease in Rubisco activity, photosynthetic carbon reduction cycle enzymes activities, mesophyll conductance, etc.

In general, studies about the effect of elevated CO_2 on plant growth and photosynthesis reveal that the photosynthesis acclimation is usually associated with a decline in plant carboxylation activity, mainly due to Rubisco activity decrease. This decrease in Rubisco activity could be due to lower Rubisco specific activity, linked to Rubisco inactivation by specific inhibitors, and/or lower levels of Rubisco protein in the chloroplast, which also affect Rubisco activity and then photosynthetic rates (Weis, 1981; Fellers *et al.*, 1998; Cen and Sage, 2005). Rubisco protein decrease is generally associated with the reduction in the concentrations of the Rubisco subunits (RbcL, large

subunit of Rubisco containing CO₂ carboxylation center of Rubisco, and RbcS, the small subunit of Rubisco with more regulatory functions). Rubisco large subunit (RLS) and Rubisco small subunit (RSS) are highly correlated with net carbon exchange at any CO₂ concentration (Sanz-Sáez, 2011). Rubisco protein can represent around 50% of the alfalfa leaf total soluble proteins (Figures 26 and 27). Therefore a reduction in Rubisco subunit concentrations may be reflected in a significant decrease in leaf total soluble proteins (Figure 24). According to some authors (Bowes, 1993), it is well known that elevated CO₂ reduces leaf total soluble protein concentrations. However, in present study leaf total soluble proteins (Figure 24) were not affected by the elevated CO₂ treatments with the exception of the elevated CO₂ and 15 mM treatment in Europe. The same behavior is observed in the large subunits concentrations (RbcL, large subunit, Figure 26). Aragón variety showed a reduction in Rubisco small subunit concentrations (RbcS, small subunit, Figure 27). However, no changes in Europe Rubisco small subunit concentrations were observed. Therefore from leaf protein analysis (Rubisco subunit concentrations and total soluble proteins) it can be concluded, that not appreciable changes in leaf soluble proteins were observed in some applied treatments (elevated CO₂ and 15 mM NH₄⁺NO₃) with the exception of elevated CO₂ and 15 mM in Europe, where a significant reduction in leaf soluble proteins was observed (Figure 24) associated with a reduction in Rubisco large subunit concentration (Figure 26).

In general, photosynthetic rates measured in the different treatments (Figure 18) agree with Rubisco extractable activity (Figure 25), Rubisco subunit concentrations, especially with Rubisco large subunit concentrations (Figures 26), and with leaf total soluble concentrations (Figure 24), since there were no remarkable changes. However the question is how the leaves of both alfalfa varieties with almost the same amount of Rubisco (activity and quantity), have the same photosynthetic rates in ambient and in elevated CO₂, with significantly higher CO₂ availability (500 $\mu\text{mol mol}^{-1}$ in elevated CO₂ and 300 $\mu\text{mol mol}^{-1}$ in ambient CO₂ treatments). The only explanation is a specific inactivation of the Rubisco enzyme activity or a supply limitation in the Ribulose-1, 5-bisphosphate (RuBP), CO₂ carboxylation acceptor. In fact, several studies in plants grown under elevated CO₂ have demonstrated changes in Rubisco activation state and photosynthetic carbon reduction cycle activity (cycle controlling the CO₂ RuBP acceptor synthesis) (Rogers, *et al.*, 1998; Stitt *et al.*, 2010; Parry *et al.*, 2011).

One of the explanations for photosynthesis acclimation is the photosynthetic final product accumulation (soluble sugars as sucrose or/and insoluble sugars like starch accumulation in the leaves). Sugar accumulation in the leaves associated with growth at elevated CO₂, could induce Rubisco inactivation and Rubisco protein synthesis inhibition. According to Aranjuelo *et al.*, (2005b) and Urban (2003), the decrease in photosynthesis was directly related to decreases in Rubisco protein. In the present experiment, accumulation of soluble sugars was only observed in Aragón leaves subjected to elevated CO₂ and grown with 0 mM NH₄⁺NO₃ (Figure 23), and leaf starch accumulation in elevated CO₂ plants of Aragón and Europe alfalfa varieties, with the exception of Europe grown at elevated CO₂ and 15 mM nitrogen (Figure 23). Therefore, from those data it can be concluded that the photosynthetic equal or lower rates measured in elevated CO₂ treated plants in comparison with ambient CO₂ treatments should be associated with sugars accumulation (soluble or insoluble sugars) which may induce a Rubisco enzyme inactivation (Moore *et al.*, 1999). However this Rubisco inactivation was not corroborated by the extractable Rubisco activity (Figure 25)(not significant changes were reported). This is probably because the inactivation state of the Rubisco *in situ* was reversed, and the enzyme was activated during the Rubisco extraction and assay (more than one hour of interval between extraction from the leaf and the enzyme activity measurement).

Sugars accumulation and leaf protein decline (including Rubisco protein reduction), frequently associated with photosynthesis acclimation induced by long term CO₂ enrichment (like in alfalfa plants growing during one month in elevated CO₂), leads to an increase in the ratio between carbon and nitrogen (C/N) in the plant or at least in the leaves (Aranjuelo *et al.*, 2007; Erice *et al.*, 2006a). In this experiment the ratio C/N in the leaves of the two alfalfa varieties, significantly increase in Aragón elevated CO₂ treatments (0 and 15 mM NH₄⁺NO₃) (Figure 28). On the contrary in Europe leaves, the C/N ratios did not change. Therefore, according to this parameter, apparently Aragón seems to be more acclimated to CO₂ than Europe.

In summary, from the study with the two alfalfa varieties subjected to elevated CO₂ under 0 and 15 mM of NH₄⁺NO₃, it can be concluded that in general Aragón is more responsive than Europe to the elevated CO₂ and nitrogen treatments. Aragón whole plant growth was stimulated by elevated CO₂ under 0 and 15 mM nitrogen

supply (NH_4^+NO_3), although the stimulation was more significant in high nitrogen supply (15 mM). These data agreed with those reported by *Sánchez et al.*, (2010) in the same alfalfa variety. According to Sanz-Sáez and coworkers, alfalfa growth improvement induced by elevated CO_2 , is limited by nitrogen supply from the nodules in 0 mM treatments. The application of extra nitrogen as ammonium nitrate (15 mM), enhances the nitrogen availability and then the response to elevated CO_2 is the highest. The beneficial effect of elevated CO_2 was better observed in the aboveground plant organs like dry matter accumulation (leaves and stems) than in belowground ones (roots and nodules). Even if the elevated CO_2 growth stimulation at the whole plant level was only observed in Aragón, the aboveground organs, especially leaf organs, showed significant growth stimulation in both varieties (Aragón and Europe). The reason for the elevated CO_2 growth stimulation (dry matter accumulation) in Aragón was not associated with an improvement in leaf photosynthesis rates or in leaf area. In the case of Europe, the lack of elevated CO_2 growth stimulation agreed with similar photosynthetic rates measured at growth conditions (ambient treatment at $400 \mu\text{mol mol}^{-1}$ and elevated treatments at $700 \mu\text{mol mol}^{-1}$), and similar leaf areas measured in elevated CO_2 treated plants. However similar rates of photosynthesis in ambient and elevated CO_2 treatments in both alfalfa varieties did not agree with significantly higher CO_2 intercellular concentration in leaves in the elevated CO_2 treatments, even if the leaves of enriched CO_2 had the lower stomatal conductances. Rubisco extractable activity was similar in elevated and ambient CO_2 treatments, as well as the concentrations of Rubisco subunits (RbcL and RbcS). Perhaps the small subunit concentration was more variable, but a general reduction in the subunit was only observed in Aragón but not in Europe. Evidences to support the photosynthesis acclimation in both alfalfa varieties and to explain the equal or even sometimes lower photosynthetic rates measured at growth conditions, are the soluble sugars accumulation in elevated CO_2 treatment and 0 mM NH_4^+NO_3 , as well as the accumulation of starch in the leaves, phenomena especially evident in Aragón elevated CO_2 (0 and 15 mM) plants. Another photosynthesis acclimation indicator is the ratio between carbon and nitrogen content of plant organ dry matter (C/N). Once again, the leaf C/N ratio was especially high in elevated CO_2 treatments in Aragón dry matter. Therefore according to the photosynthetic rates, both alfalfa varieties were acclimated, and photosynthesis acclimation was supported by the carbohydrate accumulation and the C/N ratio increase, especially in Aragón. However the molecular explanation for photosynthesis

acclimation should be found not in Rubisco extractable activity or Rubisco protein concentrations. Probably, as also supported by other studies, the explanation for photosynthesis acclimation in alfalfa may be related to Rubisco enzyme inactivation *in situ* and/or a supply limitation of the CO₂ acceptor Ribulose-1, 5-bisphosphate (RuBP) by the photosynthetic carbon reduction cycle (PCRC). It could be possible, as reported by other authors, that the long term elevated CO₂ treatment (one month) may down-regulate several enzyme activities of the photosynthetic carbon reduction cycle.



Chapter II

The response of nodule dry mass, apparent nitrogenase activity (ANA) and biochemical parameters measured in nodules of two varieties (Aragón and Europe) of nodulated alfalfa (*Medicago sativa* L.) grown under ambient or elevated CO₂ and fertilized with 0 mM (0N) or 15 mM (15N) NH₄⁺NO₃.

Introduction

Plants need nitrogen as an essential plant nutrient for many important vital molecules such as proteins, nucleic acids, etc. Nitrogen is one of the most abundant elements in nature, and especially abundant in the atmosphere where 78% of the composition is N_2 . However, in general, this nitrogen form can be not used by the plants to cover their needs of nitrogen. Only legumes can establish a symbiotic relationship with free living soil bacteria of the genera *Rhizobium* that they can grow in plant roots, where they can use the atmospheric N_2 .

The bacteria are able to reduce atmospheric nitrogen into ammonia that can be used by plant. The result of symbiosis is the formation of new plant organ called “root nodule”. Nodules contain infected cells including several thousands of bacteria surrounded by a plant cell

derived membrane, called peribacteroidal membranes (PBM). These symbiotic organelle-like structures may have one (or few) bacteria and its so-called “symbiosome” (Figure 29).

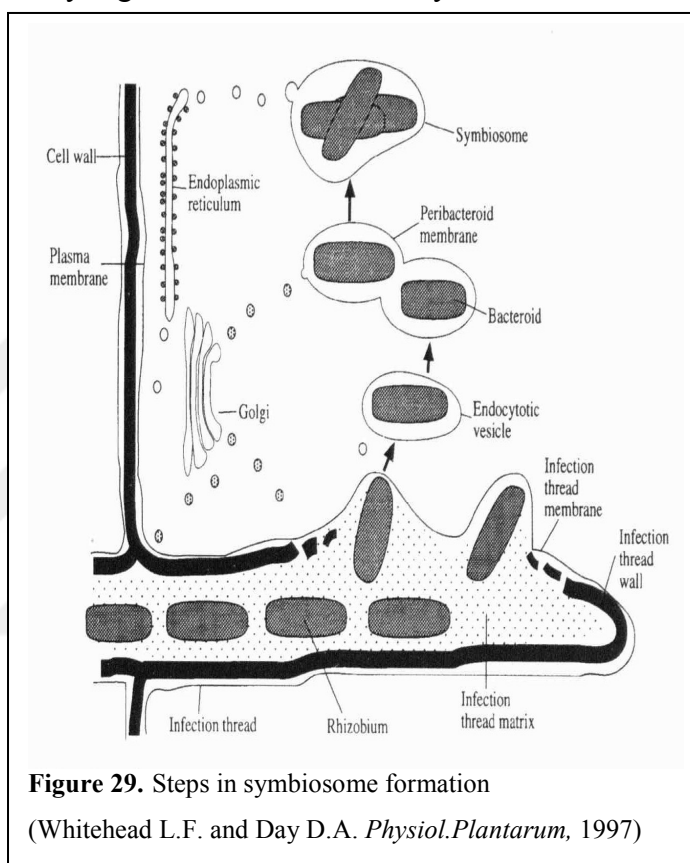


Figure 29. Steps in symbiosome formation
(Whitehead L.F. and Day D.A. *Physiol.Plantarum*, 1997)

Rhizobia is a generic term including all the bacteria that are able to form symbiotic interactions with legumes and they are classified in four genera: *Rhizobium*, *Bradyrhizobium*, *Azorhizobium*, and *Sinorhizobium* but symbiosis is mainly belonging to the alphaproteobacterial species *Sinorhizobium meliloti*.

From a functional point of view, there are three functional zones in the nodule; Zone 1, including meristematic region which is the youngest zone, most distal from the

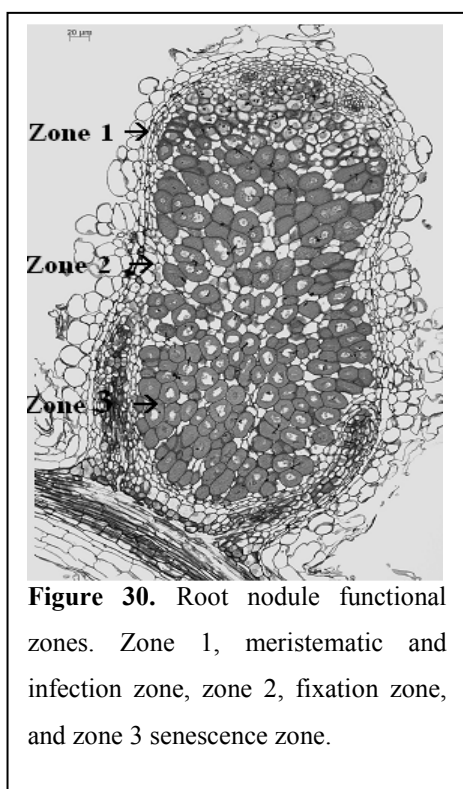


Figure 30. Root nodule functional zones. Zone 1, meristematic and infection zone, zone 2, fixation zone, and zone 3 senescence zone.

root, where the cells are actively dividing and are not yet infected, and also an infection area can be distinguished, where the bacteria are released from the infection threads and taken up into the nodule cells, forming symbiosomes that start to divide and differentiate to completely fill the host cells; Zone 2, is the fixation zone where the symbiosomes are completely differentiated and nitrogen fixation takes place; Zone 3, is the senescence area of the nodule, is closest to the root, and in this part of the nodule the bacteria are degraded and nodule cells senesce (Figure 30).

Most of the plants cover its N needs by uptaking and the assimilation of NO_3^- and NH_4^+

from the soil. However, availability of inorganic N in soil, especially in agricultural lands, is quite limited due to its soil extraction by intensive crop cultivation. Therefore, N is usually supplied in the form of N fertilizers in order to increase crop yield. Nevertheless, the extensive use of N fertilizers in agriculture has important environmental implications. The rise concentration of NO_3^- in surface water and groundwater can cause water eutrophication and as a consequence public health problems like infant methemoglobinemia and cancer due to the production of secondary amines. The production of volatile N compounds (N_2O , NO_x , NH_3 , etc.) can lead to air pollution increasing the greenhouse effect and, consequently destruction of the ozone layer and cause acid rain. Taking into account these environmental and health problems, plus the increasing energy cost required for the production of chemical N fertilizers (mainly as NH_4^+ by the Haber-Bosch process), there is clearly a need of using sustainable, environmentally-friendly alternatives. Legumes' nitrogen fixation represents a valuable alternative for certain plants, which are capable of establishing symbiotic relations with N_2 -fixing bacteria (Aparicio-Tejo *et al.*, 2008).

As reported in the introductory chapter, nitrogen availability can limit plant growth especially under elevated CO_2 conditions. Frequently when plants grown under elevated atmospheric CO_2 concentrations (perhaps $700 \mu\text{mol mol}^{-1}$), plants stimulate

photosynthesis at least in the short term application, but normally after long time elevated CO₂ exposure, photosynthesis acclimates or down regulates, as we observe and analyze in the previous chapter. In many cases the main reason for such acclimation is the nitrogen shortage. As carbon availability increases (atmosphere enriched in CO₂ and stimulated photosynthesis) the C/N ratio of the plants grown under these conditions, increases and photosynthesis acclimation occurs. Previous studies in the alfalfa (*Medicago sativa* L.) symbiotic relationship with *Sinorhizobium meliloti* showed that N₂ fixation increase at elevated CO₂ due to an increase in nodule size and nodule number per plant (Rogers *et al.*, 2009). Under elevated CO₂, nodules are probably better supplied with carbohydrates from the leaves and then a more profuse development of root and nodules can be expected. On the other hand, there is less evidence that specific nitrogenase activity increases at elevated CO₂ (Cen and Layzell, 2004; Vance and Heichel, 1991).

Photosynthesis provides organic carbon skeletons to the nodules (mainly organic acids as malate, succinate, etc.), where they were used by the bacteroid to furnish energy requirements (ATP and NADPH) of nitrogenase enzyme to fix N₂. N₂ fixation products as amines/amides (glutamate, glutamine, aspartic or asparagine) or ureids are exported to the plant via xylem (Aranjuelo *et al.*, 2007). In order to investigate the effect of elevated CO₂ on nitrogenase specific activity, apparent nitrogenase activity (ANA) and several nodule enzymes involved in C and N metabolism were analyzed. Nodule tissues were fractionated by differential centrifugation and two fractions were obtained: plants fraction and bacterial fraction (see Materials and Methods section for more details). For the metabolic analysis of both fractions, key enzymes were chosen. Phosphoenolpyruvate carboxylase (PEPC) and malate dehydrogenase (MDH) as a measure of malate synthesis capacity, isocitrate dehydrogenase activity (ICDH) as NADPH production will be tested, and glutamate-oxaloacetate transaminase (GOT) will be measured as a way to quantify the NH₄⁺ fixed by the bacteroid that can be assimilated by the plant in the nodule (Udvardi and Day, 1997). The activities of PEPC, MDH, ICDH and GOT will be measured in the nodule plant fraction, and activities of MDH and ICDH in the bacterial fraction. At the same time, total soluble sugars and starch concentrations in the plant fraction will be evaluated as well as the total soluble proteins in both fractions. Sugar and starch measurements will evaluate the supply of

carbohydrates from the plant, and the protein concentration an indicator of the metabolic activity.

Several studies have revealed that *Sinorhizobium* efficiency (Bertrand *et al.*, 2007; Sanz-Sáez, 2011), environmental factors like temperature (Sanz-Sáez, 2011), can alter the response of alfalfa to elevated CO₂ concentration, and therefore to the climate change conditions, greatly defined by elevated CO₂ and temperature. Aranjuelo *et al.* (2008) reported an interesting interaction between the plant and the bacteria in the symbiotic relationship, which may condition photosynthetic acclimation of N₂-fixing alfalfa plants exposed to elevated CO₂.

As in the previous chapter (Chapter I), analyzing the response of alfalfa forage production in two nodulated alfalfa varieties an acclimation response was reported at least in the photosynthetic rates, the possible implication in this acclimation of the nodule activity will be evaluated. The nitrogenase specific activity and several plant and bacteria key enzymes of the nodule metabolism will be analyzed, in order to establish possible connections between leaves and nodules response to elevated CO₂ in nodulated alfalfa plants. Therefore, the aim of the present study was to analyze the response of nodule dry mass, apparent nitrogenase activity (ANA) and biochemical parameters measured in nodules of two varieties (Aragón and Europe) of nodulated alfalfa (*Medicago sativa* L.) grown under ambient or elevated CO₂ and fertilized with 0 mM or 15 mM NH₄⁺NO₃.

Materials and Methods

Experimental Design, Plant Material and Growth Parameters

Experimental design, plant material and growth parameters are exactly the same as mentioned in the *Chapter I* (see pages 42-44).

Apparent Nitrogenase Activity

The apparent nitrogenase activity (ANA) was estimated by measuring the production of H_2 from nodules. This estimation is processed according to nitrogenase reaction; $N_2 + 10H^+ + 8e^- + 16ATP \xrightarrow{\text{(nitrogenase)}} 2NH_3^+ + H_2 + 16(ADP + P_i)$.

Apparent nitrogenase activity was evaluated in the intact plants with root system placed inside a hermetic pot located in an open flow-through system under $N_2:O_2$ (79:21%) according to Witty and Minchin (1998), using an electrochemical H_2 sensor (Qubit System[®] Inc., Canada) as described by González *et al.* (2001) with a flow rate of 500 ml min^{-1} (Figure 31). The detector was calibrated with high purity gases (Praxair, Madrid, Spain) using a gas mixer (Air Liquid, Madrid, Spain) flowing at the same rate as the sampling system. ANA was expressed as μmol of H_2 produced per gram of nodule DM in one minute ($\mu\text{mol } H_2 \text{ g}^{-1} \text{ nodule DM min}^{-1}$). The ANA measurements were only performed in the nodulated alfalfa of both varieties (Aragón and Europe) fertilized with $0 \text{ mM } NH_4^+NO_3$, because the 15 mM treatments maintained just few nodules, those already formed the first month of growth without nitrate. The $15 \text{ mM } NH_4^+NO_3$ treatment inhibited new nodules formation, but those already formed remained active.



Figure 31. Apparent nitrogenase activity (ANA) estimated with an electrochemical H_2 sensor (Qubit System[®] Inc., Canada). Intact alfalfa nodulated plants in the open flow system.

Extraction of Nodule Plant and Bacteroid Fractions

Organic compound concentrations (soluble proteins and sugars, and starch) and enzyme activities were quantified from nodule extracts by grinding and filtering 0.25 g of fresh nodules mass collected at the same time that the leaves and other alfalfa organs at the harvest data (see page 43), in cold mortar using an extraction buffer containing 50 mM K-phosphate (pH 7,8) and 0.2 % (v/v) β -mercaptoethanol with 10 % (p/p) PVPP. The extract was filtered and centrifuged at 120 g (1,000 rpm) for 10 minutes at 4 ° C. The pellet was resuspended with 1 M KOH and stored in a freezer at -80 ° C until later starch analysis. Isolation of plant and bacteroid fractions were performed as described by Irigoyen *et al.* (1992). A differential centrifugation of soluble extract of the nodule, after the pellet isolation for starch measurements, allowed the isolation of two fractions: the plant fraction, which was the red color soluble fraction (presence of leghemoglobin), and pellet where the bacteroids were located. After two cleaning process of the pellet with extraction buffer, the bacteroids were resuspend again in the extraction buffer and they were subjected to a sonication To ensure no contamination between plant and bacterial fractions, phosphoenolpyruvate carboxylase was used as a plant fraction enzyme marker. No PEPC activity was detected for the bacteroid fraction.

Biochemical Measurements

Total soluble protein was determined in both plant and bacteroid fraction by the method of Bradford (1976). Starch was only evaluated in the pellet of the first centrifugation, corresponding to plant fraction material. Starch was measured according to Jarvis & Walker (1993). Total soluble sugars was only determined in plant fractions according to procedure of Yemm and Wills (1954). Total soluble proteins and sugars and starch determinations were performed with same protocols as for the leaf samples (see *Chapter I* page 46). The concentrations were expressed per g of nodule dry mass.

Nodule Enzymatic Determinations

Phosphoenolpyruvate carboxylase (PEPC, E.C. 4.1.1.31) activity was assayed spectrophotometrically in the nodule plant fraction by NADH oxidation. The reaction was performed as described by Deroche *et al.* (1983). PEPC activity was only detected

in the plant fraction of the nodule, and therefore it has been used as a positive plant fraction marker. Malate dehydrogenase (MDH, E.C. 1.1.1.37) and isocitrate dehydrogenase (ICDH, E.C. 1.1.1.42) enzymes were analyzed in both plant and bacteroidal fractions. The reaction medium and assay conditions were based on those by Vance and Stade (1984). MDH was measured via NADH oxidation at 340 nm, whereas ICDH was determined by tracing the NADPH change at 340 nm. Glutamate-oxaloacetate transaminase (GOT, E.C.2.6.1.1) was assayed only in the plant fraction as described by Bergmeyer (1974). All the enzyme activities were expressed on nodule dry matter basis and per minute.

Statistical Analysis

Statistical analysis of the data was made by three factor analyses (CO₂: ambient or elevated; nitrogen supply: 0 or 15 mM; and alfalfa variety: Aragón or Europe) of variance (ANOVA). Means $\pm$ standard errors (S.E.) calculated and significant differences between factors and interactions were calculated at 5% level of significance. When significant differences between treatments were significant according to the ANOVA analysis, least significant differences were evaluated by a Tukey's *t*-test, as found in the Statistical Package for Social Science (SPSS) (SPSS Inc., Chicago, USA) version 15.0 programs for Windows XP.

Results

Nodule Dry Matter

Effect of CO₂ and nitrogen supply (0 or 15 mM NH₄⁺NO₃) on plant nodule total dry matter in nodulated alfalfa Aragón and Europe during growing period is represented in the figure 32. It clearly shows the inhibitory effect of 15 mM NH₄⁺NO₃ application in new nodule formation (high nitrogen fed plants are in the lower part of the Aragón and Europe figures). The increase of CO₂ only enhanced the amount of nodules in the 0 mM of Aragón in the second harvest (52 days old plants).

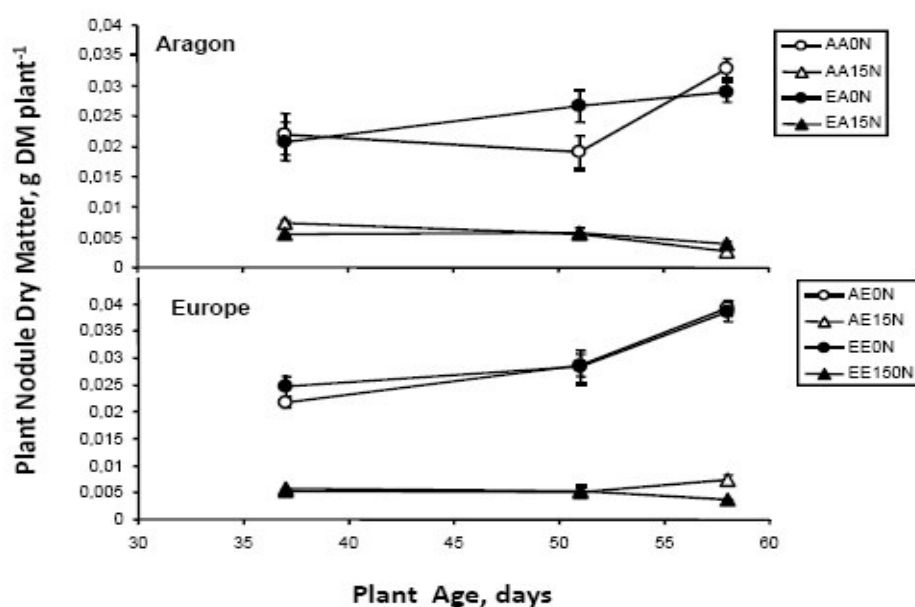


Figure 32. Effect of CO₂ (ambient, around 400 $\mu\text{mol mol}^{-1}$ or elevated 700 $\mu\text{mol mol}^{-1}$) and nitrogen (0 or 15 mM NH₄⁺NO₃) on plant nodule dry matter (g DM plant⁻¹) in two varieties (Aragón and Europe) of nodulated alfalfa (*Medicago sativa* L.) grown in greenhouse conditions during one month. Three harvests were performed when plants were 37, 52 and 58 days old. Abbreviations of the treatments were: AA0N, Ambient CO₂, Aragón alfalfa variety supplied with 0 mM; AA15N, Ambient CO₂, Aragón alfalfa variety supplied with 15 mM; EA0N, Elevated CO₂, Aragón alfalfa variety supplied with 0 mM; EA15N, Elevated CO₂, Aragón alfalfa variety supplied with 15 mM; AE0N, Ambient CO₂, Europe alfalfa variety supplied with 0 mM; AE15N, Ambient CO₂, Europe alfalfa variety supplied with 15 mM; EE0N, Elevated CO₂, Europe alfalfa variety supplied with 0 mM; EE15N, Elevated CO₂, Europe alfalfa variety supplied with 15 mM. Each value is the mean of 4 plants $\pm$ SE. Data were analysed statistically by three factors analyses of variance (ANOVA) test and least significant differences were evaluated by a Tukey's *t*-test.

Apparent Nitrogenase Activity (ANA)

The next parameters included in this chapter have only been measured in the 0 mM treatments, since the 15 mM ones showed just few nodules, as reported in the Figure 33. From the analyses of the first and second harvests not different conclusions were reached than those obtained from the third harvest analyses (58 days old plants). Therefore, in this chapter too, only the data of the third harvest are presented and discussed. Effect of CO₂ and the variety on ANA activity of nodulated alfalfa during the third harvest is shown in figure 33. The combined nitrogen (NH₄⁺NO₃), as expected, inhibited the production of nodules (figure above) and its ANA values were close to 0 (data not shown). Elevated CO₂ had a slight stimulatory effect in ANA values in both varieties (Aragón and Europe), but the differences were not significant. Aragón alfalfa variety always showed significantly higher ANA activities.

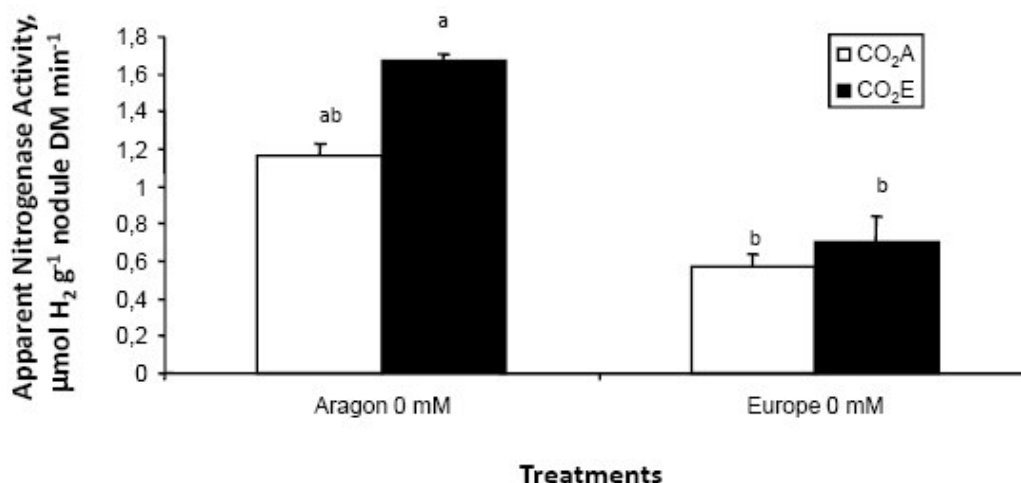


Figure 33. Effect of CO₂ (ambient, around 400 $\mu\text{mol mol}^{-1}$ or elevated 700 $\mu\text{mol mol}^{-1}$) on apparent nitrogenase activity ($\mu\text{mol H}_2 \text{ g}^{-1} \text{ nodule DM min}^{-1}$) in two varieties (Aragón and Europe) of nodulated alfalfa (*Medicago sativa* L.) grown in greenhouse conditions during one month. Data correspond to the third harvest (58 days old plants). Otherwise as for figure 32.

Nodule Plant Fraction Starch

Effect of CO₂ and variety on nodule plant fraction starch concentrations in the third harvest is represented in the figure 34. As in the case of the ANA activity, elevated CO₂ enhanced nodular starch concentration in cultivar Aragón. Nodule starch levels of alfalfa cultivar Europe were significantly lower than Aragón ones.

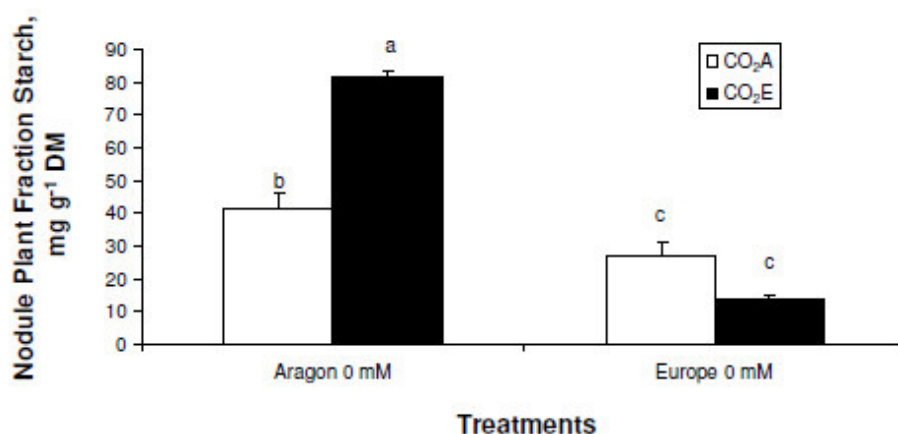


Figure 34. Effect of CO₂ (ambient, around 400 $\mu\text{mol mol}^{-1}$ or elevated 700 $\mu\text{mol mol}^{-1}$) on nodule plant fraction starch concentration (mg g⁻¹ DM) in two varieties (Aragón and Europe) of nodulated alfalfa (*Medicago sativa* L.) grown in greenhouse conditions during one month. Data correspond to the third harvest (58 days old plants). Otherwise as for figure 32.

Nodule Plant Fraction Total Soluble Sugars

Effect of CO₂ and variety on the total soluble sugars of the alfalfa nodule plant fraction (also called cytosol) in the third harvest is shown in figure 35. Not significant effect of the elevated CO₂ or of the varieties was detected in the soluble sugar concentration in the plant fraction.

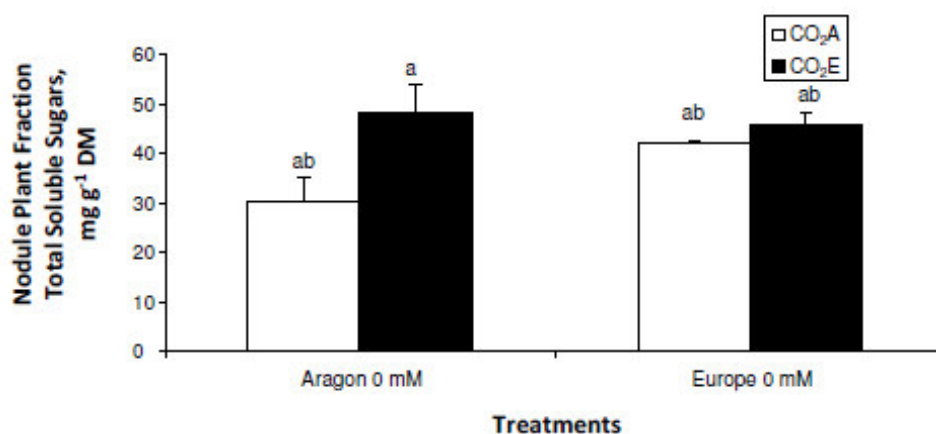


Figure 35. Effect of CO₂ (ambient, around 400 μmol mol⁻¹ or elevated 700 μmol mol⁻¹) on nodule plant fraction total soluble sugars (mg g⁻¹ DM) in two varieties (Aragón and Europe) of nodulated alfalfa (*Medicago sativa* L.) grown in greenhouse conditions during one month. Data correspond to the third harvest (58 days old plants). Otherwise as for figure 32.

Nodule Plant Fraction Total Soluble Proteins

Effect of CO₂ and the variety on total soluble proteins of the nodule plant fraction in the third harvest are represented in figure 36. Total soluble protein concentrations in the alfalfa nodule plant fraction were similar in all the treatments and in both varieties.

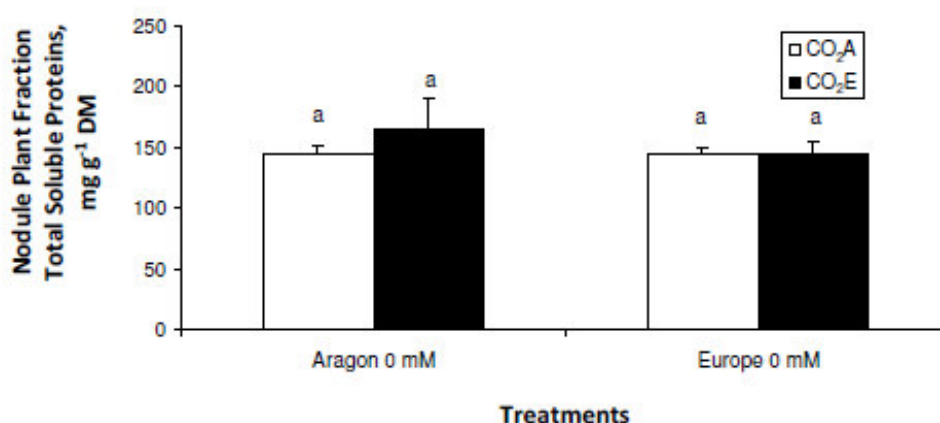


Figure 36. Effect of CO₂ (ambient, around 400 μmol mol⁻¹ or elevated 700 μmol mol⁻¹) on nodule plant fraction total soluble proteins (mg g⁻¹ DM) in two varieties (Aragón and Europe) of nodulated alfalfa (*Medicago sativa* L.) grown in greenhouse conditions during one month. Data correspond to the third harvest (58 days old plants). Otherwise as for figure 32.

Nodule Plant Fraction Malate dehydrogenase (MDH)

Total activity of malate dehydrogenase (MDH, E.C. 1.1.1.37) in the nodule cytosol or plant fraction in both alfalfa varieties in ambient and elevated CO₂ are presented in figure 37. As for total soluble proteins and sugars, MDH did not show remarkable changes.

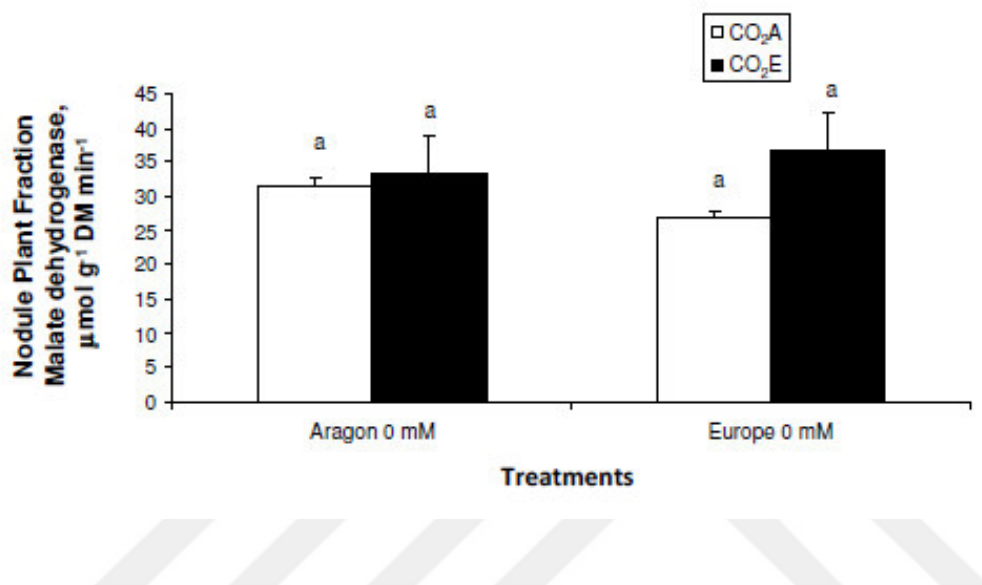


Figure 37. Effect of CO₂ (ambient, around 400 μmol mol⁻¹ or elevated 700 μmol mol⁻¹) on nodule plant fraction malate dehydrogenase activity (μmol g⁻¹ DM min⁻¹) in two varieties (Aragón and Europe) of nodulated alfalfa (*Medicago sativa* L.) grown in greenhouse conditions during one month. Data correspond to the third harvest (58 days old plants). Otherwise as for figure 32.

Nodule Plant Fraction Isocitrate dehydrogenase (ICDH)

Isocitrate dehydrogenase activity (ICDH, E.C. 1.1.1.42) of alfalfa nodule plant fraction in two varieties in the third harvest is shown in figure 38. The ICDH activities are slight higher in Europe nodule than in Aragón. However not effect of the elevated CO₂ was observed in any of both varieties.

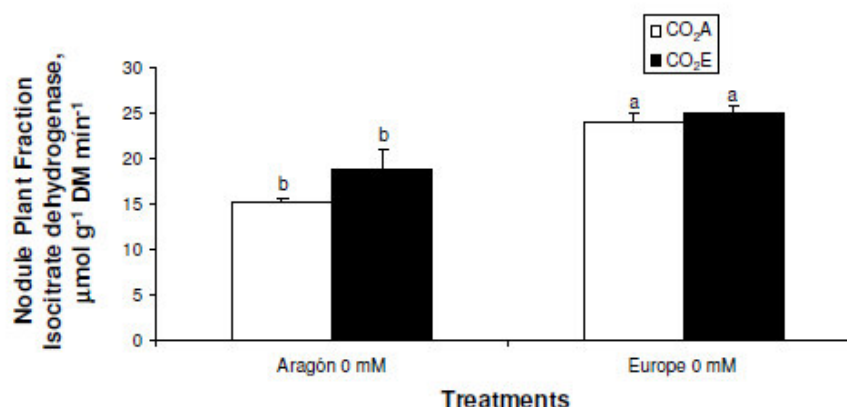


Figure 38. Effect of CO₂ (ambient, around 400 μmol mol⁻¹ or elevated 700 μmol mol⁻¹) on nodule plant fraction isocitrate dehydrogenase activity (μmol g⁻¹ DM min⁻¹) in two varieties (Aragón and Europe) of nodulated alfalfa (*Medicago sativa* L.) grown in greenhouse conditions during one month. Data correspond to the third harvest (58 days old plants). Otherwise as for figure 32.

Nodule Plant Fraction Phosphoenolpyruvate Carboxylase (PEPC)

Effect of CO₂ and variety on alfalfa nodule plant fraction phosphoenolpyruvate carboxylase activity (PEPC, E.C. 4.1.1.31) is represented in figure 39. PEPC activities are slight higher in Europe nodule than in Aragón. However not effect of the elevated CO₂ was observed in any of both varieties.

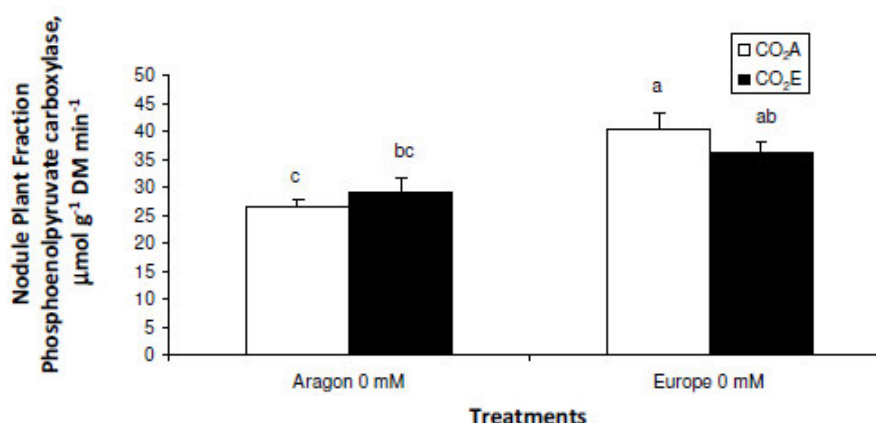


Figure 39. Effect of CO₂ (ambient, around 400 μmol mol⁻¹ or elevated 700 μmol mol⁻¹) on nodule plant fraction phosphoenolpyruvate carboxylase activity (μmol g⁻¹ DM min⁻¹) in two varieties (Aragón and Europe) of nodulated alfalfa (*Medicago sativa* L.) grown in greenhouse conditions during one month. Data correspond to the third harvest (58 days old plants). Otherwise as for figure 32.

Nodule Plant Fraction Glutamate-Oxaloacetate Transaminase (GOT)

Glutamate-oxaloacetate transaminase activity (GOT, E.C.2.6.1.1) of nodule plant fraction (vegetal fraction) of alfalfa Aragón and Europe varieties in the third harvest under ambient and elevated CO₂ conditions is represented in figure 40. GOT activity showed similar values in both alfalfa varieties and under both CO₂ conditions.

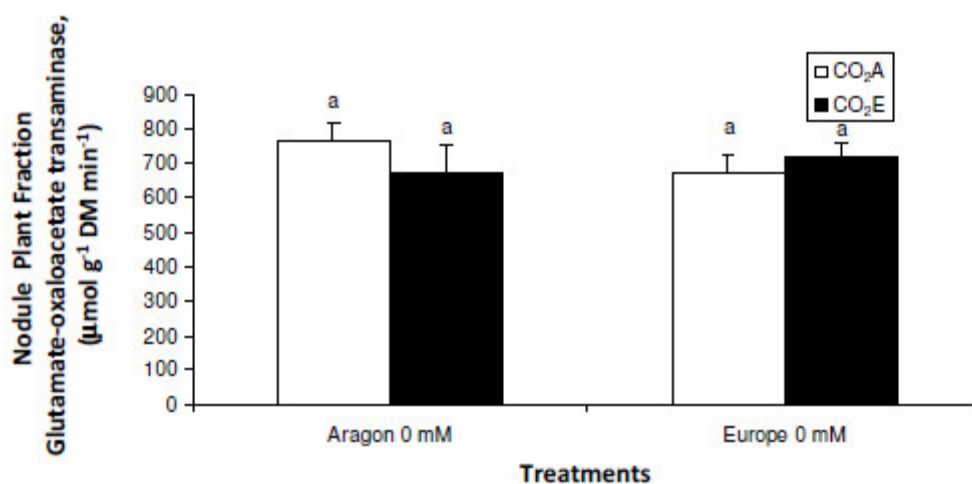


Figure 40. Effect of CO₂ (ambient, around 400 μmol mol⁻¹ or elevated 700 μmol mol⁻¹) on nodule plant fraction glutamate-oxaloacetate transaminase activity (μmol g⁻¹ DM min⁻¹) in two varieties (Aragón and Europe) of nodulated alfalfa (*Medicago sativa* L.) grown in greenhouse conditions during one month. Data correspond to the third harvest (58 days old plants). Otherwise as for figure 32.

Nodule Bacteroid Total Soluble Proteins

Effect of CO₂ and alfalfa varieties on total soluble proteins of alfalfa nodule bacteroid fraction in the third harvest (58 days old plants) is shown in figure 41. The concentrations of soluble proteins in the bacteroid fraction isolated from alfalfa nodules are significantly higher in Aragón than in Europe, and not appreciable effect of CO₂ is observed in any of both varieties.

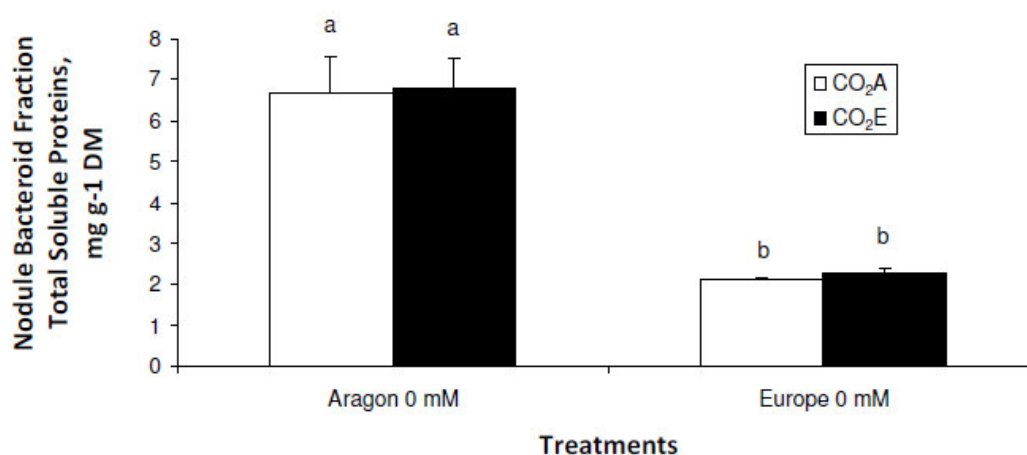


Figure 41. Effect of CO₂ (ambient, around 400 $\mu\text{mol mol}^{-1}$ or elevated 700 $\mu\text{mol mol}^{-1}$) on nodule bacteroid fraction total soluble protein (mg g^{-1} DM) in two varieties (Aragón and Europe) of nodulated alfalfa (*Medicago sativa* L.) grown in greenhouse conditions during one month. Data correspond to the third harvest (58 days old plants). Otherwise as for figure 32.

Nodule Bacteroid Malate dehydrogenase (MDH)

The malate dehydrogenase activity (MDH, E.C. 1.1.1.37) of the alfalfa nodule bacteroid fraction in the third harvest is represented in figure 42. MDH total activity was similar in all the treatments. No effect of the CO₂, or variety was observed.

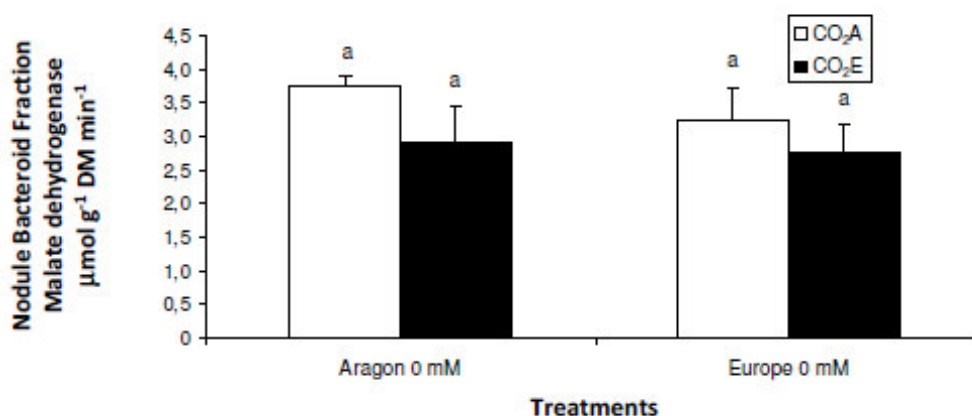


Figure 42. Effect of CO₂ (ambient, around 400 $\mu\text{mol mol}^{-1}$ or elevated 700 $\mu\text{mol mol}^{-1}$) on nodule bacteroid fraction malate dehydrogenase activity ($\mu\text{mol g}^{-1}$ DM min^{-1}) in two varieties (Aragón and Europe) of nodulated alfalfa (*Medicago sativa* L.) grown in greenhouse conditions during one month. Data correspond to the third harvest (58 days old plants). Otherwise as for figure 32.

Nodule Bacteroid Isocitrate dehydrogenase (ICDH)

Effect of CO₂ and alfalfa variety on isocitrate dehydrogenase activity (ICDH, E.C. 1.1.1.42) of alfalfa nodule bacteroid fraction in the third harvest is represented in figure 43. Total ICDH activities were similar in ambient CO₂ treatment in both varieties, however in elevated CO₂ conditions the response was opposite in the two varieties. While in Aragón ICDH was stimulated, in Europe significantly declined, reaching the lowest values.

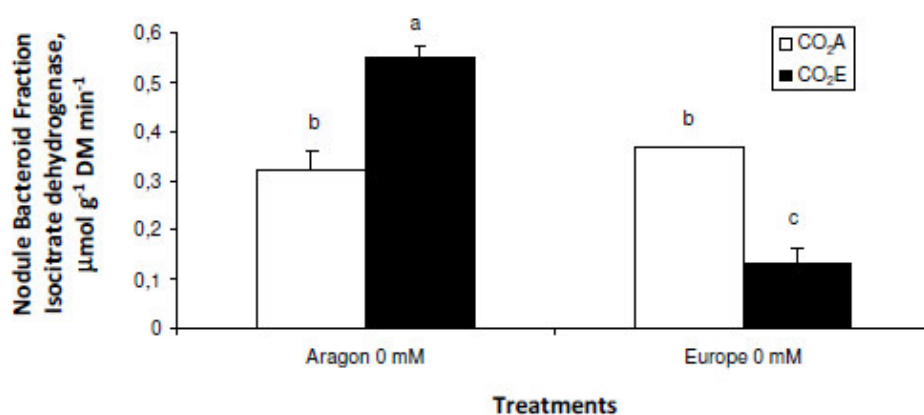


Figure 43. Effect of CO₂ (ambient, around $400 \mu\text{mol mol}^{-1}$ or elevated $700 \mu\text{mol mol}^{-1}$) on nodule bacteroid fraction isocitrate dehydrogenase activity ($\mu\text{mol g}^{-1} \text{DM min}^{-1}$) in two varieties (Aragón and Europe) of nodulated alfalfa (*Medicago sativa* L.) grown in greenhouse conditions during one month. Data correspond to the third harvest (58 days old plants). Otherwise as for figure 32.

Discussion

Previous study conducted by Sanz-Sáez *et al.*, (2010) and Erice *et al.*, (2006a), have demonstrated that the nitrogen supply from the nodules, in strictly N₂ fixing alfalfa plants, is limiting plant growth response to elevated CO₂. However when the nodulated alfalfa plants were fed with 15 mM NH₄⁺NO₃, the growth response to elevated CO₂ increased, as also has been seen in this study with Aragón (see Figure 11 page 49). As expected, the presence of 15 mM NH₄⁺NO₃ inhibited new nodule formation (Figure 32). The combined nitrogen application reduced by nearly 90% the nodule dry mass accumulation per plant.

When plants grow under elevated CO₂, it is expected a better supply of carbohydrates from the leaves to the rest of plant organs including roots. Therefore when nodulated plants were grown under CO₂ enriched atmosphere, the carbohydrates of the nodule tissues may be enhanced. This is the case of Aragón nodules; those nodules showed a significant increase in starch content in the plant fraction (Figure 34) and even total soluble sugars in the same fraction also increase, although the augmentation was not significant. Europe nodules, showed significant lower starch content, contrariwise to that expected when plants grow under elevated CO₂. Total soluble sugars in the plant fraction of Europe nodules showed similar values in ambient and elevated CO₂ treatment, and also similar to the values observed in Aragón. From those results it can be concluded, that the soluble sugar availability (sucrose, glucose, fructose, etc.), that are the immediate substrates for the nodule metabolism (glucolysis, oxidative pentose phosphate pathway, etc.), must maintain a critical level for correct functioning of the nodule. Nodule concentrations of starch, a storage carbohydrate, are more labile and therefore more susceptible to suffer changes under different CO₂ concentration growth conditions.

It could be expected that the nitrogen fixation activity, occurring in the infected cell of the nodules, might be enhanced as the carbohydrate supply from the plant to the nodules is improved under elevated CO₂ (Lee *et al.*, 2003; Cen and Layzell, 2004). Nitrogenase activity take place in the bacteroid fraction of the nodules and high supply of ATP and reducing power (NADPH) are needed for the enzyme complex. The higher supply of oxidable sugars to the nodules (high starch concentration in Aragón nodules)

is expected to ensure a better supply of organic acids to nourish bacteroid needs, and therefore improvement in N_2 fixation activity is a possible feature. However, apparent nitrogenase activity (ANA), was not affected by elevated CO_2 in any of both varieties of alfalfa (Figure 33). Similar results were reported by Sanz-Sáez *et al.*, (2012a) and Aranjuelo *et al.*, (2008) in Aragón variety. It is very remarkable the higher values of ANA measured in Aragón nodules compared with Europe (Figure 33). According to Twary and Heichel (1991), differences in ANA activity with similar nodule DM could be linked to differences in the C cost of N_2 fixation.

Low N_2 fixation activity (ANA) in the nodules could be due to many factors as: low nitrogenase activity, low nodule metabolism in plant and/or bacterial fractions, etc.). Therefore, the lower ANA activity observed in Europe (Figure 33), should be due to one of those factors or to the interaction between them. To verify which of those factors is implicated or explains the differences in ANA activity between the two alfalfa variety, the study included the analyses of soluble protein levels (as an indicator of the metabolic activity) and several enzymatic assays related to organic acid production (PEPC and MDH activities) (Udvardi and Day, 1997), ATP and NADPH production (MDH and ICDH) and the assimilation of the ammonia (GOT) produced by the nitrogenase activity.

Nodule plant fraction analysis included total soluble proteins, PEPC, MDH, ICDH and GOT enzymes activities. Soluble protein concentrations were similar in both alfalfa varieties and no effect of the elevated CO_2 treatment was observed (Figure 36). Therefore we can conclude that apparently the nodule plant metabolism showed adequate metabolic activities and the differences in ANA activities between Aragón and Europe nodules were not associated with this aspect of the nodule biochemistry. In fact, the activities of PEPC and MDH involved in malic organic acid production appear to supply adequate carbon substrates to the bacteroidal fraction (Figures 37 and 39). The same is true for the ammonia assimilation capacity of the plant fraction estimated by GOT activity (Figure 40). Both Aragón and Europe showed similar values of GOT activity, therefore the feedback negative regulation of nitrogenase activity by N_2 fixation end products (mainly ammonia or aminoacids) is discarded in Aragón and Europe nodules subjected to the applied treatments (Hartwig *et al.*, 1994; Almeida *et al.*, 2000). The reduction of isocitric acid to oxoglutarate in the nodule plant fraction

catalized by ICDH was also in good levels in both alfalfa varieties. Therefore, the lower apparent nitrogenase activity (ANA) in Europe nodules is not associated apparently with lower metabolic activities in the nodule plant fraction. Even if the starch concentrations were lower in Europe nodules, however the soluble sugars, intermediates used in the metabolism, remained high (Figure 35).

The bacteroidal fraction analyses revealed a very interesting fact. The soluble protein in the bacteroids of Europe nodules, was significantly lower than that measured in Aragón (Figure 41). This means, that the bacteroid general metabolism is less active in Europe bacteroids. This reduction in the bacteroid soluble proteins probably means significant lower levels of nitrogenase. Even if malate dehydrogenase activity (MDH) in the Europe nodule bacteroids (Figure 42) remained in similar levels as those observed in Aragón, however ICDH activity tended to be lower in Europe bacteroids, especially in the elevated CO₂ treatment (Figure 43). Therefore it can be concluded that the lower nitrogen fixation activity (estimated ANA) observed in Europe nodules as compared with Aragón ones, is probably associated with a lower activity (lower soluble protein levels) in the bacteroids of Europe nodules. Similar reduction in nodule nitrogen fixation activity associated with an inhibition in bacteroid metabolism has been reported by Aranjuelo *et al.*, (2008) in Aragón alfalfa nodules. However, a specific analysis of the protein profile in the bacteroid (by Western blot or PAGE techniques) should be performed in the future to confirm the hypothesis.

In summary, from the study it can be concluded that 15 mM NH₄NO₃ inhibited new nodules formation in both alfalfa varieties (Aragón and Europe). As the amount of nodule was very low in high nitrogen irrigated plants (15 mM), all the metabolic analysis were only performed in the 0 mM treatments. Elevated CO₂ treatment stimulated sugar supply to the nodules in Aragón, and nodule starch was increased. However no remarkable effects of elevated CO₂ on nodule metabolism (plant and bacteroid fractions) were observed, with the exception of a significant reduction in ICDH activity in Europe bacteroids. Nodules of Europe appear less efficient to fix N₂, according to the ANA rates. The main reason for such lower apparent nitrogenase activity seems to be the lower soluble protein in the bacteroids, probably affecting metabolic activity including nitrogenase. However this conclusion should be confirmed with additional and more specific measurements (western or PAGE analyses). Perhaps

one of the reasons to explain the lower responsiveness of Europe plants to elevated CO_2 may be the lower N_2 fixing efficiency of its nodules. However, the absence of response to elevated CO_2 in this variety when plants were supply with 15 mM NH_4NO_3 seems to be some how contradictory with the previous assessment.



General Conclusions

- 1- Generally, it is seen that elevated CO₂ (from 700 to about 400 $\mu\text{mol mol}^{-1}$) stimulated plant growth, especially in combination with nitrogen (15 mM NH₄⁺ NO₃) in Aragón alfalfa variety. In general the plant aboveground organs (mainly leaves) were more responsive to the applied treatments, especially elevated CO₂, than belowground organs (roots and nodules).
- 2- Europe alfalfa variety seems to be less sensitive to the applied treatments (elevated CO₂ and NH₄⁺ NO₃ supply) than Aragón. However, despite the positive effect of elevated CO₂ was not visible in the total plant biomass accumulation, it was significant in the leaves of Aragón and Europe.
- 3- Net photosynthetic rates of both alfalfa varieties measured at growth conditions (A_{growth}), that is at 400 $\mu\text{mol mol}^{-1}$ for ambient treatments and at 700 $\mu\text{mol mol}^{-1}$ for elevated CO₂ treatments, showed similar values, therefore it is concluded that Aragón and Europe nodulated alfalfa plants suffer a photosynthesis acclimation after 28 days of elevated CO₂ treatment in both nitrogen supplies (0 and 15 mM NH₄⁺ NO₃). Stomatal closure induced by elevated CO₂, may partly explain some photosynthesis limitation. Nevertheless, the significantly high CO₂ concentrations in the leaf mesophyll tissues, suggest that other non stomatal limitations to the photosynthetic activity may be acting (such as Rubisco activity, Rubisco concentration, RuBP regeneration, etc.).
- 4- Rubisco extractable activity isolated from the leaves, Rubisco subunits (large and small subunits of Rubisco, RbcL and RbcS respectively), and total soluble protein of the leaves, did not show any appreciable sign of photosynthesis acclimation. Therefore, the possible reasons for photosynthesis acclimation could be the inactivation of the Rubisco enzyme *in situ*, and/or supply limitation of the CO₂ acceptor RuBP.
- 5- Nitrogenase activity, estimated by the apparent nitrogenase activity (ANA), showed consistent differences between nodules of both varieties of alfalfa. Aragón, according the highest ANA values, appeared the most efficient fixing

N₂. The lower values of ANA activity in Europe nodules were probably due to the lower activity of the bacteroids. Europe nodule bacteroids showed 50% lower soluble proteins than Aragón ones. This low protein concentration, probably affect nitrogenase protein. No remarkable effect of elevated CO₂ was evident in the nodule metabolism, with the exception of a higher starch concentration in Aragón nodules.

- 6- The lower responsiveness of Europe alfalfa variety to the applied treatments, especially elevated CO₂, may be related to the lower efficiency to fix N₂.
- 7- The absence of differences between biomass accumulation in Europe growing in 0 or 15 mM NH₄⁺ NO₃ under elevated CO₂ made the N limitation hypothesis weak for this alfalfa variety



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