Universidad de Navarra

Facultad de Ciencias

ESTUDIO DEL EFECTO DE LA INTERACCIÓN ENTRE AUMENTO DE CO_2, TEMPERATURA Y SIMBIOSIS CON DIFERENTES CEPAS DE *Sinorhizobium meliloti* EN LA FOTOSÍNTESIS, FIJACIÓN DE N_2 Y CALIDAD DE LA ALFALFA (*Medicago sativa* L. cv. Aragón)

Tesis presentada por
Álvaro Sanz Sáez de Jáuregui
para optar al Grado de Doctor por la
Universidad de Navarra

Servicio de Publicaciones de la Universidad de Navarra

ISBN 84-8081-121-8

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Memoria presentada por D. Álvaro Sanz Sáez de Jáuregui para aspirar al grado de Doctor en Ciencias Biológicas por la Universidad de Navarra

El presente trabajo ha sido realizado bajo nuestra dirección en el Departamento de Biología Vegetal, Sección Biología Vegetal y autorizamos su presentación ante el Tribunal que lo ha de juzgar.

Pamplona, 1 de Septiembre de 2011

Dr. Juan José Irigoyen Iparrea

Dr. Gorka Erice Soreasu

AGRADECIMIENTOS

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En primer lugar, me gustaría agradecer al Departamento de Biología Vegetal de la Universidad de Navarra y en especial a su director, Dr. Manuel Sánchez-Díaz por haberme brindado la oportunidad de realizar esta tesis doctoral. Gracias por depositar su confianza en mi, por su paciencia en los momentos más difíciles y por preocuparse en todo momento de la correcta realización de esta tesis doctoral.

Asimismo, me gustaría agradecer a la Asociación de Amigos de la Universidad de Navarra, al Ministerio de Ciencia e Innovación (MICINN BFU2008-01405) y a la Fundación Caja Navarra por financiar esta investigación.

A mi director de tesis, el Dr. Juan José Irigoyen Iparrea por permitirme la oportunidad de realizar esta tesis bajo su dirección. Gracias por su atención diaria, por su paciencia, dedicación y compromiso y por estar siempre cuando se le necesita. Gracias por enseñarme los valores de trabajo en equipo, respeto a los demás y a ser positivo ante los problemas derivados del trabajo.

Al Dr. Gorka Erice Soreasu también director de esta tesis, por todo su apoyo, enseñanzas y tiempo invertido y porque sin él esta tesis no hubiese sido posible. Gracias por su ayuda en los invernaderos, laboratorio y en Granada y sobre todo, gracias por su amistad.

Al Dr. Ricardo Aroca Álvarez por permitir mi estancia en la Estación Experimental del Zaidín (CSIC) en Granada y por su preocupación en que la estancia fuese una de las mejores etapas del doctorado. Gracias por su esfuerzo, paciencia y amistad.

Al Dr. Iker Aranjuelo Mitxelena por su colaboración en esta tesis, por su esfuerzo, enseñanzas, correcciones y por los momentos pasados en el trabajo y fuera de él, gracias.

Al Dr. Fernando Muñoz del Centro de Investigación y Tecnología Agroalimentaria de Aragón por su ayuda prestada al realizar los análisis de calidad del forraje de alfalfa.

A la Dra. Jone Aguirreolea por su implicación en esta tesis, por su paciencia y por sus consejos a la hora de escribir.

A los Doctores Nieves Goicoechea, Inmaculada Pascual, María del Carmen Antolín y Fermín Morales por sus consejos y atención.

A Amadeo Urdiain, Mónica Oyarzun y Laura González porque sin su ayuda técnica en los invernaderos de gradiente y sin su amistad esta tesis no habría alcanzado su fin.

Al resto de mis compañeros, Marouane, Carmentxu, Mafer, Johan, Iñaki, Ivan, María y Maria Laura por la ayuda que me han prestado y por hacer agradables estos cuatro años.

A los compañeros de Granada, Ana, José, Miguel, Bea, Gloria, Bea Japón, Jacob y Chon por toda su ayuda en el laboratorio y por hacerme pasar unos bonitos meses en esa ciudad.

Mi especial agradecimiento a mis padres, Mamen y Deme, por su apoyo incondicional, su cariño y por estar siempre ahí para escucharme y aconsejarme.

A mis abuelos, los que están y los que no, por su tiempo jugando y enseñándome a ser buena persona, gracias por haberme dado tanto cariño.

A mis tíos de verdad y a los de mentira, a mis primos y amigos de la familia por vuestro apoyo, cariño y preocupación. A mi tío Fernando, que ya no está, porque sin él no hubiese estudiado en Pamplona y no hubiese realizado esta tesis.

A mis amigos de Vitoria, Khorne, Rober, Iñigo, Borja, JJ, Paco, Iker, Isa, Barbara, Silvia, Patri y Esti, y a los de Pamplona, Marta, Ana, África, Clara, Ixai, Laura, Rubén, Itxaso, Diego, Escalada, Elisa, Amaya, Mikel, Dani, Iñigo y Javi por vuestras horas de diversión, charlas y viajes.

Y a todas aquellas personas que aunque no están nombradas explícitamente aquí han formado parte de mi vida hasta este momento.

Gracias a todos,

Álvaro

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INTRODUCCIÓN GENERAL

Introducción General

Según el informe de la División de Población de Naciones Unidas (UNPD, 2010), el crecimiento de la población mundial aumentará, de 7000 millones en la actualidad, a 9300 millones en el año 2050, esperándose 10000 millones para finales de este siglo. Para sustentar este aumento demográfico, la disponibilidad de alimentos tendrá que incrementarse de la misma manera. Para asegurar el suministro de alimentos a la población mundial desde el punto de vista del crecimiento sostenible, la producción de alimentos debería aumentarse sin incrementar la superficie cultivada (Kern, 2002). Desde el inicio de la revolución industrial en el siglo XVIII, el desarrollo económico y demográfico ha ido de la mano del aumento de la concentración de CO_2 atmosférico (Krausmann *et al.,* 2009). La concentración de CO_2 ha ido incrementándose desde el año 1750, cuando era de 280 μmol mol^{-1}, hasta la actualidad, en la que alcanza los 392 μmol mol^{-1}, y sigue aumentado a un ritmo de 1,9 μmol mol^{-1} por año (Intergovernmental Panel on Climate Change; IPCC, 2007). De seguir esta progresión, el IPCC, prevé que para finales de este siglo la concentración atmosférica de CO_2 será cercana a las 700 μmol mol^{-1}. El CO_2 es considerado uno de los gases invernadero que tiene más efecto sobre el aumento de la temperatura y junto con otros gases de efecto invernadero podría provocar que para finales de este siglo la temperatura media del planeta subiera 4 ºC (IPCC, 2007).

La exposición de plantas C_3 a periodos cortos de CO_2 elevado, suele derivar en un aumento de la fotosíntesis y, por tanto, de la producción (Daepp *et al.,* 2000). Esto es debido a que la enzima encargada de fijar el CO_2 en las plantas, la ribulosa-1,5-bisfosfato carboxilasa/oxigenasa, esta limitada por la concentración actual de CO_2 (Drake *et al.,*1997). El CO_2 elevado favorece la carboxilación de la ribulosa-1,5-bisfosfato (RuBP) sobre la fotorrespiración, potenciando así la eficiencia de esta enzima y, por tanto, de la fotosíntesis (Andrews and Lorimer, 1987).

La exposición de las plantas a largos periodos de CO_2 elevado suele dar como resultado un descenso de la fotosíntesis (Long *et al.,* 2004; Aranjuelo *et al*., 2005; 2009; Erice *et* al., 2006; Ainsworth and Rogers, 2007), también conocido como aclimatación fotosintética (Saralabai *et al*., 1997). Esta aclimatación puede deberse a dos causas: 1)

limitaciones estomáticas debidas a un descenso de la conductancia foliar en condiciones de CO_2 elevado (Sánchez-Díaz *et al*., 2004), o 2) limitaciones metabólicas, normalmente asociadas a una caída de la actividad de carboxilación de la rubisco (Aranjuelo *et al*., 2005; Erice *et al*., 2006) y/o a un descenso en el contenido de rubisco en condiciones de CO_2 elevado (Urban, 2003; Aranjuelo *et al*., 2005).

La respuestas de las plantas al CO_2 elevado no siempre es idéntica; algunos autores han encontrado que la fotosíntesis de plantas C_3 se estimulaba (Curtis et al. 2000; Ellsworth *et al.,* 1995; Jackson *et al.,* 1995), mientras que otros han observado plantas fuertemente aclimatadas (Ainsworth and Rogers 2007; Aranjuelo *et al.,* 2009; Erice *et al.,* 2006; Lee *et al.,* 2001; Rogers and Ellsworth, 2002). Estas diferencias pueden ser debidas a que en distintas especies vegetales en diferentes condiciones, responden de manera dispar al enriquecimiento en CO_2. Si bien también puede responder a que no existe un consenso claro en qué parámetro utilizar para definir la aclimatación.

La respuesta de las plantas al CO_2 elevado, depende de su capacidad para generar nuevos sumideros o ampliar los ya existentes, y eliminar el exceso de carbono que se genera en estas condiciones (Ceulemans, 1997; Aranjuelo *et al*., 2009). Cuando la planta no es capaz de evitar la acumulación de carbono se produce el descenso de la fotosíntesis para equilibrar el balance entre las fuentes y sumideros de carbohidratos (Thomas and Strain, 1991). Muchos autores sugieren que la aclimatación fotosintética debida a una capacidad sumidero insuficiente de la planta se debe a un aporte deficiente de nitrógeno (Rogers and Ainsworth, 2006; Aranjuelo *et al*., 2005; 2008). Se ha observado, que este aumento de carbono en condiciones de CO_2 elevado, se corresponde con la acumulación de carbohidratos no estructurales en la hoja, que inhibirían la expresión de genes fotosintéticos, entre ellos los de la rubisco, reduciendo así la fotosíntesis (Geiger *et al*., 1999).

En plantas superiores la rubisco es un octámero, formado por cuatro subunidades de alto peso molecular (RLS, subunidad grande) y cuatro de bajo peso molecular (RSS, subunidad pequeña) y solo es activa cuando se unen 4 a 4 (Jordan and Ogren, 1981). Teniendo en cuenta que la subunidad pequeña de la rubisco, suele estar en menor concentración que la grande y que tiene menos afinidad por el CO_2, descensos en la expresión génica y contenido de la subunidad pequeña, como ocurre en ocasiones

en condiciones de CO_2 elevado, contribuiría a la caída de la fotosíntesis (Andersson and Blacklund 2008). El comportamiento de la expresión génica y contenido de la rubisco en condiciones de CO_2 elevado, es dependiente de la especie (Moore *et al.,* 1999). En alfalfa no se han realizado experimentos en los que se estudie el efecto del enriquecimiento en CO_2 sobre la expresión y concentración de la rubisco, a excepción de Bertrand *et al.* (2007b) que estudiaron el efecto del CO_2 sobre la expresión de la subunidad pequeña de la alfalfa y sus resultados no fueron concluyentes. Por lo tanto, es necesario profundizar en el estudio de la expresión y contenido de las dos subunidades de rubisco en condiciones futuras de CO_2 elevado.

Como se ha mencionado anteriormente, el nitrógeno es un factor que limita la respuesta de la planta al CO_2 elevado. Un suministro deficiente de N, en exposiciones a largo plazo de CO_2 elevado, puede causar una menor disponibilidad de N en la planta, produciendo un descenso en la fotosíntesis y reduciendo, por lo tanto, su crecimiento potencial (Peterson *et al*., 1999; Luo *et al*., 2004). El descenso de la concentración de N en los tejidos vegetales crecidos en condiciones de CO_2 elevado, puede deberse a una dilución del N inducida por la acumulación de carbohidratos, de material estructural en la hoja, y/o por el aumento en la demanda de N por parte de la planta. (Ellsworth *et al*., 2004). La baja disponibilidad de N en la planta causa la inmovilización del carbono, su acumulación en forma de carbohidratos y la aparición de la aclimatación fotosintética (Ellsworth *et al*., 2004). Otro factor que limita la respuesta de la planta al CO_2 elevado, es la temperatura. La temperatura influye en el crecimiento modificando el metabolismo vegetal, especialmente la respiración y la fotosíntesis. La respiración a la oscuridad se incrementa de forma lineal hasta los 25-30 ºC. El aumento sostenido por encima de estas temperaturas haría que las reacciones metabólicas se acelerasen produciéndose un agotamiento de sustratos y metabolitos que llevaría a una reducción de la respiración. Un exceso de temperatura puede llevar a la desnaturalización de proteínas, inhibición enzimática y variación en la fluidez de las membranas que llevaría a la inhibición de las reacciones metabólicas. Sin embargo, a temperaturas por debajo de las óptimas, se dan limitaciones en la reacciones metabólicas, entre las que se incluye la fotosíntesis, debido a que las enzimas no alcanzan la energía mínima de activación. Las altas temperaturas afectan negativamente a la fijación de CO_2, debido a que reducen su solubilidad respecto a la del O_2 y descienden la especificidad de la rubisco por el CO_2, aumentando

la fotorrespiración con respecto a la asimilación de CO_2. Sin embargo, el CO_2 elevado inhibe competitivamente la fotorrespiración, mostrándose clave a la hora de aumentar la fotosíntesis neta en condiciones de temperatura elevada. En estas condiciones (temperatura ambiente + 4 ºC), se g cha observado que la alta temperatura provoca un aumento en el contenido de almidón en hojas, que se relaciona con la bajada de la fotosíntesis (Erice *et al.,* 2006).

La alfalfa está distribuida por todo el mundo, y es un cultivo importante desde el punto de vista ecológico y económico por su uso como forraje (Bagavathiannan and Van Acker, 2009). Es una leguminosa que establece simbiosis con bacterias del género *Sinorhizobium* capaces de fijar N_2. En esta asociación la planta proporciona carbohidratos a la bacteria a cambio de compuestos nitrogenados derivados de la reducción de N_2 atmosférico. Esta relación es beneficiosa para el medio ambiente puesto que supone un aporte extra de N para la planta y el suelo, mejorando así su estructura y aumentando el aporte de materia orgánica (Bourgeois, 1990). Algunas leguminosas fijadoras de N_2 suelen mostrar una mayor estimulación de la fotosíntesis en condiciones de CO_2 elevado. Esto es debido a que la simbiosis es un potente sumidero de carbono que podría evitar la acumulación de carbohidratos en la hoja y por lo tanto el desequilibrio entre fuentes y sumideros que lleva a la aclimatación (Udvardi and Day, 1997). Además, el aumento en la disponibilidad de carbono para el nódulo en condiciones de CO_2 elevado, podría conllevar un mayor aporte de energía, que aumentaría la actividad nodular incrementando la cantidad de N disponible para el crecimiento de la planta (Vance and Heichel, 1991). Sin embargo, algunos estudios en los que se relaciona la fotosíntesis con la fijación de N_2 (Hartwig and Sadowsky, 2006), sugieren que un descenso en la actividad rubisco como consecuencia de la exposición a largo plazo al CO_2 elevado, podría estar asociado a una caída en la demanda de N que provocaría un descenso de la actividad nodular (Erice *et al.,* 2011a, b). Bertrand et al. (2007a, b) comprobaron que la interacción entre distintas cepas de *Sinorhizobium meliloti* con diferentes variedades de alfalfa podía variar la respuesta de la planta a diferentes factores ambientales como la tolerancia al frío o la respuesta de la planta al CO_2 elevado. Por otro lado, Kulkarni y Nautiyal, (1999), comprobaron cómo la respuesta del algarrobo a las altas temperaturas era dependiente de la cepa de *Sinorhizobium* con la que se inoculaba.

La disponibilidad de N no solo tiene relación con la respuesta de la planta al CO_2 elevado, sino también con la calidad del forraje (Owensby *et al.,* 1996; Erice *et al.,* 2011a, b). La reducción en el contenido de N de la parte aérea en condiciones de CO_2 elevado, conduce a una disminución en la calidad forrajera, entendida como pérdida de contenido en proteína cruda (Campbell *et al.,* 2000; Owensby *et al.,* 1996; Wand *et al.,* 1999). Según Morgan *et al.* (2001) el CO_2 elevado también afecta negativamente a la digestibilidad del forraje, a través del aumento del contenido de fibras poco digestibles como la lignina (Owensby *et al.,* 1996). El incremento en el contenido de fibras, puede significar el descenso en la digestibilidad y palatabilidad del forraje, así como la necesidad del aumento de la ingesta para conseguir la misma nutrición (Van Soest, 1982).

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OBJETIVOS GENERALES

Objetivos Generales

El objetivo general del estudio realizado, fue investigar la respuesta de plantas de alfalfa inoculadas con diferentes cepas de *Sinorhizobium meliloti* (102F78, 102F34 y 1032GMI) frente al aumento de CO_2 y temperatura. Los objetivos parciales planteados y desarrollados en los distintos capítulos de la memoria han sido:

1. Comprobar si plantas de alfalfa exclusivamente fijadoras de N_2 inoculadas con la cepa 102F78 tienen un aporte deficiente de N desde el nódulo que limita la respuesta al CO_2 elevado, desencadenando así la aclimatación fotosintética y examinar si la adición de compuestos nitrogenados (NH_4NO_3) es capaz de inhibir la aclimatación fotosintética. .....….….....Capítulo 1

2. Estudiar la relación existente entre el metabolismo de la hoja y del nódulo, así como la proteómica nodular en condiciones de CO_2 elevado durante la aclimatación fotosintética en plantas de alfalfa inoculadas con la cepa 102F78. …….…Capítulo 2

3. Comprobar si la inoculación con diferentes cepas de *S. meliloti* modifica la eficiencia fijadora de N_2 de la simbiosis *Sinorhizobium*-alfalfa, y si dicha eficiencia resulta afectada en condiciones de CO_2 elevado y alta temperatura, y cuál es su repercusión en la producción en dos estaciones diferentes (verano y otoño). ……..…Capítulo 3

4. Estudiar si la inoculación con diferentes cepas de *S. meliloti* evita la aclimatación fotosintética al CO_2 elevado, y definir el parámetro de intercambio gaseoso y bioquímico más sensible para detectar dicha aclimatación. ….....Capítulo 4

5. Analizar las variaciones diarias de la fotosíntesis, así como el contenido y expresión génica de la rubisco en plantas de alfalfa crecidas en CO_2 ambiente y elevado durante la aclimatación fotosintética. …….……..…….……..Capítulo 5

6. Estudiar si el CO_2 elevado, la alta temperatura y la inoculación con diferentes cepas de *S. meliloti* afectan la calidad y digestibilidad del forraje de alfalfa. ...…Capítulo 6

CAPÍTULO 1

Photosynthetic down-regulation under elevated CO_2 exposure can be prevented by nitrogen supply in nodulated alfalfa

La aclimatación fotosintética en condiciones de CO_2 elevado puede evitarse con la aplicación de nitrógeno en plantas de alfalfa noduladas

Publicado en *Journal Plant Physiology*
doi:10.1016/j.jplph.2010.06.015

Photosynthetic down-regulation under elevated CO_2 exposure can be prevented by nitrogen supply in nodulated alfalfa

Resumen

Una concentración de CO_2 atmosférico mayor que la actual puede aumentar la fotosíntesis y el crecimiento de las plantas. Sin embargo, después de una larga exposición, las plantas se aclimatan y muestran una reducción en la actividad fotosintética (llamada regulación a la baja o aclimatación fotosintética), que puede causar una merma en la producción. Algunos autores, sugieren que esta regulación a la baja está relacionada con la disponibilidad de nutrientes y más específicamente con una insuficiencia en la capacidad sumidero de C, debida a un aporte limitante de N. En este trabajo, hemos investigado si la disponibilidad de N puede prevenir o reducir la aclimatación fotosintética de la alfalfa (*Medicago sativa* L.) nodulada. Para alcanzar dicho objetivo, estudiamos el efecto de la adición de tres niveles diferentes de NH_4NO_3 (0, 10, 15 mM) a plantas de alfalfa noduladas de 30 días de edad, que se expusieron durante un mes en cámaras de crecimiento a CO_2 ambiente (aproximadamente 400 μmol mol^{-1}) o elevado (700 μmol mol^{-1}). Después de 2 semanas de exposición al CO_2 elevado, no se observaron diferencias significativas ni en el crecimiento, ni en la fotosíntesis en ninguno de los tratamientos. Sin embargo tras 4 semanas de crecimiento, las plantas exclusivamente fijadoras de N_2 (0 mM NH_4NO_3), mostraron descensos en la fotosíntesis y velocidad máxima de carboxilación de la rubisco (Vc_{max}). La aclimatación fotosintética en estas plantas se asoció con un desequilibrio de la relación C/N, como lo reflejan las concentraciones de carbohidratos no estructurales y el N. Por otro lado, las plantas regadas con 15 mM NH_4NO_3 y crecidas en condiciones de CO_2 elevado, mantuvieron altas tasas fotosintéticas gracias a un mejor ajuste de la relación C/N. El tratamiento con dosis intermedias de N, 10 mM NH_4NO_3, también mostró aclimatación fotosintética, pero en menor grado que el de 0 mM NH_4NO_3. Este estudio demuestra claramente cómo el aporte externo de N, puede reducir o incluso eliminar la aclimatación fotosintética al CO_2 elevado, al aumentar la capacidad sumidero de C e incrementar la disponibilidad de N.

Summary

Increasing atmospheric CO_2 concentration is expected to enhance plant photosynthesis and yield. Nevertheless, after long-term exposure, plants acclimate and show a reduction in photosynthetic activity (called down-regulation), which may cause a reduction in potential yield. Some authors suggest that down-regulation is related to nutrient availability and more specifically to an insufficient plant C sink strength caused by limited N supply. In this paper we have tested whether or not N availability prevents down-regulation of photosynthesis in nodulated alfalfa plants (*Medicago sativa* L.). To do so, we have studied the effect of the addition of different levels of NH_4NO_3 (0, 10, 15 mM) on 30 days-old nodulated alfalfa plants exposed to ambient (approximately 400 μmol mol^{-1}) or elevated CO_2 (700 μmol mol^{-1}) during one month in growth chambers. After two weeks of exposure to elevated CO_2 no significant differences were observed in plant growth and photosynthesis rates. After 4 weeks of treatment, exclusively N_2 fixing alfalfa plants (0 mM NH_4NO_3) showed significant decreases in photosynthesis and Vc_{max}. Photosynthetic down-regulation of those plants was caused by the C/N imbalance as reflected by the carbohydrate and N data. On the other hand, plants supplied with 15 mM NH_4NO_3 grown under elevated CO_2 maintained high photosynthetic rates thanks to their better C/N adjustment. The intermediate N treatment, 10 mM NH_4NO_3, also showed photosynthetic down-regulation but to a lower degree than 0 mM treatment. The present study clearly shows that external N supply can reduce or even avoid acclimation of photosynthesis to elevated CO_2 as a consequence of the increase in C sink strength associated with N availability.

Key words: Carbon dioxide, isotopic composition, nitrogen, *Medicago sativa* (alfalfa), photosynthetic down-regulation.

Abbreviations: $\delta^{13}C$, C isotope composition; DW, dry weight; C_i, intercellular CO_2; IPCC, Intergovernmental Panel on Climate Change; A_{net}, net photosynthesis; A_{growth}, net photosynthesis measured in growth CO_2 conditions concentration; C_{new} %, percentage of new C; PPFD, photosynthetic photon flux density; Vc_{max}, rubisco maximum velocity of carboxylation; SLA, specific leaf area; TOM, total organic matter; TSP, total soluble protein; TSS, total soluble sugars.

Introduction

Continued emissions of CO_2 by burning of fossils fuels are expected to increase global atmospheric CO_2 concentrations. The Intergovernmental Panel on Climatic Change (IPCC) predicts that the CO_2 concentration may be between 660 and 790 μmol mol^{-1} from 2060 to 2090 (IPCC, 2007). Human activities do not only affect the CO_2 concentration, but also alter the global nitrogen (N) cycle by increasing the inputs of fixed forms of N, mainly because of the extensive use of chemical fertilizers (Vitousek *et al.,* 1997).

This increase in CO_2 concentration may enhance the potential net photosynthesis for C_3 plants, because ribulose-1,5-bisphophate carboxylase/oxygenase (rubisco) is not CO_2 saturated at the current concentration (Drake *et al.,*1997). This enzyme catalyses photosynthesis and photorespiration reaction, but the current atmospheric CO_2 concentration is insufficient to saturate rubisco in C3 plants. Thus, an increase in ambient CO_2 increases the leaf internal CO_2 concentration and the CO_2/O_2 ratio at the rubisco site, which favours carboxylation rather than oxygenation of ribulose-1,5-bisphosphate (RuBP) (Andrews and Lorimer, 1987). When analyzing the effect of CO_2 concentration on plant growth, it must be considered that the photosynthetic response will depend on the genetically determined potential and on the relative availability of other co-limiting environmental factors. Moreover, other environmental variables such as temperature, soil N and water content, atmospheric humidity, and solar radiation (PPDF intensity), will interact with the CO_2 effect (Ainsworth and Long, 2005; Aranjuelo *et al*., 2005a, b).

Furthermore, many studies have shown that photosynthesis acclimates to elevated CO_2 over long-term experiments (Long *et al.,* 2004; Aranjuelo *et al*., 2005b; 2009; Erice *et al.,* 2006a; Ainsworth and Rogers, 2007). This process, often referred as 'down regulation' (Saralabai *et al*., 1997), may be due to two main causes: 1) Stomatal limitations like those resulting from lower leaf conductance at an elevated CO_2 concentration (Sánchez-Díaz *et al*., 2004) or 2) Metabolic limitation, usually attributable to a reduced carboxylation activity (Aranjuelo *et al*., 2005b; Erice *et al*., 2006b) and/or a reduced rubisco amount at elevated CO_2 (Urban, 2003; Aranjuelo *et al*., 2005b).

One of the parameters that could affect photosynthetic down-regulation is the modification of the source-sink ratio (Urban, 2003). Growth responses to elevated CO_2 depend on the ability of plants to develop new sinks or expand the existing ones (Ceulemans, 1997; Aranjuelo *et al*., 2009). Thomas and Strain (1991) suggested that when plants exposed to elevated CO_2 have limitations to increasing C sink strength, plants decrease their photosynthetic rates to balance C source activity and sink capacity. From this point of view, the reduction in photosynthetic rates would be conditioned by plant ability to develop new sinks (e.g. new vegetative or reproductive structures, enhanced respiratory rates), or to expand the storage capacity or growth rate of existing sinks (Lewis *et al.,* 2002). Many studies suggest that down-regulation is the consequence of an insufficient sink plant capacity (Ainsworth *et al*., 2004; Morgan *et al*., 2001) caused by a limited N supply (Rogers and Ainsworth, 2006). In this context it has also been reported that leaves grown under elevated CO_2 concentration show higher carbohydrate content than those grown under ambient CO_2 (Geiger *et al*., 1999). The enhancement of non-structural carbohydrates and the inhibition of the expression of genes that encode for different photosynthetic apparatus compounds are suppressed through the possible increased hexose cycling within the leaf, resulting in decreased photosynthetic capacity and a notable decrease in the amount of rubisco (Drake *et al*., 1997; Moore *et al*., 1999). A very useful tool to investigate the source-sink strength, developed during the last decades, is the stable isotope analysis. In fact labelling with $^{13}C/^{12}C$ isotopes has emerged as an optimum tool to study the allocation of C compounds between different parts of plants, especially in herbaceous species grown under controlled environment conditions (Avice *et al*., 1996; Gebbing *et al*., 1998; Nogés *et al*., 2004; Aranjuelo *et al*., 2008). Plants grown in environments with modified isotopic composition will incorporate the tracer into C-containing compounds (Avice *et al.,* 1996; Nogues *et al*., 2004; Aranjuelo *et al*., 2008) providing essential information about the C sinks to which the recently fixed C is delivered. The C labelling method provides, in a non-invasive and reversible way, information about the role of source-sink strength of the different plant tissues.

N availability is a critical factor, limiting plant growth and increasing the response to elevated CO_2 conditions. As it has been reported, at elevated CO_2 concentration, a low soil N supply could limit photosynthesis, leading to diminished plant N availability in the long term (Peterson *et al*., 1999; Luo *et al*., 2004). A decrease in leaf N under elevated CO_2

is a consequence of dilution by carbohydrate accumulation, leaf structural material and increases in plant internal demands for N (Ellsworth *et al*., 2004). Furthermore, N dilution may cause the immobilization of C in source tissue leading to carbohydrate accumulation. This is why many authors have related photosynthesis acclimation to a low nutrient supply. Alfalfa (*Medicago sativa* L.) is a forage crop that establishes a symbiotic relationship with N_2-fixing bacterium (*Sinorhizobium meliloti* L). According to Udvardi and Day (1997), because legumes form this symbiotic association there is an extra sink for exchange of additional C with the nodule to enhance N_2 fixation in these plants. The larger C sink strength implies that legumes will have a lower tendency towards photosynthetic down-regulation (Ainsworth and Rogers, 2007). As previously described by Aranjuelo *et al.* (2008), strictly N_2-fixing alfalfa (Aranjuelo *et al*., 2005*b*) grown under elevated CO_2 concentrations showed down-regulated photosynthesis and reduced leaf N concentration, probably because of the absence of strong sinks. The fall in leaf N content suggests that nodulated alfalfa plants are not competent to support the N demand at elevated CO_2 concentrations and therefore the N deficiency could cause the absence of strong sinks, and lead to down-regulation. In fact, previous data in nodulated alfalfa growth under elevated CO_2 showed that nodule activity is not improved and even some parameters such as plant and bacteroid soluble proteins and key metabolic activities like malate dehydrogenase, phosphoenolpyruvate carboxylase, and others, declined significantly in elevated CO_2 grown plants (Aranjuelo *et al*., 2008).

In order to test if higher N availability prevents photosynthetic down-regulation in alfalfa, we studied the effect of the addition of increasing doses of NH_4NO_3 (0, 10, 15 mM) to nodulated alfalfa plants grown at elevated CO_2, and thus tested if nodule activity is sufficient to prevent down-regulation in strictly N_2-fixing conditions. Some studies demonstrated that elevated doses of NH_4NO_3 (until 20 mM) had a negative impact on new nodule development, but did not have any effect on nodule structure or activity when they were already formed (Becana *et al*., 1985a; b). Because of these studies, we applied increasing doses to find a NH_4NO_3 dose that could be compatible with nodule activity and the disappearance of down-regulation. The $^{13}CO_2$ labelling was conducted at the plant level in order to obtain a more real understanding of C partitioning and sink strength in the whole plant. To achieve these objectives, we studied N and C mobilization and composition by isotopic discrimination. Also, we analysed the A/C_i curves, net

photosynthesis and total soluble protein concentration to prove the effect of NH_4NO_3 on photosynthetic down-regulation. The accumulation of carbohydrates was checked by measuring total sugar and starch concentration and the ^{13}C isotopic composition ($\delta^{13}C$) of total organic matter (TOM) over a period of one month.

Materials and methods

Plant material and experimental design

Alfalfa (*Medicago. sativa* L. cv. Aragón) seeds were sterilized in a solution of $HgCl_2$ (0.1% w/v) and germinated in Petri dishes. One week later, 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, plants were inoculated three times with *Sinorhizobium meliloti* strain 102F78 (The Nitragin Co. Milwaukee, WI, USA) and irrigated alternatively with N-free Hoagland solution (Hoagland and Arnom, 1950) and distilled water (three times per week) to avoid salt accumulation in the substrate. Plants were grown in a greenhouse at 25/15 ºC (day/night) with a 14h photoperiod under natural daylight, supplemented with fluorescent lamps (Sylvania Decor 183, Professional-58W, Germany) providing a photosynthetic photon flux density (PPFD) of 400 µmol m^{-2} s^{-1}.

When plants were 30 days old, they were transferred to growth chambers (Conviron PGV 36, Winnipeg, Canada) and randomly assigned to six treatments according to the NH_4NO_3 concentration (0, 10 and 15 mM) and atmospheric CO_2 concentration (Ambient- approximately 400 µmol mol^{-1} or -Elevated- 700 µmol mol^{-1}). Therefore the following treatments were considered: 0 mM of NH_4NO_3 and 400 µmol mol^{-1} (0A), 0 mM of NH_4NO_3 and 700 µmol mol^{-1} (0E), 10 mM of NH_4NO_3 and 400 µmol mol^{-1} (10A), 10 mM of NH_4NO_3 and 700 µmol mol^{-1} (10E), 15 mM of NH_4NO_3 and 400 µmol mol^{-1} (15A), 15 mM of NH_4NO_3 and 700 µmol mol^{-1} (15E). The NH_4NO_3 was added as a supplement to the Hoagland N-free solution. In order to avoid salt accumulation in the pots, every third irrigation was deionised water. The conditions in the growth chambers were 25/15 ºC (day/night), 40% RH, 14h photoperiod and 600 µmol m^{-2} s^{-1} PPFD.

Growth parameters

One month-old plants were harvested after two, and four weeks (37, 44, 51 and 60 day-old plants respectively). Plant organs were separated into leaves, stems, roots and nodules and weighed. Dry mass (DM) of each organ was obtained after drying in an oven at 60 ºC for 48h. Leaf area was measured using an automatic leaf area meter (Li-3000, LICOR, NE, USA). The specific leaf area (SLA) was calculated as leaf area/leaf dry mass.

Gas exchange parameters

Gas exchange parameters were measured in fully expanded young leaves corresponding to the second and fourth weeks using a LI-COR 6400 portable photosynthesis system (LICOR biosciences. Lincoln, Nebraska, USA). The gas exchange response to CO_2 was measured from 60 to 1400 µmol mol^{-1} CO_2 at 1400 µmol m^{-2} s^{-1} PPFD provided by LED light. Measurements started at 400 µmolmol^{-1} of CO_2, decreased stepwise until 250, 100, 0. µmol mol^{-1} and restarted at 400 and increased stepwise until 700, 850, 1000 and 1400. µmol mol^{-1}. Net photosynthesis (A_{net}) and leaf conductance (g) were calculated as described by Long & Hallgreen (1985). The A_{net}/Ci curve measurements were used to assess the maximum rate of carboxylation (Vc_{max}), employing the mathematic model developed by Ethier and Livingston (2004) and Sharkey et al. (2007). The photosynthetic N use efficiency (PNUE) was measured as the ratio between net photosynthesis (A_{net}, µmol CO_2 m^{-2} min^{-1}) and N content (g N m^{-2}).

Biochemical measurements

Leaf total soluble proteins (TSP), total soluble sugars (TSS) and starch concentration were quantified by grinding and filtering 200 mg of leaf fresh weight from leaves harvested from the second and fourth weeks of treatment, in a cold mortar using an extraction buffer containing 50 mM K-phosphate (pH 7.5). The extract was filtered and centrifuged at 28,710 g for 15 min at 4ºC. The TSP and TSS quantifications were performed in supernatant, whereas starch was measured using the pellet as described by Jarvis & Walker (1993). TSP levels were measured using the method of Bradford (1976), while the TSS determinations followed the procedure of Yemm and Willis (1954).

Since reduction of SLA by elevated CO_2 could underestimate the physiological and biochemical parameters when represented per mass unit, we have expressed all measured parameters on area basis.

C labelling procedure

The C labelling was conducted during the second month where the plants were exposed to elevated CO_2 conditions in the above described growth chambers. The isotopic composition ($\delta^{13}C$) of the growth chamber in the elevated CO_2 module was modified by mixing of commercial CO_2 ($\delta^{13}C$ *ca.* -44.2±0.2‰) provided by Air Liquide S.A. (Zamudio, Spain) with the ambient air ($\delta^{13}C$ *ca.* -10.3±0.3‰) resulting in a $^{13}CO_2$ isotopic composition of $\delta^{13}C$ *ca* -23.45±0.2‰ (see below for details on $\delta^{13}C$ collection and measurement). The procedure for air (ambient and source bottle) sampling and the subsequent $\delta^{13}C$ analyses is described in detail by Nogués *et al*. (2008).

Plant tissue total organic matter (TOM) measurements: C isotopic composition ($\delta^{13}C$, %C and %N)

Leaf, stem, root and nodule samples were collected corresponding to the second and fourth weeks of treatment, and therefore given data correspond to whole plant. Samples were dried at 60ºC for 48 h and analysed for the isotopic composition together with the %C and %N of TOM. One mg of ground sample was used for each determination, and 8 replicates were analysed for each treatment (4 experimental and 2 laboratory replicates). The $^{13}C/^{12}C$ ratios (*R*) of plant material were determined using an elemental analyzer (EA1108, Series 1, Carlo Erba Instrumentazione, Milan, Italy) coupled to an isotope ratio mass spectrometer (Delta C, Finnigan, Mat., Bremen, Germany) operating in continuous flow mode.

The $^{13}C/^{12}C$ ratios (*R*) of air samples were determined by Gas Chromatography-Combustion-Isotope Ratio Mass Spectrometry (G12C-C-IRMS). Briefly, water vapour and oxygen from gas samples were removed and the carbon dioxide, argon, and nitrogen gases were separated by gas chromatography (Agilent 6890 Gas Chromatograph) coupled to an isotope ratio mass spectrometer Delta^{plus} via a GC-C Combustion III interphase (ThermoFinnigan). The column used was a 30 m x 0.32 mm i.d. GS-GASPRO (J. and W. Scientific, USA). The carrier gas was helium at a flow rate of 1.2 mL min^{-1}. The injection port temperature was 220^{o}C. The oven temperature was kept at 60^{o}C during the entire run. Injection was conducted in the split mode (injected volume 0.3 mL, split flow 20 mL min^{-1}).

Results of both the TOM and air samples were expressed as $\delta^{13}C$ values, using a secondary standard calibrated against Vienna Pee Dee Belemnite calcium carbonate (VPDB) and the analytical precision was about 0.1‰:

$$\delta^{13}C = \left(\frac{R_{sample}}{R_{standard}} \right) - 1$$

Carbon isotope discrimination ($\Delta^{13}C$) of the analysed tissues was calculated according to the description of Farquhar *et al*. (1989).

$$\Delta^{13}C = (\delta^{13}C_a - \delta^{13}C_p)/[1 + (\delta^{13}C_p/1000)]$$

where $\delta^{13}C_a$ and $\delta^{13}C_p$ refer to air and the plant carbon isotope compositions, respectively.

The proportion of "new" carbon (C_{new}) from the TOM analysed samples was calculated as:

$$p = \frac{\delta^{13}C_{labelled} - \delta^{13}C_{non\text{-}labelled}}{\delta^{13}C_{fixed} - \delta^{13}C_{non\text{-}labelled}}$$

where $\delta^{13}C_{non\text{-}labelled}$ and $\delta^{13}C_{lebelled}$ are the carbon isotope compositions of TOM non-labelled and labelled plants, respectively; $\delta^{13}C_{fixed}$ is the isotope composition of fixed CO_2, which is given by $\frac{\delta_o - \Delta^{13}C}{1 + \Delta^{13}C}$, where δ_o is the isotope composition of the outlet air (Nogués *et al.,* 2004).

Statistical analysis

Statistical analysis was made by two-factors analyses of variances (ANOVA, factorial 2 x 3) (SPSS v.15.0). Taking CO_2 as the first factor (ambient CO_2, around 400 µmol mol^{-1}; and elevated, 700 µmol mol^{-1}) and NH_4NO_3 doses as the second factor (0 mM, 10mM and 15 mM), six treatments in total, with four experimental replicates per treatment . Significant differences between factors and interactions were calculated at 5%, 1% and 0.1%. When differences between treatments (CO_2 and/or NH_4NO_3 and/or interactions)

were significant according to the ANOVA analysis, least significant difference were evaluated using low significant different test (LSD) ($P < 0.05$).

Results

Elevated CO_2 did not modify plant dry weight (DW) at second week (44 days-old), but at the fourth week (60 days-old) plant production was enhanced by elevated CO_2 (Table 1) (*CO_2 F = 7.945, P = 0.011*). NH_4NO_3 increased DW at the second and fourth weeks (Table 1) (*NH_4NO_3 F = 8.85, P = 0.002* and *NH_4NO_3 F = 24.64, P < 0.001* respectively). Increasing doses of NH_4NO_3 reduced nodule DW in both weeks (second and fourth) of treatment (*NH4NO3 F = 21.29, P < 0.001* and *NH4NO3 F = 27.66, P < 0.001* respectively). At the second and fourth weeks, total leaf area was increased by NH_4NO_3 (*NH_4NO_3 F = 29.9, P < 0.001 and NH_4NO_3 F =123.90, P < 0.001* respectively), but elevated CO_2 did not affected it (Table 1). At the second and fourth weeks of treatment the root/shoot ratio was not modified by elevated CO_2 or NH_4NO_3, with the exception of the 0E versus 0A treatment in the fourth week of treatment (Table 1). At the second week, the specific leaf area (SLA) decreased with elevated CO_2 (Table 1) except for plants irrigated with 15 mM NH_4NO_3 (*CO_2 x NH_4NO_3 F = 7.13, P = 0.005*). Nevertheless, at the fourth week, the SLA decreased with elevated CO_2 in all treatments (*CO_2 F = 137.08, P < 0.001*), but was enhanced with increasing doses of NH_4NO_3 (*NH_4NO_3 F = 7.57, P = 0.004*).

TREATMENTS	WEEK 2					WEEK 4				
	Total Dry Weight	Nodule Dry Weight	Total Leaf Area	Root/shoot ratio	SLA	Total Dry Weight	Nodule Dry Weight	Total Leaf Area	Root/shoot ratio	SLA
0A	0.32 ± 0.11 **b**	7.6 ± 1.5 **a**	17.7 ± 2.2 **c**	0.44 ± 0.02	311 ± 12.9 **bc**	1.04 ± 0.21 **d**	31,1 ± 8,5 **a**	88.11 ± 9.8 **c**	0.46 ± 0.02	339.4 ± 18.8 **a**
0E	0.14 ± 0.02 **c**	4.6 ± 1.2 **b**	11.7 ± 2.1 **c**	0.41 ± 0.03	196.7 ± 3 **d**	1.37 ± 0.40 **d**	34,1 ± 2,5 **a**	69.5 ± 7.2 **c**	0.31 ± 0.09	183 ± 12.5 **c**
10A	0.44 ± 0.03 **ab**	1.1 ± 0.2 **c**	56.2 ± 3.6 **a**	0.31 ± 0.04	386.7 ± 19.4 **a**	2.83 ± 0.29 **c**	0,9 ± 0,1 **b**	312.5 ± 22 **ab**	0.36 ± 0.04	335.8 ± 7.9 **a**
10E	0.62 ± 0.04 **a**	0.8 ± 0.1 **c**	56.6 ± 6 **a**	0.39 ± 0.03	277.3 ± 7.1 **c**	4.08 ± 0.15 **ab**	11,3 ± 0,7 **b**	293 ± 9.8 **ab**	0.36 ± 0.02	227.2 ± 9.1 **b**
15A	0.47 ± 0.10 **ab**	1.3 ± 0.4 **c**	39.1 ± 8.4 **b**	0.26 ± 0.07	324.9 ± 7.8 **b**	3.28 ± 0.16 **bc**	0,01 ± 0,0014 **b**	321.4 ± 19.4 **a**	0.38 ± 0.01	360.3 ± 5.3 **a**
15E	0.48 ± 0.07 **ab**	1 ± 0.04 **c**	49.3 ± 7.4 **ab**	0.35 ± 0.04	294.6 ± 16.5 **bc**	4.56 ± 0.82 **a**	0,2 ± 0,0025 **b**	264.9 ± 27.1 **b**	0.35 ± 0.03	261.9 ± 18.9 **b**
CO_2	ns	ns	ns	ns	***	*	ns	ns	ns	***
NH_4NO_3	**	***	***	ns	***	***	***	***	ns	**
CO_2*NH_4NO_3	ns	ns	ns	ns	**	ns	ns	ns	ns	ns

Table 1: Effect of the interaction between CO_2 (A, Ambient CO_2 = approximately 400 µmol mol^{-1} and E, Elevated CO_2 = 700 µmol mol^{-1}) and different doses of NH_4NO_3 (0, 10 and 15 mM) in total dry weight (g $plant^{-1}$), nodule dry weight (g $plant^{-1}$), total leaf area (cm^2 g^{-1}), root/shoot ratio and specific leaf area (SLA, cm^2 g^{-1}) in nodulated alfalfa plants at the age of 44 (Week 2) and 60 days (Week 4). Values represent the mean ± SE; n = 4. Statistical analysis was made by a two factors Analysis of the Variance (ANOVA), see the results in the bottom of the table. The meaning of symbols used in ANOVA were: *, Significant difference at 5%. ** Significant difference at 1%, *** significant difference at 0.1%. When significant differences were detected in ANOVA, LSD analysis was applied. Means followed by the same letter are not significantly different (P > 0.05) according to LSD test parameters When no significances were detected in ANOVA analyses, no LSD test was applied, and therefore no significance letters were included.

Net photosynthesis (A_{net}) measured at 400 µmol mol^{-1} CO_2 after the second week of treatment (44 days-old plants) (Fig. 1A) was decreased by NH_4NO_3 *(NH_4NO_3 F = 4.97, P = 0.028*) but not significantly affected by CO_2 (Fig. 1A). When A_{net} was measured at 700 µmol mol^{-1} (Fig. 1C) no effect of CO_2 or NH_4NO_3 was observed. After 4 weeks of treatments (60 days-old plants) A_{net} measured at 400 µmol mol^{-1} (Fig. 1B), was unaffected by NH_4NO_3, but decreased in plants grown under elevated CO_2 (*CO_2 F = 16.99, P = 0.002*). At 700 µmol mol^{-1}, A_{net} (Fig. 1D) was not significantly modified by NH_4NO_3, but decreased in plants grown under elevated CO_2 (*CO_2 F = 11.89, P = 0.005*).

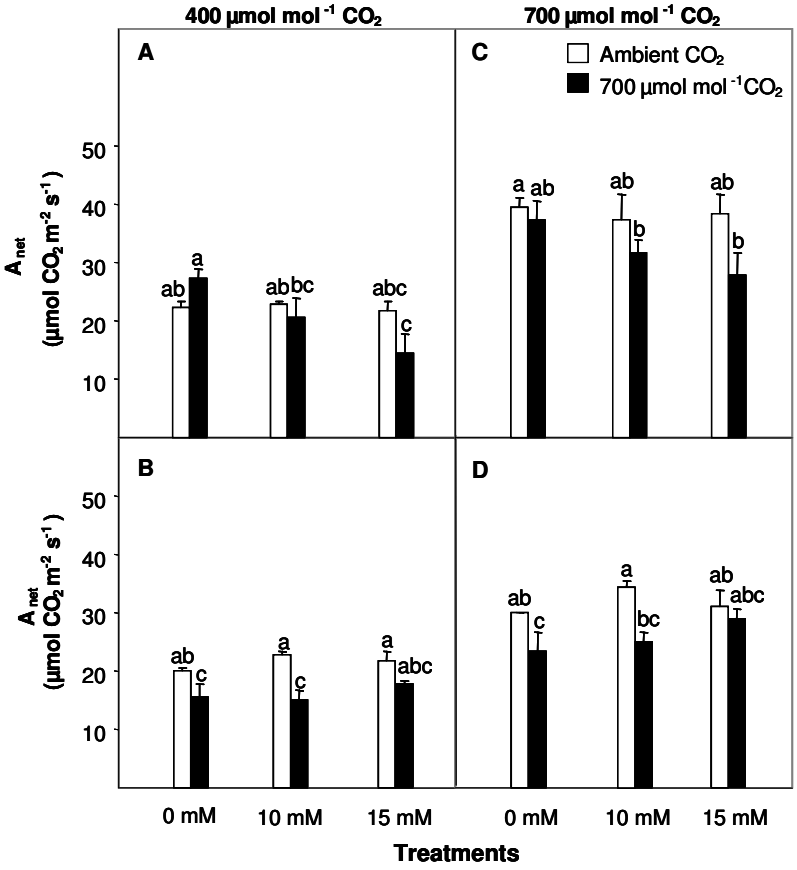

Figure 1: Effect of CO_2 (ambient CO_2, approximately 400 µmol mol^{-1} and elevated CO_2, 700 µmol mol^{-1}) and increasing doses of NH_4NO_3 (0, 10 and 15 mM) on net photosynthesis (A_{net}, µmol CO_2 m^{-2} s^{-1}) of nodulated alfalfa at the age of 44 (**A** and **C**, corresponding to 2 weeks of treatment) and 60 days (**B** and **D**, corresponding to 4 weeks of treatment), at 400 and 700 µmol mol^{-1} CO_2. Bars represent the mean ± SE; n = 4. Bars with the same letter are not significantly different (P > 0.05) according LSD test.

Net photosynthesis measured in growth CO_2 conditions (A_{growth}) at the second and fourth weeks was enhanced by elevated CO_2 (*CO_2 F = 14.12, P = 0.001* and CO_2 *F = 8.31, P < 0.015* respectively) (Table 2).

	WEEK 2			WEEK 4		
TREATMENTS	**Vc_{max}**	**A_{growth}**	**Ci**	**Vc_{max}**	**A_{growth}**	**Ci**
0A	116.5 ± 6.3	21.5 ± 6.5 **b**	150.1 ± 6.2 **c**	94.6 ± 2.9 **b**	20 ± 6.5 **b**	259.5 ± 18.5 **b**
0E	98.1 ± 3.7	34.1 ± 9.6 **a**	448.1 ± 6.9 **ab**	82.2 ± 6.7 **c**	24.6 ± 9.6 **ab**	476.2 ± 26.3 **a**
10A	111.6 ± 6.5	21 ± 1.2 **b**	192.5 ± 27.6 **c**	121.6 ± 5 **a**	22.6 ± 1.2 **ab**	223.3 ± 16.2 **b**
10E	116.1 ± 12	27.8 ± 3.6 **ab**	492.4 ± 17.1 **a**	97.6 ± 6 **ab**	25.1 ± 3.6 **ab**	411 ± 39 **a**
15A	122.7 ± 7,8	20.3 ± 8.8 **b**	172.3 ± 12.0 **c**	108.9 ± 15.4 **a**	21 ± 8.8 **b**	221.3 ± 19.7 **b**
15E	113.6 ± 8,8	27.2 ± 1.8 **ab**	429.6 ± 13.9 **b**	102.4 ± 5.9 **a**	29.1 ± 1.8 **a**	412 ± 10 **a**
CO_2	ns	***	***	*	**	***
NH_4NO_3	ns	*	*	*	ns	ns
CO_2*NH_4NO_3	ns	ns	ns	ns	ns	ns

Table 2: Effect of the interaction between CO_2 (A, Ambient CO_2 = approximately 400 µmol mol^{-1} and E, Elevated CO_2 = 700 µmol mol^{-1}) and different doses of NH_4NO_3 (0, 10 and 15 mM) in leaf carboxylation capacity (Vc_{max}, µmol CO_2 m^{-2} s^{-1}), net photosynthesis at growth CO_2 concentration (A_{growth}, µmol CO_2 m^{-2} s^{-1}) and mesophyll CO_2 concentration (Ci, µmol CO_2 mol^{-1}), in nodulated alfalfa plants at the age of 44 (Week 2) and 60 days (Week 4). Values represent the mean ± SE; n = 4. Statistical analysis was made by a two factors Analysis of the Variance (ANOVA), see the results in the bottom of the table. The meaning of symbols used in ANOVA were: *, Significant difference at 5%. ** Significant difference at 1%, *** significant difference at 0.1%. When significant differences were detected in ANOVA, LSD analysis was applied. Means followed by the same letter are not significantly different (P > 0.05) according to LSD test parameters. When no significances were detected in ANOVA analyses, no LSD test was applied, and therefore no significance letters were included.

At the second week, rubisco maximum velocity of carboxylation (Vc_{max}) was not modified by CO_2 or the different NH_4NO_3 doses (Table 2). Nevertheless, at the fourth week, elevated CO_2 reduced Vc_{max} in the 0 mM NH_4NO_3 treatment (*CO_2 x NH_4NO_3 F = 4.73, P = 0.047*) but increase in the others NH_4NO_3 treatments (*NH_4NO_3 F = 3.93, P = 0.049*). Elevated CO_2 at the second and fourth weeks enhanced intercellular CO_2 concentration (Ci; *CO_2 F = 415.64, P < 0.001; CO_2 F = 99.77, P < 0.001,* respectively) (Table 2). At the second week the Ci was enhanced by NH_4NO_3 (*NH_4NO_3 F = 3.83, P = 0.023*). Opposite to this, at the fourth week NH_4NO_3 had no effect on Ci (Table 2).

At the second week, the TSP concentration (Table 3) was enhanced by elevated CO_2 when the plants were irrigated with 15 mM NH_4NO_3. Nevertheless, when plants were irrigated with 0 mM NH_4NO_3, TSP concentration decreased (*CO_2 x NH_4NO_3 F = 7.24, P = 0.001*). At the fourth week, TSP was not modified by CO_2 or NH_4NO_3. At the second week of treatment, photosynthetic N use efficiency (PNUE) (Table 3) was enhanced in plants irrigated with 0mM NH_4NO_3 and grown under elevated CO_2 (*CO_2 x NH_4NO_3 F = 61.149, P = 0.02*). At the fourth week of treatment, plants irrigated with 0mM NH_4NO_3 and grown at elevated CO_2 had an enhanced PNUE (Table 3). Conversely, plants irrigated with 15 mM NH_4NO_3 and grown at elevated CO_2 decreased PNUE (*CO_2 x NH_4NO_3 F = 4.851, P = 0.029*). At the second week of treatment, the total soluble sugar (TSS) concentration was enhanced by CO_2 with 10 mM NH_4NO_3 (Table 3). Plants grown at ambient CO_2 and irrigated with 10 and 15 mM NH_4NO_3 showed lower TSS concentrations than irrigated with 0 mM NH_4NO_3 (*CO_2 x NH_4NO_3 F = 7.14, P = 0.005*).

TREATMENTS	WEEK 2				WEEK 4			
	TSP	PNUE	TSS	Starch	TSP	PNUE	TSS	Strach
0A	3.2 ± 0.2 **ab**	14.3 ± 0.6 **b**	0.9 ± 0.10 **bc**	2.8 ± 0.10 **b**	2.3 ± 0.1	13.3 ± 0.3 **bc**	1.3 ± 0.02 **b**	1.4 ± 0.10 **b**
0E	1.8 ± 0.2 **c**	24.3 ± 2.3 **a**	1.1 ± 0.08 **ab**	5.5 ± 0.30 **a**	2.0 ± 0.3	16.0 ± 1.0 **ab**	1.8 ± 0.09 **a**	2.4 ± 0.60 **a**
10A	1.8 ± 0.2 **c**	15.3 ± 2.8 **b**	0.6 ± 0.04 **e**	1.8 ± 0.07 **c**	2.8 ± 0.3	14.4 ± 1.4 **a**	0.8 ± 0.05 **d**	0.6 ± 0.20 **c**
10E	2.3 ± 0.4 **bc**	15.0 ± 1.2 **b**	1.3 ± 0.10 **a**	2.3 ± 0.30 **b**	2.5 ± 0.2	15.0 ± 0.4 **ab**	1.7 ± 0.07 **a**	2.7 ± 0.10 **a**
15A	2.0 ± 0.3 **c**	13.5 ± 1.5 **b**	0.7 ± 0.03 **de**	1.4 ± 0.06 **c**	2.5 ± 0.1	14.2 ± 0.6 **ab**	1.0 ± 0.05 **cd**	0.6 ± 0.20 **c**
15E	3.6 ± 0.4 **a**	14.8 ± 1.5 **b**	0.9 ± 0.03 **cd**	1.8 ± 0.03 **c**	1.9 ± 0.1	10.8 ± 0.3 **c**	1.1 ± 0.10 **bc**	0.2 ± 0.06 **c**
CO_2	ns	*	***	***	ns	ns	***	***
NH_4NO_3	ns	*	*	***	ns	*	***	***
CO_2*NH_4NO_3	***	*	***	**	ns	*	***	***

Table 3: Effect of the interaction between CO_2 (A, Ambient CO_2 = approximately 400 µmol mol^{-1} and E, Elevated CO_2 = 700 µmol mol^{-1}) and different doses of NH_4NO_3 (0, 10 and 15 mM) in leaf total soluble protein (TSP, g m^{-2}), photosynthetic N use efficiency (PNUE, µmol CO_2 g^{-1} N min^{-1}) total soluble sugars (TSS, g m^{-2}), starch concentration (g m^{-2}) in nodulated alfalfa leaves at the age of 44 (Week 2) and 60 days (Week 4). Values represent the mean ± SE; n = 4. Statistical analysis was made by a two factors Analysis of the Variance (ANOVA), see the results in the bottom of the table. The meaning of symbols used in ANOVA were: *, Significant difference at 5%. ** Significant difference at 1%, *** significant difference at 0.1%. When significant differences were detected in ANOVA, LSD analysis was applied. Means followed by the same letter are not significantly different (P > 0.05) according to LSD test

parameters. When no significances were detected in ANOVA analyses, no LSD test was applied, and therefore no significance letters were included.

At the fourth week, the TSS concentration (Table 3) was enhanced by CO_2, except in plants irrigated with 15 mM NH_4NO_3, which showed similar TSS concentrations irrespective of CO_2 level (*CO_2 x NH_4NO_3 F = 10.13, P = 0.001*). Starch concentration (Table 3) was enhanced by CO_2 at the second and fourth weeks, except in plants irrigated with 15 mM NH_4NO_3. The NH_4NO_3 doses reduced starch concentration in both the second and fourth weeks (*CO_2 x NH_4NO_3 F = 8.72, P = 0.003; F = 22.52, P < 0.001* respectively).

Leaf N concentration at the second week was enhanced by elevated CO_2 with 10 and 15 mM NH_4NO_3 treatments (Fig. 2A), but decreased with 0 mM NH_4NO_3 (*CO_2 x NH_4NO_3 F = 619.26, P < 0.001.*). At the fourth week (Fig. 2B), elevated CO_2 and NH_4NO_3 doses enhanced N concentration, especially in plants grown (*CO_2 x NH_4NO_3 F = 6392, P < 0.001*) in the 15 mM treatment and under elevated CO_2.

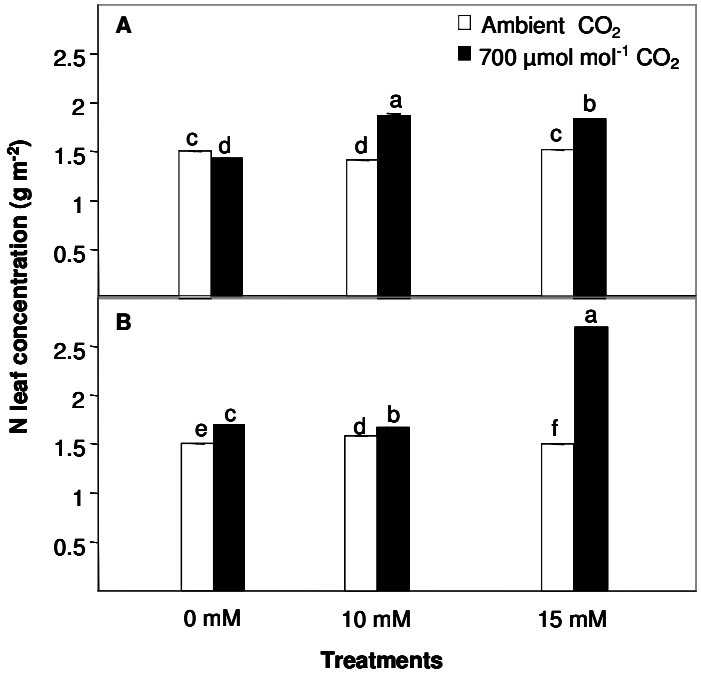

Figure 2: Effect of CO_2 (ambient CO_2, approximately 400 µmol mol^{-1}, and elevated CO_2, 700 µmol mol^{-1}) and increasing doses of NH_4NO_3 (0, 10 and 15 mM) on N leaf concentration (g m^{-2}) of nodulated alfalfa at the age of 44 (**A**, corresponding to 2 weeks of treatment) and 60 days (**B**, corresponding to 4 weeks of treatment). Bars represent the mean ± SE; n = 4. Bars with the same letter are not significantly different (P > 0.05) according LSD test.

After two weeks of treatment, elevated CO_2 increased the C/N ratio (Fig. 3A), except in plants irrigated with 15 mM NH_4NO_3 and grown at elevated CO_2, which also showed a lower C/N ratio than plants grown at ambient CO_2 (*CO_2 x NH_4NO_3 F = 6888.75, P < 0.001)*. At the fourth week, the C/N ratio was enhanced by CO_2 in all treatments (*CO_2 F = 4003.788, P <0.001*), although increasing NH_4NO_3 doses decreased this ratio (Fig. 3b) (*CO_2 F = 4003.78, P < 0.001*).

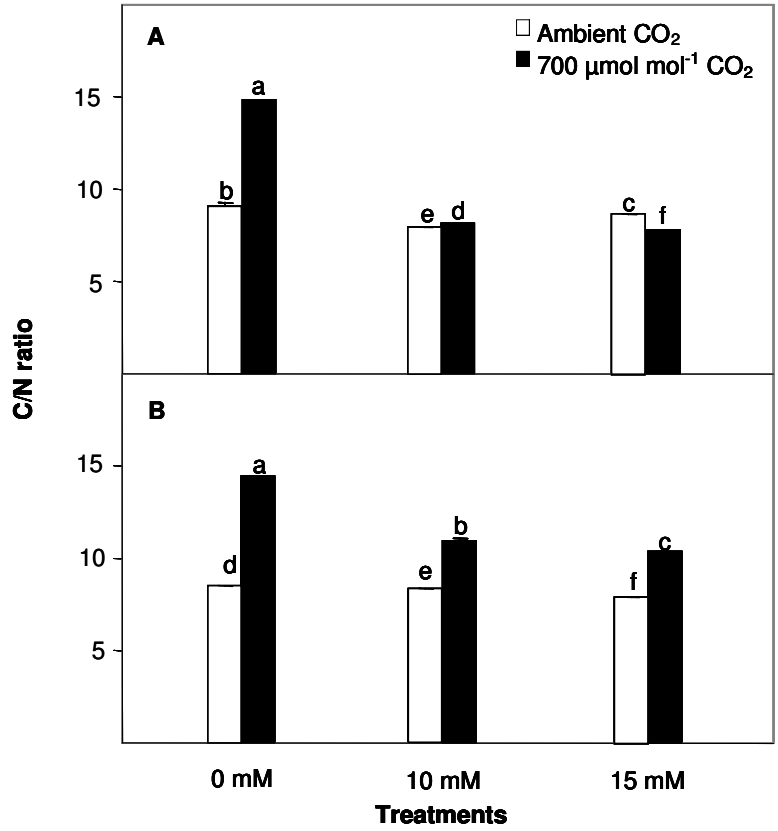

Figure 3: Effect of CO_2 (ambient CO_2, approximately 400 µmol mol^{-1}, and elevated CO_2, 700 µmol mol^{-1}) and increasing doses of NH_4NO_3 (0, 10 and 15 mM) on leaf C/N ratio of nodulated alfalfa at the age of 44 (**A**, corresponding to 2 weeks of treatment) and 60 days (**B**, corresponding to 4 weeks of treatment). Values represent the mean ± SE; n = 4. The SE was less than 1%. Bars represent the mean ± SE; n = 4. Bars with the same letter are not significantly different (P > 0.05) according LSD test.

At the second and fourth weeks, elevated CO_2 treatment reduced the $\delta^{13}C$ composition of total organic matter (Fig. 4A) (TOM) (CO_2 *F = 2813.67, P < 0.001* and CO_2 *F = 703.011, P < 0.001* respectively). The new C percentage (C_{new} %) of TOM was enhanced by elevated CO_2 at the second and fourth weeks of treatments (*CO_2 F = 900409.5, P < 0.001 and CO_2 F = 439249.8, P < 0.001 respectively*). The C_{new}% mean values at elevated CO_2 were 91.61 ± 0.16 and 91.40 ± 0.23 for second and fourth week

treatments respectively. The NH_4NO_3 treatment did not affect the $\delta^{13}C$ composition and C_{new} % during the second and fourth weeks of treatment.

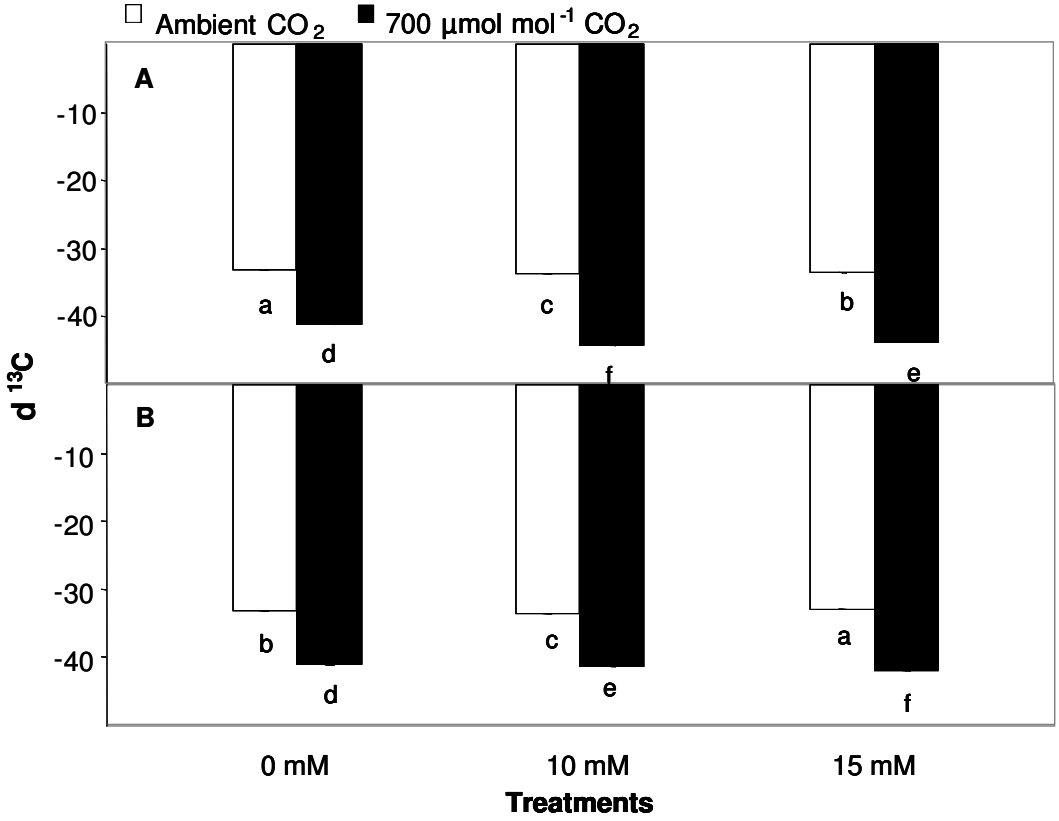

Figure 4: Effect of CO_2 (ambient CO_2, approximately 400 µmol mol^{-1} and elevated CO_2, 700 µmol mol^{-1}) and increasing doses of NH_4NO_3 (0, 10 and 15 mM) on $\delta^{13}C$ in nodulated alfalfa at the age of 44 (**A**, corresponding to 2 weeks of treatment) and 60 days (**B**, corresponding to 4 weeks of treatment). Measures were made using samples of whole plant. The SE was less than 1%. Bars represent the mean ± SE; n = 8. Bars with the same letter are not significantly different (P > 0.05) according LSD test.

Discussion

As described by the Intergovernmental Panel on Climate Change (IPCC, 2007) the expected increase in the atmospheric CO_2 concentration by the end of this century may enhance photosynthesis in C_3 plants. This increase in photosynthesis by elevated CO_2 leads to the enhancement of dry weight (DW) (Drake *et al*., 1997) (Table 1).

Our data revealed that **after two weeks of elevated CO_2 treatment**, there were no significant differences in biomass production associated with N or CO_2 levels. Interestingly, elevated CO_2 did not diminish the photosynthetic activity of the plants. Indeed, in plants without N fertilization (0 mM NH_4NO_3), elevated CO_2 increased photosynthetic activity (Table 2). As shown in Table 2, such stimulation of A_{net} by elevated CO_2 at the second week (Table 2) possibly was due to the increased photosynthetic N use efficiency (PNUE, Table 3). Plants grown under elevated CO_2 and irrigated with 0 mM NH_4NO_3 showed a decrease in TSP content (Table 3) but their photosynthetic rates were high. These data suggest that rubisco content or activity may increase to the detriment of other leaf proteins in order to maintain high photosynthetic rates. Therefore, 0 mM NH_4NO_3 treatment did not down regulate photosynthesis but other biochemical characteristics associated with acclimation were observed, like decreases of TSP (Table 3) and N content (Fig. 2A) under elevated CO_2 (Luo *et al*., 1994; Geiger *et al.,* 1999; Drake *et al.,* 1997; Moore *et al.,* 1999). As previously described by other authors (Ainsworth and Rogers, 2007; Aranjuelo *et al*., 2007), when N availability is limited, sink development is restricted leading to C accumulation and affects on the C/N ratio. In our experiment, the C/N ratio was higher in plants grown at elevated CO_2 and 0 mM NH_4NO_3 than in plants grown at ambient CO_2 (Fig. 3A), whereas plants irrigated with 10 and 15 mM NH_4NO_3 did not show significant differences.

Nevertheless, after **four weeks of elevated CO_2 treatment**, the responsiveness of the photosynthetic apparatus and plant growth differed from the observations after two weeks of exposure (Table 1). Our data showed that N irrigation (10 and 15 mM NH_4NO_3) increased plant biomass to a larger extent than CO_2 enhancement. Interestingly, the plant growth data showed that the CO_2-associated stimulatory effect

was only observed in alfalfa plants watered with 10 and 15 mM NH_4NO_3. These data suggest that plant growth was more conditioned by N rather than CO_2 availability. This DW enhancement observed in the 10 mM and 15 mM treatments after the fourth week was caused by increased area (table 1), and consequently, total photosynthesis enhancement (Fig. 1 A, C and Table 2) (Aranjuelo *et al.,* 2005a; 2008; Craine *et al*., 2003; Luo *at al*., 2004; Niklaus *et al*., 2001). The addition of NH_4NO_3 (10 and 15 mM) reduced nodule dry matter in both weeks (harvests) of treatment (Table 1). These plants have few nodules, but they seemed to be of normal size and colour. The nodules were formed possibly before the application of NH_4NO_3 treatment, thus plants irrigated with elevated doses of NH_4NO_3 do not develop new nodules but maintain activity in the existing ones. Gas exchange analyses revealed that 0 and 10 mM NH_4NO_3 plants grown under elevated CO_2 showed down-regulation as observed by $A_{net\ 400\text{-}700}$, Vc_{max} and A_{growth} (Fig. 1B,D, Table 2) (Aranjuelo *et al*., 2005a, b; 2008; Erice *et al*., 2006b). That is, plants grown at elevated CO_2 and irrigated with 0 mM NH_4NO_3 always showed down-regulation in the photosynthetic parameters. Conversely, plants irrigated with 15 mM NH_4NO_3 did not acclimate to elevated CO_2 and did not show decreases in $A_{net,}$ Vc_{max} and A_{growth}. The reduced carboxylation efficiency is (Long *et al*., 2004) due to a reduced amount/activity of rubisco in plants grown under elevated CO_2. In the present study, the observed down-regulation was not caused by lower intercellular CO_2 concentration (Ci) as result of stomatal closure because during all experiments, plants grown at elevated CO_2 showed higher Ci (Table 2) than those grown at ambient CO_2 (Aranjuelo *et al*., 2005b).

Some of the changes in metabolism that occur under elevated CO_2 can indirectly be affected by N limitation (Stitt and Krapp, 1999). When plants are N limited, down-regulation has been related to reallocation of N away from the photosynthetic apparatus (Aranjuelo *et al*., 2005b) or the effect on particular photosynthetic enzymes (Moore *et al*., 1999; Rogers and Ellsworth, 2002). According to our results, down-regulated plants showed the same TSP levels (Table 3), but their photosynthetic rates were lower than plants grown under ambient CO_2 (Fig. 1B, D).

As previously described, the decrease in photosynthetic capacity under elevated CO_2 has been associated with end product inhibition, in which the demand for

carbohydrates is insufficient to offset the enhanced carbohydrate supply (Jifon and Wolfe, 2002; Aranjuelo *et al*., 2008). ^{13}C isotopic composition data ($\delta^{13}C$) highlighted the fact that after two weeks of exposure to elevated CO_2 conditions, regardless of N source, almost all the C present in the total organic matter (TOM) of plants grown at 700 μmol mol^{-1} CO_2 was incorporated during the previous 14 days (C_{new}% at elevated CO_2 = 91.61 ± 0.16). Furthermore, the $\delta^{13}C$ data revealed that C fixed during the last two weeks replaced completely C fixed during the first month. In the analysis of DM at 0 mM, 10 mM and 15 mM NH_4NO_3 only 9.03%, 2.12% and 2.73% of the DM, respectively, was produced during the previous month. This confirmed that almost all the biomass was produced during the elevated CO_2 exposure (Aranjuelo *et al*., 2008). Absence of old (pre-labelling) C in the TOM of plants exposed to 700 μmol $mol^{-1}CO_2$ suggests that this C was respired and that, one month later no old C was present in the TOM (C_{new}% at elevated CO_2 = 91,4 ± 0,23). Several authors (Avice *et al*., 1996; Aranjuelo *et al*., 2009) have explained how leaf respiration consumes ~50% of the recently assimilated C. These data suggest that although almost all the pre-labelling C was replaced by C fixed during the last two weeks, plants exposed to 700 μmol mol^{-1} CO_2 were not capable of increasing C sink strength and consequently to balance C sink/source.

In a similar way, TSS and starch concentrations (Table 3) were significantly enhanced by elevated CO_2, with the exception of plants irrigated with the highest NH_4NO_3 concentration (15 mM). The maintenance of starch and TSS concentration at control levels could be related to avoidance of photosynthetic down-regulation. Jifon & Wolfe (2002) observed that the effect of N on acclimation to elevated CO_2 depends on the balance between the availability and demand for N, and the effect on biomass allocation and source-sink carbon balance. Low N availability may affect plant growth and thus, the capacity to develop new sinks (Erice *et al*., 2006b). A parameter that may indicate the source/sink balance is the C/N ratio, which increases when plant sink capacity is not strong enough to consume or mobilize carbohydrates. In our experiment, at the fourth week of treatment, the C/N ratio was enhanced by elevated CO_2 (Fig. 3B) in all treatments, although increased NH_4NO_3 reduced these differences. In plants irrigated with 10 and especially 15 mM NH_4NO_3, leaf N content (Fig. 2B) increased and

carbohydrate accumulation (TSS and starch) decreased, avoiding photosynthetic down regulation.

Plants irrigated with 0 mM NH_4NO_3, and with abundant active nodules, showed the largest C/N imbalance under elevated CO_2 (Fig. 3B). Such behaviour may be indicative of two problems causing down-regulation: first that N_2-fixation was insufficient to maintain satisfactory N availability for the plants; second, that nodule sink strength was insufficient to avoid leaf C accumulation caused by elevated CO_2. These data suggest that the largest N supply of 15 mM NH_4NO_3 (Fig. 2B) enabled the development of strong C sinks that would eliminate photosynthetic down-regulation.

In this paper we showed the effect of elevated CO_2 after short and long-term exposure on nodulated alfalfa plants supplied with an external N source (NH_4NO_3). As a summary, we can conclude that according to the photosynthetic parameters (net photosynthesis rates and Vc_{max}), nodulated alfalfa plants with or without the addition of exogenous N did not show photosynthetic down-regulation during the short-term exposure to elevated CO_2 (second week). In contrast, under longer exposure to elevated CO_2 (fourth week), nodulated plants not supplied with external N (0 mM NH_4NO_3) showed photosynthetic (reduction in net photosynthesis and Vc_{max}) and biochemical behaviour (leaf soluble protein reduction, carbohydrate accumulation, and high C/N ratio) indicating a clear down-regulation. Plants supplied with 15mM NH_4NO_3 did not show photosynthetic down-regulation. The 10 mM NH_4NO_3 treatment showed less down-regulation than the 0 mM NH_4NO_3 treatment because 10 mM NH_4NO_3 treatment decreased photosynthesis but not Vc_{max}. From all these results we can conclude that N supply by the nodules in alfalfa is insufficient to cover N plant demand, especially in long-term elevated CO_2 exposure when higher growth and biomass accumulation occur, and therefore photosynthesis down-regulation takes place. Therefore, it is clearly concluded that the highest availability of N (15 mM NH_4NO_3) enabled the avoidance of photosynthetic down-regulation.

Acknowledgements

This work was supported by Ministerio de Ciencia e Innovación (MICINN BFU2008-01405), Fundación Universitaria de Navarra (PIUNA-2008) and Fundación Caja Navarra. A. Sanz-Sáez was the recipient of a research grant from Asociación de Amigos de la Universidad de Navarra and Iker Aranjuelo was the recipient of a Juan de la Cierva grant from Ministerio de Ciencia e Innovación. The assistance of A. Urdiain and M. Oyarzun is also appreciated.

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CAPÍTULO 2

Photosynthetic down-regulation of N_2-fixation in alfalfa under elevated CO_2 alters rubisco content and decreases nodule metabolism *via* nitrogenase and tricarboxylic acid cycle

La aclimatación fotosintética de plantas noduladas de alfalfa altera el contenido de rubisco y disminuye el metabolismo nodular y el ciclo de los ácidos tricarboxílicos

Enviado a *Phytochemistry*

Photosynthetic down-regulation of N_2-fixation in alfalfa under elevated CO_2 alters rubisco content and decreases nodule metabolism *via* nitrogenase and tricarboxylic acid cycle

Resumen

Después de la exposición al CO_2 a largo plazo, el estímulo inicial de la fotosíntesis tiende a descender. En leguminosas, esta aclimatación fotosintética al CO_2 elevado ha sido relacionada con la acumulación de carbohidratos en la hoja y con una menor actividad nodular. El descenso de la actividad nodular se asocia con una reducción en la demanda de N de la parte aérea. El objetivo de este trabajo ha sido estudiar la relación entre el metabolismo de la hoja y del nódulo durante la aclimatación fotosintética de plantas de alfalfa estrictamente dependientes del nitrógeno fijado por los nódulos, profundizando en el efecto de la exposición a largo plazo al CO_2 elevado sobre la fijación de nitrógeno. Para alcanzar dicho objetivo, se realizaron medidas fisiológicas y bioquímicas, incluyendo el análisis proteómico del nódulo de plantas de alfalfa crecidas en condiciones de CO_2 ambiente (400 μmol mol^{-1}) o elevado (700 μmol mol^{-1}). Después de 30 días de exposición al CO_2 elevado, las plantas mostraron síntomas de aclimatación fotosintética, como la reducción en la fotosíntesis en condiciones de luz saturante (A_{sat}) y en la velocidad máxima de carboxilación de la rubisco (Vc_{max}). A pesar de los descensos en la expresión del gen que codifica para la subunidad pequeña de la rubisco (*RbcS*), el contenido de proteína de la subunidad pequeña de la rubisco (RSS) aumentó con el CO_2 elevado, demostrándose que el contenido final de RSS está regulado por procesos post-transcripcionales. En condiciones de CO_2 elevado, el contenido en subunidad grande de la rubisco (RLS) limitó el potencial de la fotosíntesis en un 12% aproximadamente, esto representó la mayor parte (70%) de la caída observada en la Vc_{max}. En estas condiciones, la actividad nodular específica (SNA) disminuyó debido al efecto negativo del CO_2 sobre el metabolismo nodular, que se tradujo en un menor contenido de nitrogenasa reductasa. También se vio alterado el ciclo de los ácidos tricarboxílicos por una reducción de la proteína isocitratosintasa. Al mismo tiempo se observó una relajación del sistema antioxidante en condiciones de CO_2 elevado, que se manifestó con un descenso en el contenido de las enzimas catalasa e isoflavona reductasa.

Summary

After long-term exposure to elevated CO_2, the initial increase of C_3 plant photosynthesis tends to decline. In legumes, this acclimation to rising CO_2 has been related to leaf carbohydrate accumulation and to reduced nodule activity resulting from the lower N demand of shoots. The aim of our study was to deepen our understanding of the relationship between leaf and nodule metabolism of N_2-fixing alfalfa plants after long-term exposure to elevated CO_2. We examined a combination of physiological and biochemical measurement with proteomic analysis of the nodules of alfalfa plants grown at ambient (400 μmol mol^{-1}) or elevated CO_2 concentrations (700 μmol mol^{-1}). After 30 days exposure to elevated CO_2, plants showed photosynthetic down-regulation with reductions in the light-saturated rate of CO_2 assimilation (A_{sat}) and the maximum rate of rubisco carboxylation (Vc_{max}). Despite the drop in the rubisco small subunit mRNA (RbcS), rubisco small subunit protein content (RSS) increased, revealing that post-transcriptional processes affected the final RSS content. Under elevated CO_2 conditions, the rubisco large subunit (RLS) limited potential photosynthesis by around 12%, which represented the majority (70%) of the observed fall in Vc_{max}. In this condition, specific nodule activity (SNA) was reduced due to an effect on nodule metabolism that manifested as a lower amount of nitrogenase reductase. At elevated CO_2, the tricarboxylic acid cycle was also altered with a reduced amount of isocitrate synthase protein. This metabolic repression was related to relaxation of the antioxidant system as shown by a decline in the amount of catalase and isoflavone reductase proteins.

Key words: Alfalfa, carbon dioxide, N_2 fixation, photosynthetic down-regulation, proteome, rubisco.

Abbreviations: 2-DE, two-dimensional electrophoresis; A_{sat}, light-saturated rate of CO_2 assimilation; Ci/Ca, leaf-to-ambient CO_2 concentration; DM, dry matter; PPFD, photosynthetic photon flux density; R/S, root to shoot ratio; RbcS, rubisco small subunit mRNA; RH, relative humidity; RLS, rubisco large subunit; RSS: rubisco small subunit; SNA, specific nodule activity; TCA, tricarboxylic acid; TNC, total non-structural carbohydrates; V_{cmax}, rubisco maximum carboxylation capacity.

Introduction

Since the beginning of the industrial revolution in the 18th century, the increase in atmospheric CO_2 has been mainly of anthropic origin. This has been the consequence of industrial development and a quadrupling of the human population during the last one hundred years (Krausmann *et al.*, 2009). In 2009, atmospheric CO_2 concentration reached 387 μmol mol^{-1} and, according to the predictions of the Intergovernmental Panel on Climate Change, at the end of the present century this concentration may reach 700 μmol mol^{-1} (IPCC, 2007). The primary effect of increasing CO_2 in C_3 plants is a short term photosynthetic enhancement, and consequently, an increase in plant productivity (Daepp *et al.*, 2000). Nevertheless, this response to CO_2 is frequently not maintained over the long term and photosynthesis declines (Erice *et al.*, 2006a; Ainsworth and Rogers, 2007; Aranjuelo *et al.*, 2009), a phenomenon named acclimation of photosynthesis. Previous studies with alfalfa showed that this photosynthetic down-regulation was attributed to a decreased photosynthetic efficiency without limitation of CO_2 supply to mesophyll cells (Aranjuelo *et al.*, 2005a; Erice *et al.*, 2006b).

In the context of elevated CO_2 environments, legumes are particularly interesting due to their symbiotic relationship with N_2-fixing bacteria, provide nitrogen (N) autonomy to them. Symbiotic N_2 fixation is narrowly related to photosynthesis (Hartwig and Sadowsky, 2006). Many studies have confirmed that most N_2-fixing legumes increase their level of N_2 fixation per plant under elevated CO_2 conditions (Hartwig, 1998; Rogers *et al.*, 2006). However, some studies suggest that reduced rubisco activity after long-term exposure to CO_2 is associated with lower leaf N demand resulting in nodule decline. A common plant response under elevated CO_2 is the enhancement of non-structural carbohydrate concentrations. This is a consequence of the inhibition of the expression of genes that encode different proteins belonging to the photosynthetic apparatus, resulting in decreased photosynthetic capacity and notable declines in the amount of rubisco (Drake *et al.*, 1997; Moore *et al.*, 1999) and in photosynthetic capacity. This rubisco depletion has been associated with significant decreases in the transcript levels of *RbcS* genes that encode the small subunit of rubisco (Nie *et al.*, 1995; Van Oosten and Besford, 1994; 1995). Nevertheless, the link between carbohydrate repression of gene expression and control of photosynthetic acclimation remains elusive (Cheng *et al.*, 1998). This coupling between the

C and N cycles causes biological symbiotic N_2 fixation to be regulated by photosynthesis (carbon supply) and N demand (Aranjuelo *et al.*, 2007).

Some authors have reported that the decrease in rubisco content and acclimation to elevated CO_2 is accompanied by a lower leaf N content (Nakano *et al.*, 1997), which may suggest that photosynthetic down-regulation is due to N status (Farage *et al.*, 1998; Geiger *et al.*, 1999). Other studies have shown that elevated CO_2 concentration increases legume nodule growth (Phillips *et al.*, 1976; Murphy, 1986; Aranjuelo *et al.*, 2009) despite the fact that specific nodule activity (SNA) could remain unchanged (Cabrerizo *et al.*, 2001).

The mechanisms that determine the response of photosynthesis to the predicted increased CO_2 concentration in the atmosphere are of crucial interest in predicting the impact of global climate change on the Earth's terrestrial ecosystems (Cheng *et al.*, 1998). To achieve a more comprehensive picture of the proteins related to elevated CO_2 responses in nodules, we performed protein separation by two-dimensional electrophoresis (2-DE). This proteomic approach in association with physiological studies was developed with successful results for investigation of plant responsiveness under limited growth conditions (Desclos *et al.*, 2008, 2009; Aranjuelo *et al.*, 2011). Djordjevic (2004) reviewed the proteomic characterization of *Sinorhizobium meliloti* as free-living cells and bacteroids, but knowledge of the plant proteome in the *Medicago* root nodule remains scarce (Larrainzar *et al.*, 2007).

A number of studies have reported photosynthetic down-regulation and some of them have related this phenomenon to carbohydrate accumulation and the plant's demand for N, but less attention has been paid to the role of both rubisco subunits under elevated CO_2 in relation to nodule metabolism. The aim of the present study was to determine the quantitative changes in the rubisco large (RLS) and small subunits (RSS) and their significance to the elevated CO_2 acclimation context in alfalfa. The relationship between leaf and nodule metabolism has also been studied by 2-DE to discover proteins of interest involved in nodule responses to long-term alfalfa CO_2 exposure. The present work combines physiological and biochemical measurements with proteomic analysis of nodules in order to contribute to the understanding of plant photosynthetic acclimation to elevated CO_2 in strictly N_2-fixing alfalfa i.e. in plants without any source of nitrate or ammonium.

Materials and methods

Plant material

Alfalfa (*Medicago sativa* L. cv. Aragón) seeds were sterilized in a solution of $HgCl_2$ (0.1%, w/v) and germinated in Petri dishes. 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, plants were inoculated three times with *Sinorhizobium meliloti* strain 102F78 (The Nitragin Co. Milwaukee, WI, USA) and irrigated with either Hoagland N-free solution (Hoagland and Arnon, 1950) or distilled water (three times per week) to avoid salt accumulation in the substrate. Plants were grown in a greenhouse at 25/15 ºC (day/night) with a 14h photoperiod under natural daylight, supplemented with fluorescent lamps (Sylvania Decor 183, Professional-58W, Germany) providing a photosynthetic photon flux density (PPFD) of 400 μmol m^{-2} s^{-1}. When plants were 30 days old, they were transferred to a growth chamber (Conviron PGV 36, Winnipeg, Canada) and randomly assigned to the appropriate atmospheric CO_2 concentration (ambient -approximately 400 μmol mol^{-1}- or elevated - 700 μmol mol^{-1}). The conditions in the growth chamber were 25/15 ºC (day/night), 40% RH, 14h photoperiod and 600 μmol m^{-2} s^{-1} PPFD.

Growth parameters

After one month exposure to ambient or elevated CO_2 concentrations, 60 day-old plants were separated into leaves, stems, roots and nodules. The dry mass (DM) of each organ and the root-to-shoot ratio (R/S) were obtained after drying in an oven at 80 ºC for 48h. Fresh weight aliquots of leaves and nodules were also stored at -80°C for further biochemical and molecular analysis.

Gas exchange parameters

Gas exchange parameters were measured in fully expanded young apical leaves after 30 days exposure to CO_2 treatments (60-days-old plants) using a LI-COR 6400 portable photosynthesis system (LICOR Biosciences, Lincoln, Nebraska, USA). The A-Ci determinations were conducted from 60 to 1400 μmol mol^{-1} CO_2 at a saturating PPFD of

1200 μmol m^{-2} s^{-1}. The light-saturated rate of CO_2 assimilation (A_{sat}) was estimated using equations developed by von Caemmerer and Farquhar (1981). Measurements from net photosynthesis and intercellular CO_2 were used to assess the maximum rate of rubisco carboxylation (Vc_{max}) employing the mathematical model developed by Ethier & Livingstone (2004) and Sharkey *et al.* (2007). Leaf-to-ambient CO_2 concentration (Ci/Ca) was also calculated for both ambient (400 μmol mol^{-1}) and elevated (700 μmol mol^{-1}) CO_2 concentrations.

Analysis of non-structural carbohydrates

Leaf total soluble sugars (TSS) and starch concentration were quantified from leaves harvested after one month of treatment by grinding and filtering 200 mg of leaf fresh weight in a cold mortar using an extraction buffer containing 50 mM K-phosphate (pH 7.5). The extract was filtered and centrifuged at 28,710 × g for 15 min at 4 ºC. The TSS quantification was performed with the supernatant, whereas starch was measured using the pellet as described by Jarvis and Walker (1993). TSS levels were measured using the method of Yemm and Willis (1954). Total non-structural carbohydrates (TNC) were calculated as the addition of starch and total soluble sugar concentration. So as to avoid possible data underestimation derived from differences in leaf thickness, we have expressed TNC measurements on an area basis (Sanz-Sáez *et al.*, 2010).

Analysis of N and determination of specific nodule activity (SNA)

Nitrogen concentration corresponding to leaf, stem, root and nodule samples was determined with 1.5 mg of ground dry subsamples (4 replicates for each sample). All these determinations were conducted at the Laboratoire d'Ecophysiologie Végétale, Agronomie et nutritions N, C, S, Université de Caen Basse-Normandie (France) using an isotopic ratio mass spectrometer (IRMS, IsoPrime, GV Instrument, Manchester, UK) linked to an elemental analyzer (EA 3000, EuroVector, Milan, Italy).

Specific nodule activity (SNA) was calculated as described by Brioua & Wheeler (1994), being the ratio between plant total nitrogen content and nodule DM.

RNA isolation, synthesis of cDNA and Quantitative real-time RT-PCR

Total RNA was isolated from alfalfa leaves by phenol/chloroform extraction (Kay *et al.*, 1987). *RbcS* gene expression was studied by real-time PCR by using an iCycler (Bio-Rad, Hercules, California, USA). cDNAs were obtained from 2.5 µg of total DNase-treated RNA in a 20 µL reaction containing 500 ng of random hexamer primer, 0.5 mM of each dNTP, 10 mM DTT, 40 U of RNase inhibitor, 1x first strand buffer (Invitrogen, Carlsbad, California, USA) and 200 U of Superscript II Reverse Transcriptase (Invitrogen). The primer sets used to amplify *RbcS* were: primer forward 5´-TTCGGAGCCACTGATTCTTCTC-3´ and primer reverse 5´-ACTGCACTTGACGAACATTGTC-3´. Each 25 µL q-PCR reaction contained 1 µL of a 1:10 dilution of the cDNA, 200 nM dNTPs, 400 nM of each primer, 3 mM $MgCl_2$, 2.5 µL of 1x SyBR Green (Molecular Probes, Eugene, Oregon, USA), and 0.5 U Platinum *Taq* DNA polymerase (Invitrogen) in 1x PCR buffer (20 mM Tris–HCl, pH 8.4, 50 mM KCl).

The PCR program consisted of a 4 min incubation at 95 ºC to activate the hot-start recombinant *Taq* DNA polymerase, followed by 30 cycles of 45 s at 94 ºC, 45 s at 69 ºC, and 50 s at 72 ºC, where the fluorescence signal was measured. The results obtained for the different treatments were standarized according to tubulin gene expression levels, which were analyzed with primer forward 5´-GAAGCAAGCGGTGGAAGATATG-3´ and primer reverse 5´-CCAAATGGACCAGAACGCAAAC-3´, and showed stable expression under the conditions tested in this study

Real-time PCR experiments were carried out with at least four independent RNA samples, with the threshold cycle (C_T) determined in triplicate. The relative levels of transcription were calculated by using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). Negative controls without cDNA were used in all PCR reactions.

Rubisco semi-quantification

Extracts from protein quantification 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, 4 μg of soluble proteins were 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 ImageQuant TL software (GE Amersham Biosciences, UK). The relative proportion of rubisco large (RLS) and small (RSS) subunits was calculated in reference to the abundance value of RLS observed in the ambient CO_2 concentration.

Nodule proteomic characterization

Frozen nodule samples (200 mg fresh weight) were ground with liquid nitrogen and resuspended in 2 mL of cold acetone containing 10% TCA (v/v). After centrifugation at 16,000 *g* for 3 min at 4 °C, the supernatant was discarded and the pellet was rinsed with methanol, acetone, and phenol solutions as previously described by Wang *et al.* (2003). The pellet was stored at –20 °C or immediately resuspended in 200 μl of R2D2 rehydration buffer [5 M urea, 2 M thiourea, 2% 3-[(3-cholamidopropyl) dimethyl-ammonio]-1-propane-sulphonate, 2% N-decyl-N,N-dimethyl-3-ammonio-1-propane-sulphonate, 20 mM dithiothreitol, 5 mM TRIS (2-carboxyethyl) phosphine, 0.5% IPG buffer (GE Healthcare, Saclay, France), pH 4 to 7 (Mechin *et al.,* 2003). The total soluble protein (TSP) concentration was determined by the method of Bradford (1976) using BSA as standard. Two-dimensional electrophoresis (2-DE) was conducted according to the method of Aranjuelo *et al.* (2011).

Image analysis of 2-DE gels

Images of the two-dimensional gels were acquired with the ProXPRESS 2D proteomic Imaging System and analyzed using Phoretix 2-D Expression Software v2004 (Nonlinear Dynamics, Newcastle upon Tyne, UK). Gels from four independent biological replicates were used. An average gel, representative of each group, was automatically selected by the software with a parameter for spots to be present on more than two-thirds of the gels. The software automatically selected the average gel with the most spots as the image for the reference gel, and unmatched spots from the remaining average gel were added to the reference gel, which was subsequently used for spot matching to average gels.

Warping and matching were performed automatically and only adjusted on those gels where darker images led to both incorrect warping and matching. *M*r and p*I* were calculated using Samespots software calibrated with commercial molecular mass standards (precision protein standards prestained Bio-Rad) run in a separate marker lane on the 2-DE gel. ANOVA ($P < 0.05$) was performed using MiniTAB to compare the relative abundance of the total volume of all detected spots for each gel.

Protein identification by ESI-LC MS/MS

Excised spots were washed several times with water and dried for a few minutes. Peptide extracts were then dried and dissolved in starting buffer for chromatographic elution, which consisted of 3% CH_3CN and 0.1% HCOOH in water. Peptides were enriched and separated using lab-on-a-chip technology (Agilent, Massy, France) and fragmented using an on-line XCT mass spectrometer (Agilent). The fragmentation data were interpreted using the Data Analysis program (version 3.4, Bruker Daltonic, Billerica, USA). For protein identification, tandem mass spectrometry peak lists were extracted and compared with the protein database using the MASCOT Daemon (version 2.1.3; Matrix Science, London, UK) search engine. Tandem mass spectrometry spectra were searched with a mass tolerance of 1.6 Da for precursor ions and 0.8 for MS/MS fragments.

The LC MS/MS data were converted into DTA-format files which were further searched for proteins with MASCOT Daemon. Only peptides matching an individual ion score >51 were considered. Proteins with two or more unique peptides matching the protein sequence were automatically considered as a positive identification. Among the positive matches based on one unique peptide, the fragmentation spectrum from each peptide was manually interpreted using the conventional fragmentation rules. In particular, we looked for a succession of at least five *y*- and/or *b*-ions, specific immonium ions, specific fragment ions (proline and glycine), and signatures of any modifications carried by the peptides. For protein identification, two strategies were used to mine the maximum information. Measured peptides were searched in the NCBInr-protein sequence database Viridiplantae (green plants) and Bacteria. Once the proteins were identified, their functional classification was determined according to Bevan *et al.* (1998).

Statistical analysis

Statistical analysis was performed using SPSS software 12.0 (SPSS, Chicago, Illinois, USA). Data were subjected to one-way analysis of variance (ANOVA) to determine significant differences between the CO_2 treatments. The results were considered significant at $P < 0.05$.

Results

After 30 days exposure to ambient or elevated atmospheric CO_2 concentration there were no differences observed in leaf, stem, root, or nodule dry mass (DM), nor were there differences in R/S (Table 1).

	Leaf DM (g plant-1)	Stem DM (g plant-1)	Root DM (g plant-1)	Nodule DM (g plant-1)	R/S
Ambient CO_2	0.32 ± 0.04 a	0.46 ± 0.05 a	0.28 ± 0.04 a	0.036 ± 0.005 a	0.50 ± 0.03 a
Elevated CO_2	0.34 ± 0.03 a	0.41 ± 0.05 a	0.37 ± 0.04 a	0.034 ± 0.003 a	0.45 ± 0.05 a

Table 1. Effect of ambient or elevated CO_2 in leaf, stem, root, nodule dry matter (DM) and root/shoot ratio (R/S) in nodulated alfalfa plants. Values represent the mean ±SE (n=4). The different letters indicate significance differences ($P < 0.05$).

The measured gas exchange parameters (A_{sat} and Vc_{max}) significantly decreased under elevated CO_2 ($F = 25.8$; $P = 0.007$ and $F = 33.9$; $P = 0.004$ respectively) but no differences were found when comparing Ci/Ca for both 400 or 700 μmol mol^{-1} (Figure 1).

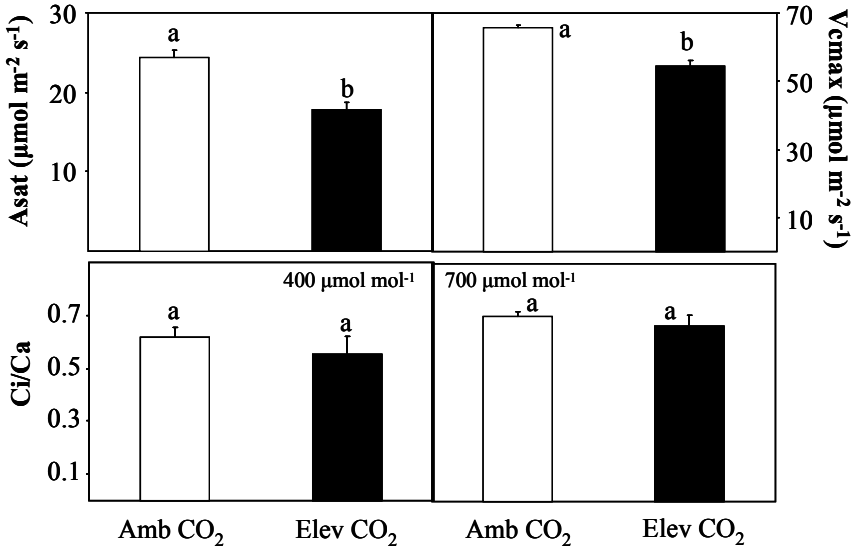

Figure 1. Effect of elevated CO_2 in saturating maximum photosynthetic rate (Asat), maximum carboxylation velocity of rubisco (Vcmax) and leaf-to-ambient CO_2 concentration (Ci/Ca) in nodulated alfalfa plants. Values represent the mean ±SE (n=4). The different letters indicate significance differences ($P < 0.05$).

Leaf TNC concentration was increased (x 1.8) by elevated CO_2 ($F = 22.5$; $P = 0.003$) (Table 2). After CO_2 exposure, leaf N concentration was decreased under elevated CO_2 ($F =$

10.8; P = 0.017) whereas stem or root N as well as total N concentration were unaffected by CO_2 (Table 2). Specific nodule activity (SNA) was significantly decreased by elevated CO_2 (F = 13.3; P = 0.011) (Table 2).

	TNC (mg m^{-2})	Leaf N (mg g^{-1} DM)	Stem N (mg g^{-1} DM)	Root N (mg g^{-1} DM)	Total N (mg $plant^{-1}$)	SNA (mg N nodule DM^{-1})
Ambient CO_2	4.09 ± 0.16 b	16.16 ± 2.14 a	9.68 ± 1.09	6.46 ± 0.24	33.37 ± 4.18	0.95 ± 0.07 a
Elevated CO_2	7.36 ± 0.78 a	9.63 ± 0.85 b	7.75 ± 0.93	6.72 ± 0.84	24.37 ± 2.56	0.66 ± 0.06 b

Table 2. Effect of ambient or elevated CO_2 in total non-structural carbohydrates (TNC), leaf, stem and root N concentration, total N and specific nodule activity (SNA) in nodulated alfalfa plants. Values represent the mean ±SE (n=4). The different letters indicate significance differences (P < 0.05).

Elevated CO_2 led to significantly decreased expression of rubisco small subunit mRNA (RbcS) (F = 221.4; P < 0.000) (Figure 2A). SDS-PAGE performed on leaf soluble proteins revealed that rubisco large subunit (RLS) content decreased significantly under elevated CO_2 concentration but rubisco small subunit (RSS) content increased (F = 15.33; P < 0.000) (Figure 2B-C). This resulted in a decrease in the RLS/RSS ratio (F = 44.52; P < 0.000) (Figure 2D).

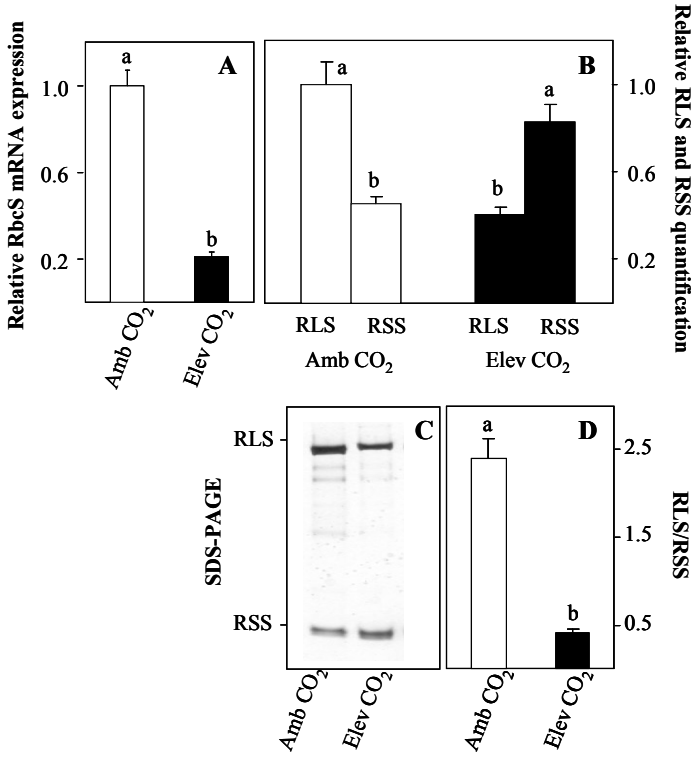

Figure 2. Effect of elevated CO_2 in relative RbcS mRNA expression (A), RLS and RSS quantification (B), leaf SDS-PAGE profile (C) and RLS/RSS ratio (D) in nodulated alfalfa plants. Values represent the mean ±SE (n=4). The different letters indicate significance differences (P < 0.05).

The effect of rising CO_2 on the nodule protein profile in alfalfa plants was studied using 2-DE (Figure 3). Twelve proteins have been identified with different expression under

ambient and elevated CO_2 concentration. Eight of those proteins were down-regulated by CO_2 and 4 were up-regulated (Table 3).

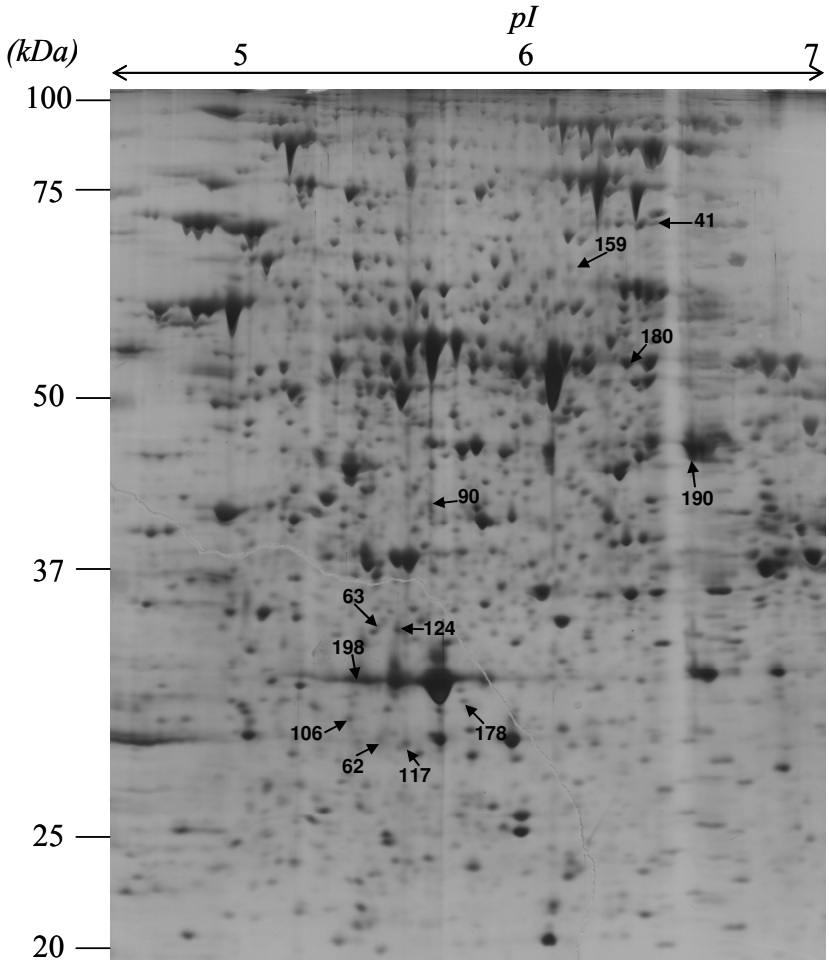

Figure 3. Effect of elevated CO_2 in silver stained two-dimensional gel of proteins extracted from *Sinorhizobium meliloti* strain 102F78 alfalfa nodules. In the first dimension, 125 mg of total protein was loaded on a 18 cm IEF strip with a linear gradient of pH 4-7. The second dimension was conducted in 12% polyacrylamide (w/v) gels (20x20 cm) (for details see Material and methods). The gel imagine analyses conducted with Progenesis SameSpots software v3.0 and the subsequent mass spectrometry analyses identified up to 12 proteins (marked by arrows) that, statistically, were involved in the nodule response to elevated CO_2.

These proteins were classified into different groups according to their presumed biological function. The down-regulated proteins were classified into six groups: metabolism (1), energy processes (1), transporters (2), disease/defence (1), secondary metabolism (1) and unclassified (2). Up-regulated proteins were essentially related to energy (2) or were unclassified (2). The roles of these proteins are discussed in the following section with regard to physiological and biochemical changes observed in nodulated alfalfa under elevated CO_2.

Down-regulated

no.	B/V	Spot % volume variations	Exp. p*I*/M_r	Theor. p*I*/M_r	PM	SC (%)	Score (p < 0.05 corresponding to score > 51)	Protein name / Organism / NCBI accesion no.	Putative Function
198	B	15%	5.11/30.01	5.35/31.99	6	33	360	nitrogenase reductase / *Sinorhizobium meliloti* 1021 / gi|16262902	**01. Metabolism**
190	B	20%	6.41/44.72	6.02/47.97	13	38	635	type II citrate synthase / *Sinorhizobium meliloti* 1021 / gi|15965262	**02. Energy**
41	B	86%	6.40/69.61	6.26/68.16	1	1	58	ABC transporter, ATP-binding protein / *Roseovarius nubinhibens* ISM / gi|83952290	**07. Transporters**
124	B	47%	5.39/31.04	4.33/10.70	1	19	51	YebA / *Klebsiella pneumoniae* / gi|38639575	**07. Transporters**
180	B	24%	6.29/58.77	6.52/62.93	4	10	194	catalase / *Sinorhizobium meliloti* / gi|1698550	**11. Disease/Defence**
90	B	61%	5.20/40.23	6.19/47.46	1	1	55	hypothetical protein Sare_4839 / *Salinispora arenicola* CNS-205 / gi|159040327	**13. Unclassified**
106	V	52%	5.21/28.65	8.61/36.35	1	2	51	predicted protein / *Chlamydomonas reinhardtii* / gi|159462486	**13. Unclassified**
63	V	71%	5.23/33.49	5.39/35.39	4	16	99	isoflavone reductase / *Medicago sativa* / gi|19620	**20. Secondary metabolism**

Up-regulated

Nº	B/V	Spot % volume variations	Exp. pI/Mr	Theor. pI/Mr	PM	SC (%)	Score (p < 0.05 corresponding to score > 51)	Protein name / Organism / NCBI accesion no.	Putative Function
62	V	71%	5.36/29.01	5.78/29.85	3	15	88	carbonic anhydrase / *Lotus japonicus* / gi|28625017	**02. Energy**
117	V	48%	5.58/29.02	6.02/30.13	5	20	164	carbonic anhydrase / *Medicago sativa* subsp. x varia / gi|1938227	**02. Energy**
159	B	38%	6.31/72.68	10.75/25.17	2	4	52	hypothetical protein NGO1977 / *Neisseria gonorrhoeae* FA 1090 / gi|59802286	**13. Unclassified**
178	V	27%	5.75/27.82	9.62/218.41	6	1	52	predicted protein / *Physcomitrella patens* subsp. Patens / gi|168015024	**13. Unclassified**

Table 3. Annotation of down/up-regulated identified spots following elevated CO_2 exposure in silver stained 2-DE gels of nodules. Spot no. represents the number of proteins assigned. Spot volume (%) is an estimation of relative protein abundance. The p*I* and molecular mass (M_r) values shown are the theoretical and experimental values. SC represents the protein sequence coverage (%) score, which is the Mascot score of the in-solution digestion protocol. Function, the predicted protein function is assigned according to the NCBInr-protein sequence database of Bacteria (B) or Viridiplantae (V).

Discussion

The primary effect of rising atmospheric CO_2 concentration (IPCC, 2007) in C_3 plants is the enhancement of photosynthesis, but after long-term exposure photosynthetic acclimation appears (Erice *et al.*, 2006a; 2006b; Aranjuelo *et al.*, 2009). Despite the theoretical initial increase in photosynthesis, in our study, plant growth measured as dry mass (DM) accumulation did not show any significant differences (Table 1) (Aranjuelo *et al.*, 2005a, 2008, 2009). According to the A_{sat} and Vc_{max} results, plants grown under elevated CO_2 concentration showed a clear acclimation that led to a reduction of 17.2% of Vc_{max} (from 66 to 54 μmol m^{-2} s^{-1}, Figure 1). The absence of significant differences in Ci/Ca (Figure 1) discarded stomatal closure as being the factor that caused photosynthetic down-regulation (Aranjuelo *et al.*, 2005a; Erice *et al.*, 2006b).

Studies about photosynthesis down-regulation have related the accumulation of total non-structural carbohydrates (TNC) with limitation of photosynthetic enzymes (Moore *et al.*, 1999; Rogers and Ellsworth, 2002) including rubisco (Aranjuelo *et al.*, 2005a, 2008). The present work shows that TNC accumulates in leaves (Table 2) in response to elevated CO_2 (Erice *et al.*, 2006b; Sanz-Sáez *et al.*, 2010) and this may be the cause of the significant decrease in rubisco small subunit gene expression (RbcS) (Figure 2A). Down-regulation of *RbcS* by elevated CO_2 has been reported repeatedly (Majeau and Coleman, 1996; Cheng *et al.*, 1998; Gesch *et al.*, 1998) but the regulation of transcriptional and/or posttranscriptional processes (e.g. mRNA stability) will determine the level of rubisco protein at elevated CO_2 (Cheng *et al.*, 1998). In the present study the reduction of *RbcS* expression was not accompanied by a lower quantity of RSS, which actually increased under elevated CO_2 conditions (Figure 2B and 2C). Similar results with post-transcriptional regulation of protein content were obtained in tomato (Van Oosten and Besford, 1995) or *Chlamydomonas reinhardtii,* probably due to the inhibition of translation of RbcS mRNA (Winder *et al.*, 1992).

It is considered that due to the relative quantity of RLS and RSS, and the lower specificity of RSS (Jordan and Ogren, 1981) that differences in the kinetic properties of rubisco and thus photosynthesis result from changes in RSS (Andersson and Backlund,

2008). At ambient CO_2 conditions RLS doubled the quantity of RSS, confirming that RSS is the subunit that may limit rubisco carboxylation capacity (Figure 2B and C). Nevertheless, at elevated CO_2 conditions the RLS/RSS ratio was inverted (Figure 2D), which suggests that the quantity of RLS was lower than RSS (Figure 2B and C). Thus, in such conditions RLS could limit photosynthesis. Under elevated CO_2, down-regulation corresponding to a limitation in the quantity of rubisco can be calculated as the decrease from RSS at ambient CO_2 to RLS at elevated CO_2. This decrease (12.1%) corresponds to the observed 70.2% total drop in Vc_{max} (17.2%). According to the results obtained, it can be concluded that most of the photosynthetic acclimation in alfalfa plants cultivated in growth chambers under elevated CO_2 is related to changes in RLS, which is the limiting subunit.

Some studies have related the decrease in rubisco protein under elevated CO_2 with limited N availability (Farage *et al.*, 1998; Geiger *et al.*, 1999; Rogers and Ainsworth, 2006). Symbiotic N_2 fixation could counterbalance the CO_2-induced N limitation in the rhizosphere (Haase *et al.*, 2007). Nevertheless, N_2-fixation in alfalfa has been revealed as insufficient to support the N demand at elevated CO_2 concentrations in previous work (Aranjuelo *et al.*, 2005a, 2008) resulting in reduced leaf N (Sanz-Sáez *et al.*, 2010). In the present study, alfalfa plants showed reduced leaf N but not stem or root N, which led to no differences in the total N (Table 2). The reduced leaf N was linked to impaired nodule functioning under elevated CO_2 conditions, as reflected by the lower specific nodule activity (SNA) (Table 2) (Aranjuelo *et al.*, 2005b). This fact may be related to a previously observed drop in nodule protein content as well as decreases in plant and bacteroid enzymatic activities like malate dehydrogenase or phosphoenolpyruvate carboxylase (Aranjuelo *et al.*, 2008). Nevertheless, variability in the response of SNA to elevated CO_2 has been extensively reported. Some authors have reported an increase of SNA in alfalfa with root CO_2 enrichment (Fischinger *et al.*, 2010), whereas in most reported experiments shoot CO_2 assimilation shows neither a short- nor a long-term effect on SNA (Vance and Heichel, 1991; Cabrerizo *et al.*, 2001).

The nodule proteomic profile under elevated CO_2 confirmed the alteration of nodule metabolism that reduced nitrogenase reductase (Figure 3; Table 3). Nitrogenase

reductase, frequently referred to as Fe-protein (Howard and Rees, 1994), is part of the nitrogenase enzyme complex and catalyzes the N_2 fixation reaction (Dixon and Kahn, 2004; Seefeldt *et al.*, 2009). The lowered leaf N demand of photosynthetically acclimated plants, as observed under water-limited conditions in soybean (Serraj *et al.*, 1998; 1999), may lead to a decline in N-transporting solutes. It also favours the accumulation of products associated with N_2 fixation in nodules leading to an inhibition of nitrogenase activity in bacteroids. The depletion in nitrogenase reductase content (Figure 3; Table 3) suggests that the lower shoot N demand negatively affected the availability of the activity of this protein and consequently affected nitrogenase activity and N_2 fixation similarly.

In nodules grown under elevated CO_2, the bacteroid citrate synthase was also repressed (Figure 3; Table 3). This enzyme catalyzes oxaloacetate condensation with acetyl-CoA, a step in citrate synthesis (Popova and Pinheiro de Carvalho, 1998), and is involved in the initial stage of the tricarboxylic acid (TCA) cycle. Citrate synthase in association with isocitrate dehydrogenase may be a crucial point in TCA cycle regulation (Wiegand and Remington, 1986; Chen and Gadal, 1990). Changes in the rate of the TCA cycle and its anaplerotic reactions may not only alter the source of redox equivalents for the electron transfer chain, but also act as a source of intermediates for lipogenesis, organic and amino acid synthesis. Thus, TCA is considered as a central point of bacteroid intracellular metabolism, decreasing energy supply to nitrogenase and other energy demanding processes (Popova and Pinheiro de Carvalho, 1998). Therefore, its depletion could have contributed to the poor nodule performance of nodules exposed to elevated CO_2. The proteomic characterization (Figure 3, Table 3) also revealed a decrease in catalase content. Aranjuelo *et al.* (2008) showed that in alfalfa plants exposed to elevated CO_2, the activity of leaf antioxidant enzymes such as catalase, superoxide dismutase and glutathione reductase significantly decreased. This relaxation of the antioxidant system was related to the lower growth rate that resulted from photosynthesis acclimation (Erice *et al.*, 2007). This observation agrees with the repression of isoflavone reductase (Figure 3) (Table 3), a protein involved in the production of isoflavone phytoalexins that accumulates under biotic or abiotic stresses (Salekdeh *et al.*, 2002; Kim *et al.*, 2003) and along with antioxidants plays a crucial role in cell signalling or maintaining the redox status of cells (Lee *et al.*, 2009). This

metabolic reduction in the nodules is also supported by the repression of a bacteroid ABC transporter (Figure 3) (Table 3) implicated in urea production (Wang *et al.*, 2008), peptide transients (Stacey *et al.*, 2002) and legume nodule development (Marx, 1996) and all of which may also be inhibited by the lower leaf N demand under elevated CO_2.

Besides, the nodules of alfalfa plants grown at elevated CO_2 concentrations showed increased carbonic anhydrase content (Figure 3) (Table 3). This enzyme with the combined activity of phosphoenolpyruvate carboxylase (Vance *et al.*, 1994) and malate dehydrogenase (Schulze *et al.*, 2002) transforms phosphoenolpyruvate into oxaloacetate and malate (Atkins *et al.*, 2001). The higher carbonic anhydrase activity is needed to maintain a continuous supply of malate (Gálvez *et al.*, 2000), and thus C and energy available for bacteroid consumption (Aranjuelo *et al.*, 2009). The up-regulation of this protein revealed that the poor nodule performance was not caused by limitations on C supply (in form of organic acids, the main C source for nodules) to the nodules. In parallel to this effect, bicarbonate/CO_2 equilibrium on convective gas flow into legume nodules would enhance O_2 transport into the central zone (Thumfort, 1996).

In summary, after long-term exposure, alfalfa plants acclimated to elevated CO_2 showed decreases in A_{sat} and Vc_{max}. This down-regulation was related to leaf TNC accumulation which may reduce RbcS expression. Nevertheless, increased CO_2 did not decrease the RSS content, probably due to posttranscriptional processes including mRNA stability, the improvement of translation of RbcS mRNA and/or the inhibition of proteolysis (resulting to a modification of RSS turn-over). Under elevated CO_2 RLS decreased at photosynthesis limiting levels, reducing the potential photosynthesis by around 12%, which is the majority (70%) of the observed total drop in Vc_{max} in these plants. Photosynthetic down-regulation has been associated with decreased N availability. Reduction in SNA suggests that the poor nodule performance was involved in the leaf N decrease. Limited bacteroid metabolism, as reflected by lower citrate synthase, would require lower O_2 demand. Increased carbonic anhydrase is related to changes in O_2 permeability, which may damage N_2-fixation through nitrogenase reductase sensitive to O_2 concentration. Oxygen permeability alteration may lead to oxidative free radical production and together with antioxidant system inhibition (catalase and isoflavone reductase) may be involved in the drop in SNA under elevated

CO_2. Moreover, a reduced bacteroid N transient capacity due to ABC transporter depletion could results in a decline in nitrogenase activity via a feedback mechanism and lead to leaf N diminution. In the light of these results, new perspectives on the study of N_2-fixing plants acclimated to elevated CO_2 have been opened. Further investigations are needed concerning the role of bacteroid isocitrate synthase as a key enzyme of the TCA cycle, the function of carbonic anhydrase in the nodule inner cortex, and its implication in regulating the O_2 concentration and the alteration of nitrogenase reductase as well as N transport from bacteroids to infected plant cells.

Acknowledgements

This work was supported by Ministerio de Ciencia e Innovación (MICINN BFU2008-01405), Fundación Universitaria de Navarra (PIUNA-2008) and Fundación Caja Navarra. Álvaro Sanz-Sáez was the recipient of a research grant from Asociación de Amigos de la Universidad de Navarra. The assistance of Amadeo Urdiain and Mónica Oyarzun is also appreciated.

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CAPÍTULO 3

Alfalfa yield under elevated CO_2 and temperature depends on the *Sinorhizobium* strain and growth season

La producción de alfalfa en condiciones de CO_2 y temperatura elevados depende de la cepa de *Sinorhizobium* y de la época de crecimiento

Enviado a *Environmental and Experimental Botany*

Alfalfa yield under elevated CO_2 and temperature depends on the *Sinorhizobium* strain and growth season

Resumen

El objetivo principal del estudio fue analizar el efecto del CO_2 elevado y la alta temperatura en plantas de alfalfa inoculadas con tres cepas de *Sinorhizobium meliloti*, crecidas en verano y en otoño en Invernaderos de Gradiente Térmico (IGT), con una acumulación similar de grados día. Para alcanzar este objetivo, se realizaron análisis de crecimiento, intercambio gaseoso y actividad nitrogenasa. La interacción entre el elevado CO_2 y la alta temperatura aumentaron la producción de la alfalfa en las dos estaciones; sin embargo, las plantas produjeron más materia seca en otoño que en verano, posiblemente debido a un efecto negativo de la temperatura sobre la fijación de N_2 en verano. En esta estación, el aumento de la producción de las plantas inoculadas con la cepa 102F78, no se relacionó con un aumento en la materia seca nodular o en la fijación de N_2 sino con un menor coste de carbono de la fijación de N_2. Por el contrario en otoño, la mayor producción en las plantas inoculadas con la cepa 102F34, se asoció a un aumento de la materia seca nodular en condiciones de CO_2 y temperaturas elevados.

Summary

The aim of the present study was to analyze the effect of elevated CO_2 and increased temperature on plants inoculated with three *Sinorhizobium meliloti* strains and grown in Temperature Gradient Greenhouses (TGG) in summer and autumn, with similar degree-days accumulation. Plant growth, gas exchange and apparent nitrogenase activity were analysed. Interaction of CO_2 and temperature enhanced alfalfa dry matter in both seasons; however, plants produced more dry matter in autumn than in summer, due to the negative effect of elevated summer temperature on N_2-fixation. Higher yield in summer corresponded to plants in symbiosis with 102F78 strain being not related to enhanced nodule dry matter or apparent nitrogenase activity but to putative lower carbon consumption for N_2 fixing process. Contrariwise, in autumn the highest yield was obtained by 102F34 as a consequence of increased nodule dry matter induced under elevated CO_2 and temperature.

Key words: Carbon dioxide, dry matter production, *Medicago sativa* (alfalfa), N_2-fixation, nitrogen, *Sinorhizobium meliloti*.

Abbreviations: ANA, apparent nitrogenase activity; DM, dry matter; C_i, intercellular CO_2; IPCC, Intergovernmental Panel on Climate Change; A, net photosynthesis; LNUE, leaf nitrogen use efficiency; TGG, temperature gradient greenhouse.

Introduction

Human activities like deforestation, intensive animal husbandry and fossil fuel burning are responsible for the considerable increase in atmospheric CO_2 over the last 150 years and this is expected to continue. The Intergovernmental Panel on Climatic Change (IPCC) predicts that elevated CO_2 concentration will be enhanced from 375 to 700 μmol mol^{-1} CO_2 in the next 100 years, leading also to an increase of 4ºC in atmospheric mean temperature (IPCC, 2007). Human activities do not only affect the CO_2 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 fertilisers (Vitousek *et al.,* 1997).

The rising CO_2 concentration enhances the potential net photosynthesis in C3 plants (Drake *et al.,* 1997) and therefore increases yield (Oliveira *et al.,* 2010) over short-term exposures. Nevertheless, many studies have shown that photosynthesis acclimates to elevated CO_2 over long-term experiments causing a reduction in potential growth (Erice *et al.,* 2006a; Ainsworth and Rogers, 2007; Aranjuelo *et al*., 2009). This process, often known as photosynthetic "down-regulation" (Saralabai *et al*., 1997) is due to a metabolic limitation usually attributable to reduced carboxylation activity (Erice *et al*., 2006b) and/or a reduced amount of rubisco at elevated CO_2 (Urban, 2003; Aranjuelo *et al*., 2005b).

Nitrogen is a factor that could limit the response of the plant to elevated CO_2, as a limited soil N supply could diminish plant N availability in the long-term, leading to lower photosynthesis and therefore yield reduction (Peterson *et al*., 1999; Luo *et al*., 2004). A decrease in leaf N content under elevated CO_2 could be a consequence of the accumulation of carbohydrates, leaf structural material and the increase in plant internal demands for N (Ellsworth *et al*., 2004). The low availability of leaf N in elevated CO_2 conditions causes the immobilisation of C, leading to carbohydrate accumulation and thus photosynthetic down-regulation and yield potential diminishment. Nevertheless, plants grown at elevated CO_2 and irrigated with N avoid photosynthetic down-regulation and thus increase plant production (Sanz-Sáez *et al.,* 2010).

Alfalfa (*Medicago sativa* L.) is an important forage crop for ecological and economical reasons, it establishes a symbiotic relationship with a N_2-fixing bacterium

(*Sinorhizobium meliloti*) providing an extra source of N for the plant and soil, improving soil structure and increasing soil organic matter (Bourgeois, 1990). N_2-fixing legumes often show a larger stimulation of growth and photosynthetic rate from elevated atmospheric CO_2 than species without this capability (Lüscher *et al.*, 1998). In this symbiotic relationship, atmospheric N_2 is fixed in exchange for plant carbohydrates (Bergersen, 1969) increasing plant sink strength and reducing C accumulation (Udvardi and Day, 1997). In this respect, increases in C availability for nodules in CO_2 elevated conditions may enhance nodular activity providing more N for plant growth (Vance and Heichel, 1991). Bertrand *et al.* (2007a) proved that the interaction between different *S. meliloti* strains and alfalfa genotypes alters plant responses to different ambient variables like freeze tolerance at elevated CO_2. Prevost *et al.* (1999) showed that the selection of cold adapted rhizobia constitutes a valuable tool to counteract the negative effect of low temperature by improving legume productivity under these conditions. Furthermore, tolerance to elevated temperature has also been reported as dependent on the strain of the inoculated *Rhizobium* in algarroba (Kulkarni and Nautiyal, 1999).

Ambient variables that influence plant growth fluctuate with circadian (Aranjuelo *et al.,* 2005a) and seasonal rhythms, affecting plant response to rising CO_2 and thus, plant production (Newton *et al.,* 1994; Tissue *et al.,* 1997). Temperature gradient greenhouses (TGG) can realistically simulate conditions of this expected future environmental change providing various CO_2 concentrations and temperature regimes under field conditions (Aranjuelo *et al.,* 2005a).

The objective of the present study was to analyze the effect of elevated CO_2 and increased temperature on plants inoculated with different *Sinorhizobium meliloti* strains and grown in Temperature Gradient Greenhouses (TGG) in summer and autumn with similar degree-days accumulation. This objective is in line with the selection of *S. meliloti* strains in order to maintain high forage yield along the year in future climate conditions.

Materials and Methods

Biological material

Alfalfa (*Medicago sativa* L. cv. Aragón) seeds were sterilised in a solution of $HgCl_2$ (0.1% w/v) and germinated in Petri dishes. One week later, seedlings were planted in pots (20 plants per pot) containing a mixture of perlite–vermiculite (2/1; v/v). Pots with a capacity of 13 L were used to avoid the possibility of becoming pot-bound. At planting, seedlings were inoculated with three different *Sinorhizobium meliloti* strains: 102F78, 102F34 (Nitragin Co., Milwaukee, WI), and 1032GMI (Biotechnology Department, Polytechnic University of Madrid, Spain) which were classified as Hup^- strains.

Experimental design and growth conditions

Plants were grown in two Temperature Gradient Greenhouses (TGG) and irrigated alternately with Evans N-free solution (Evans, 1974) and distilled water to avoid salt accumulation in the substrate during the experiment. Temperature Gradient Greenhouses (TGG) were placed at the University of Navarra campus (42.80N, 1.66W; Pamplona, Spain). The design of the TGG was similar to that described by Aranjuelo *et al.* (2005a) and Erice *et al.* (2007) based on Rawson *et al.* (1995). CO_2 concentration, temperature, relative humidity and solar radiation levels inside and outside the greenhouses were continuously monitored and controlled by a computerised system. Plants were divided into twelve treatments comprising a combination of two CO_2 levels (ambient, around 350 and elevated, 700 μmol mol^{-1}), two temperature regimens (ambient and ambient + 4ºC) and three *Sinorhizobium meliloti* strains (102F78, 102F34 and 1032GMI). The experiment was carried out in two different seasons, summer and autumn, with similar degree-days accumulation (around 750), which means eight weeks growth during summer and nine weeks growth in autumn. Accumulated degree-days over the experiment were calculated according to McMaster and Wilhelm (1997) with a base temperature of 5ºC (Confalioneri and Bechini, 2004). One greenhouse was maintained at ambient CO_2 concentration levels (338 and 276 μmol mol^{-1} CO_2 in summer and autumn, respectively), and the other was maintained at elevated CO_2 levels (697 and 688 μmol mol^{-1} CO_2 in summer and autumn, respectively). Each greenhouse was divided into three modules, thereby providing different temperature

values. The middle module was considered as a transition module, and no experimental plants were included. In each greenhouse the inlet module was maintained at ambient temperatures (medium temperature was 18 ºC in summer and 14 ºC in autumn) and the outlet module at ambient temperature + 4 ºC. The CO_2 concentration was monitored continuously using a Guardian Plus (Edinburgh Instruments Ltd., Livingston, UK) at the outlet module. The monitor's signal was fed into a proportional integrative differential controller that regulated the opening time (within a 10 s cycle) of a solenoid valve that injected CO_2 into both inlet fans. Pots were placed at inlet and outlet modules, and rotated daily in each module to avoid edge effects. The harvest was carried out before flowering on 60 day-old plants in summer and 67 day-old plants in autumn.

Plant growth parameters

Harvested plants were separated into leaves, stems, roots and nodules. Total leaf area was measured using an automatic leaf area meter (Li-3000, LiCor, NE, USA). Plant dry matter (DM) was obtained after drying at 60 ºC for 48 h. The root/shoot ratio (R/S) was calculated as root DM including nodules divided by the sum of leaf and stem DM.

Apparent Nitrogenase Activity

Apparent nitrogenase activity (ANA) was measured as H_2 evolution in the intact plants with root system sealed in 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 (QubitSystemInc., Canada) as described by González *et al.* (2001) with a flow rate of 500 ml min^{-1}. 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 per nodule DM (μmol H_2 g^{-1} nodule DM min^{-1}).

Gas exchange parameters

Gas exchange was measured in fully expanded young leaves in 60 day-old plants in summer and 67 day-old plants in autumn using a portable gas exchange system GFS-3000 (Walz, Effeltrich, Germany). Net photosynthesis (A) was measured at 350 μmol mol^{-1} CO_2

for plants grown at ambient CO_2 and at 700 μmol mol^{-1} CO_2 for plants grown at elevated CO_2 concentrations. Leaf conductance (g) and intercellular CO_2 concentration (Ci) were calculated as described by Long and Hallgreen (1985).

Nitrogen content

Leaf samples corresponding to 60 day-old plants in summer and 67 day-old in autumn were collected. Samples were dried at 60 ºC for 48 h and analysed for %N of total organic matter. One milligram of ground sample was used for each determination, and 8 replicates were analysed for each treatment (4 experimental and 2 laboratory replicates). Leaf N was determined using an elemental analyser (EA1108, Series 1, Carlo Erba Instrumentazione, Milan, Italy). Nitrogen content was calculated as g m^{-2}. Leaf nitrogen use efficiency (LNUE) was calculated as total DM divided by leaf N content.

Statistical analysis

Statistical analysis was carried out using three-factor ANOVA (factorial 2 x 2 x 3) (SPSS v.15.0). Taking CO_2 concentration as the first factor (ambient CO_2, around 400 μmol mol^{-1}; and elevated, 700 μmol mol^{-1}), temperature as the second factor (ambient T and ambient T +4 ºC) and the *Sinorhizobium meliloti* strain as the third factor (102F78, 102F34 and 1031GMI). Twelve treatments in total with four experimental replicates per treatment were performed. Significant differences between factors and interactions were calculated at 5%, 1% and 0.1% levels of significance. When differences between treatments (CO_2 and/or T and/or Strains and/or interactions) were significant according to the ANOVA analysis, least significant differences were evaluated using the least significant different test (LSD) ($P < 0.05$).

Results

In summer, plant dry matter (DM) was enhanced in plants growing in the TGG under elevated CO_2 and temperature, regardless of the *Sinorhizobium* strain (Fig.1) (*CO_2, $F= 54.23$, $P < 0.001$*) (*T, $F= 26.7$ $P < 0.001$*). Nevertheless 102F78 was the most productive under the CO_2 and temperature treatments (*strain*, *$F= 14.73$, $P < 0.001$*). In autumn, elevated CO_2 and temperature enhanced DM, especially with 102F34 strain, which was the most productive in all treatments (Fig.1) (*CO_2*T*strain, $F= 11.06$, $P < 0.001$*).

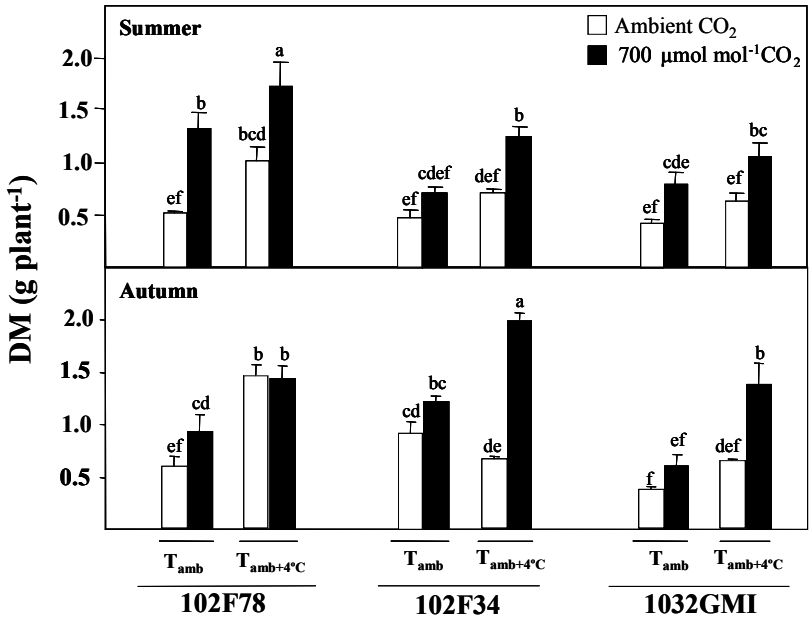

Figure 1: Effect of CO_2 (ambient CO_2, approximately 350 µmol mol^{-1} and elevated, 700 µmol mol^{-1}) and temperature (ambient and ambient +4ºC) on total dry matter (DM, g plant $^{-1}$) in alfalfa nodulated with *Sinorhizobium meliloti* strains (102F78, 102F34 and 1032GMI) after growing in the temperature gradient greenhouses in summer and autumn. Bars represent the mean ± SE; n= 4. Bars with the same letter are not significantly different (P> 0.05) according to LSD test.

Leaf area was enhanced by elevated CO_2 and temperature in summer (CO_2, *$F= 41.42$, $P < 0.001$; T, $F= 35.22$, $P < 0.001$*) and autumn (*CO_2, $F= 22.45$, $P < 0.001$; T, $F= 33.75$, $P < 0.001$*) (Table 1). Plants inoculated with 102F78 in summer, and with 102F34 in autumn presented the highest leaf area (Table 1) (Summer, *strain,* F= 10.98, P < 0.001; Autumn*, strain, $F= 21.94$, $P < 0.001$*). In summer and autumn, the R/S was not modified by CO_2, temperature, or the inoculated strain (Table 1). In summer, elevated CO_2 and temperature decreased leaf N concentration in plants inoculated with 102F78.

			Summer			Autumn		
			Leaf Area	R/S	Leaf N	Leaf Area	R/S	Leaf N
102F78	**Amb T**	Ambient CO_2	67.0 ± 9.0 ef	0.45 ± 0.03 a	1.4 ± 0.03 bcde	75.0 ± 10 cd	0.61 ± 0.07 a	1.5 ± 0.10 a
		Elevated CO_2	102 ± 11 bcd	0.68 ± 0.06 a	1.3 ± 0.04 def	77.0 ± 9.0 cd	0.72 ± 0.09 a	1.6 ± 0.16 a
	Amb T +4ºC	Ambient CO_2	114 ± 9.0 bc	0.47 ± 0.07 a	1.8 ± 0.01 a	130 ± 14 b	0.66 ± 0.07 a	1.4 ± 0.10 a
		Elevated CO_2	197 ± 29 a	0.48 ± 0.09 a	1.3 ± 0.07 cdef	132 ± 16 b	0.62 ± 0.03 a	1.1 ± 0.21 a
102F34	**Amb T**	Ambient CO_2	52.0 ± 5.0 f	0.65 ± 0.13 a	1.4 ± 0.06 bcd	121 ± 7.0 b	0.62 ± 0.10 a	1.3 ± 0.17 a
		Elevated CO_2	74.0 ± 11 def	0.63 ± 0.11 a	1.5 ± 0.02 bc	126 ± 7.0 b	0.53 ± 0.06 a	1.5 ± 0.05 a
	Amb T +4ºC	Ambient CO_2	71.0 ± 5.0 ef	0.54 ± 0.10 a	1.6 ± 0.07 ab	77.0 ± 9.0 cd	0.65 ± 0.11 a	1.3 ± 0.22 a
		Elevated CO_2	135 ± 5.0 b	0.31 ± 0.11 a	1.8 ± 0.20 a	205 ± 15 a	0.62 ± 0.09 a	1.6 ± 0.17 a
1032GMI	**Amb T**	Ambient CO_2	49.0 ± 3.0 f	0.52 ± 0.02 a	1.4 ± 0.05 bcde	46.0 ± 5.0 d	0.60 ± 0.07 a	1.2 ± 0.15 a
		Elevated CO_2	97.0 ± 13 cde	0.46 ± 0.07 a	1.2 ± 0.04 f	62.0 ± 9.0 cd	0.51 ± 0.05 a	1.5 ± 0.11 a
	Amb T +4ºC	Ambient CO_2	76.0 ± 9.0 def	0.61 ± 0.14 a	1.5 ± 0.09 bc	80.0 ± 9.0 c	0.53 ± 0.07 a	1.4 ± 0.13 a
		Elevated CO_2	111 ± 18 bcd	0.84 ± 0.17 a	1.2 ± 0.07 ef	120 ± 26 a	0.47 ± 0.05 a	1.2 ± 0.03 a
		CO_2	***	ns	**	***	ns	ns
		T	***	ns	**	***	ns	ns
		Strain	***	ns	***	***	ns	ns
		CO_2 x T x Strain	ns	ns	***	**	ns	ns

Table 1: Effect of CO_2 (ambient, approximately 350 µmol mol^{-1} and elevated, 700 µmol mol^{-1}) and temperature (ambient and ambient +4ºC) on leaf area (cm^2 $plant^{-1}$), root/shoot (R/S) ratio and leaf N content (g m^{-2}) in alfalfa nodulated with *Sinorhizobium meliloti* strains (102F78, 102F34 and 1032GMI) after growing in the temperature gradient greenhouses in summer and autumn. Values represent the mean ± SE; n = 4. Statistical analysis was undertaken with a three factor ANOVA, see the results at the bottom of the table. The symbols used in ANOVA are: *, Significant difference at 5%. ** 1% and *** 0.1%. When significant differences were detected in ANOVA, LSD analysis was applied. Means followed by the same letter are not significantly different (P > 0.05) according to LSD test parameters.

(*CO_2*T*strain, F= 10.91, P < 0.001*) (Table 1). In summer, plants inoculated with 1032GMI at elevated CO_2 had a reduced leaf N concentration regardless the temperature regime (*CO_2*T*strain, F= 10.91, P < 0.001*). In autumn, the leaf N concentration was not altered by elevated CO_2, temperature or the strain (Table 1).

Apparent nitrogenase activity (ANA) per nodule DM decreased in the interaction of CO_2 and temperature in both seasons (Fig. 2) (Summer *CO_2*T, F= 18.3, P < 0.001; autumn F= 8.35, P < 0.008*). Elevated CO_2 increased nodule DM in summer only with 102F34 (*CO_2, F= 7.42, P = 0.01*), whereas in autumn, elevated CO_2 increased nodule DM in all strains (*CO_2, F= 85.44, P < 0.001*) (Fig.2). In autumn, the interaction between elevated CO_2 and temperature increased nodule DM (*CO_2*T, F= 24.18, P < 0.001*).

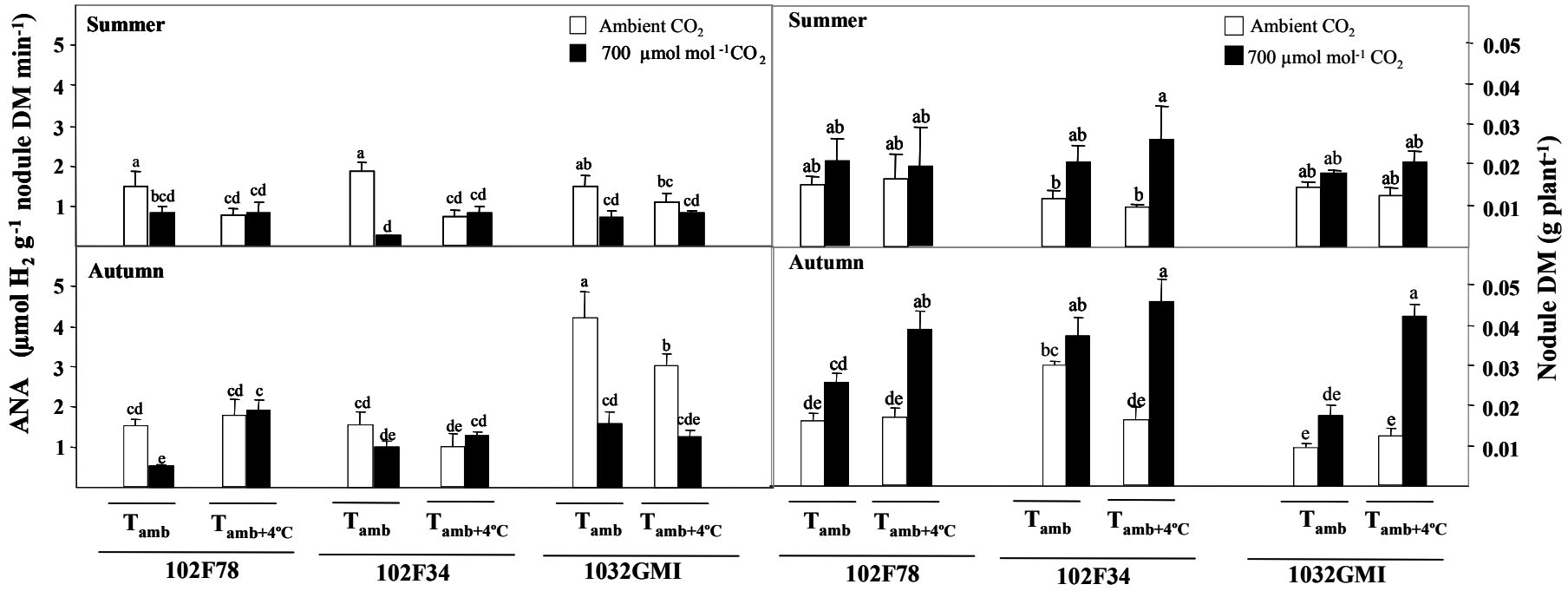

Figure 2: Effect of CO_2 (ambient, approximately 350µmol mol^{-1} and elevated, 700 µmol mol^{-1}) and temperature (ambient and ambient +4ºC) on apparent nitrogenase activity (ANA, µmol H_2 g^{-1} nodule DM min^{-1}) and nodule dry matter (g $plant^{-1}$) in alfalfa nodulated with *Sinorhizobium meliloti* strains (102F78, 102F34 and 1032GMI) after growing in the temperature gradient greenhouses in summer and autumn. Bars represent the mean ± SE; n= 4. Bars with the same letter are not significantly different (P> 0.05) according to LSD test.

Photosynthesis (A) at growth CO_2 concentrations was not modified by elevated CO_2 or temperature in summer or autumn (Table 2). Elevated CO_2 decreased leaf conductance (g) in plants inoculated with 102F78 in summer (*CO_2, $F= 6.59$, $P = 0.017$*), nevertheless in autumn, elevated CO_2 did not modify g at growth conditions (Table 2). Elevated CO_2 enhanced intercellular CO_2 concentration (Ci) in summer and autumn (*CO_2, $F= 626.9$, $P < 0.001$; CO_2, $F=945.8$, $P < 0.001$*, respectively).

			Summer			Autumn		
			A (μmol CO_2 m^{-2} s^{-1})	g (mmol m^{-2} s^{-1})	Ci (μmol CO_2 mol^{-1})	A (μmol CO_2 m^{-2} s^{-1})	g (mmol m^{-2} s^{-1})	Ci (μmol CO_2 mol^{-1})
102F78	**Amb T**	Ambient CO_2	28.9 ± 2.1 a	390 ± 40 ab	272 ± 4.0 bc	20.8 ± 1.6 a	316 ± 38 a	246 ± 15 c
		Elevated CO_2	28.3 ± 1.9 a	265 ± 45 c	500 ± 25 a	26.0 ± 2.5 a	264 ± 10 a	533 ±10 ab
	Amb T +4ºC	Ambient CO_2	27.9 ± 3.2 a	353 ± 89 abc	223 ± 45 c	20.3 ± 2.0 a	265 ± 5.0 a	219 ± 13 c
		Elevated CO_2	27.6 ± 4.7 a	273 ± 44 abc	514 ± 6.0 a	24.3 ± 1.8 a	220 ± 25 a	496 ± 24 b
102F34	**Amb T**	Ambient CO_2	24.2 ± 3.3 a	311 ± 35 abc	267 ± 5.0 bc	24.8 ± 3.0 a	276 ± 42 a	218 ±10 c
		Elevated CO_2	29.9 ± 3.4 a	274 ± 39 abc	505 ± 10 a	22.9 ± 2.2 a	231 ± 20 a	522 ± 18 ab
	Amb T +4ºC	Ambient CO_2	27.3 ± 1.0 a	379 ± 20 abc	273 ± 5.0 bc	19.9 ± 0.9 a	257 ± 28 a	218 ± 14 c
		Elevated CO_2	26.5 ± 2.1 a	272 ± 27 abc	520 ± 5.0 a	24.8 ± 1.1 a	274 ± 41 a	520 ± 23 ab
1032GMI	**Amb T**	Ambient CO_2	23.5 ± 0.9 a	357 ± 23 abc	286 ± 9.0 b	24.3 ± 1.8 a	283 ± 80 a	240 ± 12 c
		Elevated CO_2	29.1 ± 1.8 a	272 ± 39 bc	506 ± 16 a	25.1 ± 1.5 a	261 ± 43 a	500 ± 23 b
	Amb T +4ºC	Ambient CO_2	25.6 ± 1.8 a	333 ± 22 abc	267 ± 7.0 bc	23.1 ± 2.8 a	288 ± 28 a	221 ± 14 c
		Elevated CO_2	32.3 ± 4.0 a	395 ±37 a	542 ± 17 a	25.1 ± 0.9 a	307 ± 20 a	563 ± 15 a
ANOVA		CO_2	ns	*	***	ns	ns	***
		T	ns	ns	ns	ns	ns	ns
		Strain	ns	ns	ns	ns	ns	ns
		CO_2 x T x Strain	ns	ns	ns	ns	ns	ns

Table 2: Effect of CO_2 (ambient, approximately 350 µmol mol^{-1} and elevated, 700 µmol mol^{-1}) and temperature (ambient and ambient +4ºC) on net photosynthesis (A) measured at growth CO_2 concentrations, leaf conductance (g) and intercellular CO_2 concentration (Ci) in alfalfa nodulated with *Sinorhizobium meliloti* strains (102F78, 102F34 and 1032GMI) after growing in the temperature gradient greenhouses in summer and autumn. Values represent the mean ± SE; n = 4. Statistical analysis was undertaken with a three factor ANOVA, see the results in the bottom of the table. The symbols used in ANOVA are: *, Significant difference at 5%. ** 1% and *** 0.1%. When significant differences were detected in ANOVA, LSD analysis was applied. Means followed by the same letter are not significantly different (P > 0.05) according to LSD test parameters.

In summer, elevated CO_2 and temperature increased leaf nitrogen use efficiency (LNUE) in plants inoculated with the 102F78 and 102F34 strains (Fig. 3) (*CO_2, $F= 14.97$, $P = 0.001$; T, $F= 25.44$, $P < 0.001$*); however in autumn LNUE was not modified by elevated CO_2 or temperature (Fig. 3).

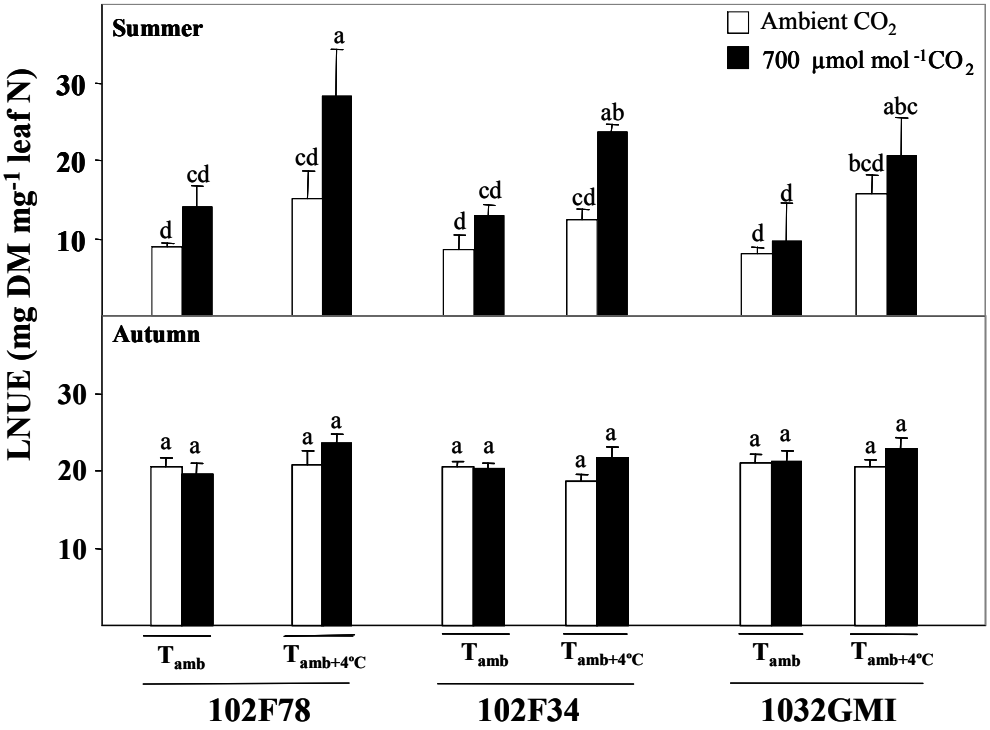

Figure 3: Effect of CO_2 (ambient, approximately 350 µmol mol^{-1} and elevated, 700 µmol mol^{-1}) and temperature (ambient and ambient +4ºC) on leaf nitrogen use efficiency (LNUE, mg DM mg^{-1} leaf N) in alfalfa nodulated with *Sinorhizobium meliloti* strains (102F78, 102F34 and 1032GMI) after growing in the temperature gradient greenhouses in summer and autumn. Bars represent the mean ± SE; n= 4. Bars with the same letter are not significantly different (P> 0.05) according to LSD test.

Discussion

The interaction between elevated CO_2 (700 μmol mol^{-1}) and temperature (ambient +4ºC) enhanced plant production in both seasons (Fig. 1). The most productive season was autumn (17.2 % more than summer), as shown previously by Aranjuelo *et al.* (2007), and this was possibly due to the negative effect of high temperature during summer. In summer, plants inoculated with 102F78 strain were the most productive under elevated CO_2 and temperature, and with significant DM increase at ambient temperature. In autumn, plants in symbiosis with 102F34 was revealed as the most productive strain, but contrariwise to summer, elevated CO_2 only increased DM production when interacted with elevated temperature. It is noteworthy that 1032 GMI is the least productive strain in all treatments and seasons.

In both summer and autumn, elevated CO_2 did not enhance photosynthesis, and therefore plants were photosynthetically down-regulated (Table 2) as was also reported previously with same-aged plants and an even shorter exposure time to elevated CO_2 (Aranjuelo *et al.,* 2005a, b; Erice *et al.,* 2006a, b). This photosynthetic acclimation was not caused by lower intercellular CO_2 concentrations (Ci) as a result of stomatal closure because throughout the experiments, plants grown at elevated CO_2 showed higher Ci (Table 2) than those grown at ambient CO_2 (Aranjuelo *et al.,* 2005b). The increase in plant DM at elevated CO_2 was a consequence of total photosynthesis enhancement, which in turn was as a result of increased leaf area in these treatments (Table 1) (Craine *et al.,* 2003; Luo *et al.,* 2004; Aranjuelo *et al.,* 2008; Sanz-Sáez *et al.,* 2010).

One of the factors that may limit photosynthesis and growth under elevated CO_2 is the N availability (Erice *et al.,* 2006b; Sanz-Sáez *et al.,* 2010). When plants are N limited, down-regulation has been related to reallocation of N away from the photosynthetic apparatus (Aranjuelo *et al.,* 2005b) or to particular photosynthetic enzymes (Moore *et al.,* 1999; Rogers and Ellsworth, 2002). Consequently, plants acclimated to elevated CO_2 usually show decreased leaf N content (Tissue *et al.,* 1997; Ellsworth *et al.,* 2004). In this experiment, elevated CO_2 decreased leaf N in summer with 1032GMI regardless of the temperature regime, and had the same effect with 102F78 under elevated temperature (Table 1). According to Ainsworth *et al.* (2003),

elevated CO_2 may partition resources away from leaves and, through increased production, sequester nutrients (like N) into organic matter. This then leads to deficiencies that indirectly cause decreases in photosynthetic capacity and diminish the potential growth as shown during summer in our study. Nevertheless, in autumn, leaf N content was not decreased by CO_2 and/or temperature (Table 1).

In the present study, alfalfa plants obtained N exclusively from biological N_2 fixation in the nodules. Usually, in the legume-*Sinorhizobium* symbiosis, the same factors that increase photosynthesis enhance N_2 fixation and *vice versa* (Aranjuelo *et al.,* 2007). Many authors suggest that elevated CO_2 could enhance N_2 fixation by increasing nodule size, number and/ or nitrogenase activity (Lee *et al.,* 2003; Cen and Layzell, 2004; Bertrand *et al.,* 2007a). In summer, elevated CO_2 only increased nodule DM in 102F34 at elevated temperature, but in autumn this increase was generalised in all strains when the CO_2 increase was combined with temperature treatments (Fig. 2). The nodule DM was lower in summer than in autumn, and this difference may have been caused by elevated temperature that occurred in summer, which could damage nodule development and activity (Hungria and Franco, 1993; Hungria and Vargas, 2000).

Besides reduced nodule DM, ANA activity per nodule DM (ANA) was also lower in summer compared with autumn (43.22%) (Fig. 2). In summer, elevated CO_2 decreased ANA for all strains at ambient temperature. In autumn, elevated CO_2 and temperature decreased ANA in plants inoculated with the 1032GMI strain. The strong diminishment of ANA in summer could provoke decreases in leaf N content (Table 1), which is usually observed in plants grown at elevated CO_2 (Aranjuelo *et al.,* 2005b). In the autumn experiment, the increase in plant DM production (Fig. 1) was more associated with an enhancement of nodule DM rather than ANA improvement (Table 1). In this sense, LNUE in the autumn showed no differences among treatments but in summer increased with the interaction between elevated CO_2 and temperature. These results may confirm that LNUE is a parameter that increases when plants suffer from N limitation derived from elevated CO_2 (Aranjuelo *et al.,* 2008) or temperature (Aranjuelo *et al.,* 2007) and it is less affected by DM production differences. Differences in plant DM produced by the three strains with similar ANA and nodule DM could be linked to differences in the C cost of N_2 fixation (Twary and Heichel, 1991). A higher C

consumption decreases the amount of photosynthates available for plant growth (Bertrand *et al.,* 2007b). Saari and Lundden (1986) reported that the C cost of N_2 fixation is mainly due to the respiratory rate. With regard to the summer results, the 102F78 strain, which had the same ANA and nodule DM but different DM yield, may consume less carbon for N_2 fixation. Therefore, in autumn, the increased plant DM production of 102F34 compared to 102F78 (Fig. 1) does not appear to be associated with enhancement of ANA or nodule DM. This result suggests a greater efficiency (C cost over N_2 fixed) under the cooler autumn temperatures. Accordingly, the 1032GMI strain showed the highest ANA at ambient CO_2 but the lowest DM production, being the least efficient strain at low temperatures.

Conclusion

In summary, greater summer production corresponded to plants in symbiosis with the 102F78 *S. meliloti* strain. The higher efficiency of this strain was not related to enhanced nodule DM or ANA but to a putative lower C consumption for the N_2 fixation process. In contrast, the highest yield in autumn was obtained by 102F34 as a consequence of increased nodule DM induced under a combination of elevated CO_2 and temperature. The interaction of CO_2 and temperature enhanced alfalfa DM production in both seasons. However, in summer, decreased ANA at elevated temperatures together with increased LNUE suggested a N limitation in the warmer season. In conclusion, alfalfa yield was dependent on the *S. meliloti* strain and the efficiency of the different strains varies according to growth conditions with 102F78 being the most efficient at the highest summer temperature and 102F34 being the most efficient at elevated CO_2 and temperature in autumn.

Acknowledgements

This work was supported by Ministerio de Ciencia e Innovación (MICINN BFU2008-01405), Fundación Universitaria de Navarra (PIUNA-2008) and Fundación Caja Navarra. A. Sanz-Sáez was the recipient of a research grant from Asociación de Amigos de la Universidad de Navarra. The assistance of A. Urdiain, M. Oyarzun and L. González is also appreciated.

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CAPÍTULO 4

Photosynthetic and molecular markers of CO_2 mediated photosynthetic down-regulation in nodulated alfalfa

Marcadores fotosintéticos y moleculares de aclimatación fotosintética en alfalfa nodulada

Enviado a *Planta*

Photosynthetic and molecular markers of CO_2-mediated photosynthetic down-regulation in nodulated alfalfa

Resumen

Ha sido ampliamente descrito en la bibliografía, que la exposición a largos periodos de CO_2 elevado provoca un descenso de la fotosíntesis que conlleva la reducción en la producción. A este proceso se le conoce como aclimatación fotosintética. Entre la comunidad científica, no hay un acuerdo claro de cuáles son los parámetros mas sensibles para detectar esta aclimatación. Por lo tanto, el objetivo de este estudio fue investigar el parámetro fotosintético y molecular mas adecuado para detectar la aclimatación fotosintética en plantas crecidas en Invernaderos de gradiente térmico en verano y otoño, en condiciones de CO_2 y temperatura elevados e inoculadas con tres cepas de *Sinorhizobium meliloti* distintas. Al final del experimento, todas las plantas mostraron aclimatación fotosintética, especialmente las crecidas en verano. Esto se debió posiblemente a la menor disponibilidad de nitrógeno asociada a la inhibición de la actividad fijadora de N_2 por las altas temperaturas del verano. Los parámetros más adecuados para detectar la aclimatación fotosintética en orden de sensibilidad, fueron: fotosíntesis medida a concentraciones de CO_2 de crecimiento, actividad *in-vitro* de la rubisco y velocidad máxima de carboxilación de la rubisco. Los casos de aclimatación más severa se correlacionaron también con descensos del contenido de nitrógeno foliar, asociado con caídas en el contenido de la proteína rubisco (subunidad grande, RLS y pequeña, RSS) y en la actividad, que provocaron en un descenso de la fotosíntesis. A pesar de la sensibilidad del contenido de rubisco como marcador de aclimatación, este no está correlacionado con la expresión del gen, debido a la posible existencia de un retraso entre la trascripción del gen y la traducción de la proteína.

Summary

It has been widely described that elevated CO_2 leads to a decrease in net photosynthesis in long-term experiments and thus to a reduction in potential growth. This process is known as photosynthetic down-regulation or acclimation. There is no agreement on the definition of which parameters are the most sensitive for detecting CO_2 acclimation. Therefore, in order to investigate the most sensitive photosynthetic and molecular markers of CO_2 acclimation, the effects of elevated CO_2, and associated elevated temperature were analyzed in alfalfa plants inoculated with different *Sinorhizobium meliloti* strains. Plants were grown in summer or autumn in Temperature Gradient Greenhouses (TGG). At the end of the experiment, all plants showed acclimation in both seasons, especially under elevated summer temperature. This was probably due to the lower N availability caused by decreased N_2-fixation under higher temperature. Photosynthesis measured at growth CO_2 concentration, rubisco *in-vitro* activity and maximum rate of carboxylation (Vc_{max}) were the most sensitive parameters for detecting down-regulation. Severe acclimation also correlated with decreased leaf N associated with declines in rubisco content (large, RLS and small, RSS subunits) and activity that resulted in a drop in photosynthesis. Despite the sensitivity of rubisco content as a marker of acclimation, it was not coordinated with gene expression due to a lag between gene transcription and protein translation.

Key words: Carbon dioxide, *Medicago sativa* (alfalfa), Photosynthetic down-regulation, *RbcL* and *RbcS*, *Sinorhizobium meliloti*.

Introduction

Continued human activities such as deforestation, intensive animal husbandry and fossil fuel burning are responsible for the considerable increase of atmospheric CO_2. The Intergovernmental Panel on Climatic Change (IPCC) predicts that the CO_2 concentration will be enhanced from 375 to 700 μmol mol^{-1} CO_2 by the end of the century. Due to its role as a greenhouse gas, this increase of CO_2 will lead to an increase of 4ºC in atmospheric mean temperature (IPCC 2007).

Some field-based studies have found strong and persistent stimulation of photosynthetic rates in C_3 species grown under elevated CO_2 (Curtis et al. 2000; Ellsworth et al. 1995; Jackson et al. 1995). In contrast, other studies have shown a significant decrease in photosynthetic response to elevated CO_2 (Ainsworth and Rogers 2007; Aranjuelo et al. 2009; Erice et al. 2006a; Lee et al. 2001; Rogers and Ellsworth 2002). High CO_2 concentrations enhance net photosynthesis and biomass production in short-term experiments (Lee et al. 2001; Oliveira et al. 2010). However, it has been widely reported that long-term exposures to elevated CO_2 lead to a decrease in net photosynthesis and thus a reduction in potential growth (Ainsworth and Rogers 2007; Aranjuelo et al. 2009; Erice et al. 2006a). This process, often known as photosynthetic "down-regulation" (Saralabai et al. 1997) is due to a metabolic limitation usually attributable to a reduced carboxylation activity (Erice et al. 2006b) and/or a reduced rubisco amount at elevated CO_2 (Aranjuelo et al. 2005a; Urban 2003). The photosynthetic down-regulation characterized by reduced carboxylation activity has usually been related to a decrease in protein content and gene expression of rubisco under elevated CO_2 conditions (Ainsworth et al. 2002; Aranjuelo et al. 2005b; Leakey et al. 2009; Urban 2003).

One factor that could limit plant responses to elevated CO_2 is the N availability (Sanz-Sáez et al. 2010). Accelerated growth under elevated CO_2 (Erice et al. 2007) increases the demand for nutrients, and in soils with low N availability (as Mediterranean region), N is usually the growth-limiting nutrient (Lüscher et al. 1998). This fact may lead to lower photosynthesis and therefore yield reduction (Luo et al. 2004; Peterson et al. 1999).

Species with an ability for symbiotic N_2 fixation often show a larger stimulation of photosynthesis and thus more growth under elevated CO_2 than plants unable to fix N_2 (Ainsworth et al. 2002; Lee et al. 2003). Alfalfa (*Medicago sativa*), an important forage crop for ecological and economical reasons, establishes a symbiotic relationship with the N_2-fixing bacterium *Sinorhizobium meliloti* providing an extra source of N (Bourgeois 1990) in exchange for plant carbohydrates (Bergersen 1969). It has been suggested by Bertrand et al. (2007) that inoculation with high N_2-fixative *S. meliloti* strains avoided high CO_2-induced photosynthetic down-regulation, and thus enhanced shoot DM in growth chamber experiments. Therefore, the selection of efficient *Sinorhizobium* strains could be of interest for improving plant performance under elevated CO_2.

It is known that plant behaviour in the field frequently differs from that in growth chambers (Aranjuelo et al. 2005a). Ambient variables that influence plant growth fluctuate with circadian (Aranjuelo et al. 2005a) and seasonal rhythms, affecting plant response to rising CO_2 (Newton et al. 1994; Tissue et al. 1997). Teixeira et al. (2008) found that shoot radiation use efficiency is higher in spring than in autumn, suggesting a strong influence of season on alfalfa growth and photosynthesis. In fact, Ainsworth et al. (2003) in Swiss FACE experiments with *Trifolium repens* L. showed photosynthetic down-regulation in autumn but not in spring. Another alternative facility to simulate the effect of projected future environmental change, including CO_2 concentration and temperature regime under near field conditions, is the use of Temperature Gradient Greenhouses (TGG) (Aranjuelo et al. 2005a).

Previous studies performed on alfalfa grown in TGG under elevated CO_2 and temperature showed photosynthetic down-regulation, possibly caused by a reduction in N_2 fixation (Aranjuelo et al. 2005a, b; Erice et al. 2006 a, b). Nevertheless, no studies have been conducted in alfalfa determining the effect of elevated CO_2 and temperature on photosynthesis, rubisco content and gene expression in near field conditions.

The aim of the study was to analyze the effect of elevated CO_2 and temperature in alfalfa inoculated with different *S. meliloti* strains and grown in TGG in summer or autumn. The first objective was to find the most sensitive gas exchange parameter to detect photosynthetic down-regulation under such variable ambient conditions. The second one

was to define the biochemical and molecular markers of photosynthetic acclimation. The third objective was to show if photosynthetic down-regulation may be altered by the *S. meliloti* strain and growth season. These objectives are in line with a wide understanding of alfalfa photosynthetic down-regulation mechanisms and characteristics and with the selection of efficient *S. meliloti* strains in order to maintain high photosynthesis under elevated CO_2, thus enabling increased production throughout the year in future climate conditions.

Material and Methods

Plant material

Alfalfa (*Medicago sativa* L. cv. Aragón) seeds were sterilized in a solution of $HgCl_2$ (0.1% w/v) and germinated in Petri dishes. One week later, seedlings were planted in pots (20 plants per pot) containing a mixture of perlite–vermiculite (2/1; v/v). Pots with a capacity of 13 L were used to avoid the possibility of them becoming pot-bound. Plants were grown in two Temperature Gradient Greenhouses (TGG) and irrigated alternatively with Evans N-free solution (Evans 1974) and distilled water to avoid salt accumulation in the substrate during the experiment. The only N source for the plants was the N_2-fixed by nodules. Plants were inoculated immediately after planting with different *S. meliloti* strains: 102F78, 102F34 (Nitragin Co., Milwaukee, WI), or 1032GMI (Biotechnology Department, Polytechnic University of Madrid, Spain). In previous studies conducted with 102F78, the tolerance of this strain to elevated temperatures has been demonstrated (Aranjuelo et al. 2005 a, b; Erice et al. 2006 a, b). In contrast, there was less information available about 102F34 and 1032GMI, although previous studies have shown that 102F34 was more efficient than 1032GMI (Muro 2009).

Experimental design and description of TGG

Alfalfa seedlings were placed into two TGG placed at the Pamplona campus of the University of Navarra, (42.80N, 1.66W; Spain). The design of the TGG was similar to that described by Aranjuelo et al. (2005a) and Pérez et al. (2005). CO_2 concentration, temperature, relative humidity and solar radiation levels inside and outside the greenhouses were continuously monitored and controlled by a computerized system. Plants were divided into 12 treatments comprising the combination of two CO_2 levels (ambient, around 387 and elevated, 700 μmol mol^{-1}), two temperature regimens (ambient and ambient + 4ºC) and three *S. meliloti* strains (102F78, 102F34 and 1032GMI). The experiment was carried out in two different seasons, summer and autumn, with similar degree-day accumulation (around 750), which means eight weeks growth during summer and nine weeks growth in autumn. According to McMaster and Wilhelm (1997), accumulated degree-days over the experiment were calculated with a base temperature of 5ºC (Confalioneri and Bechini 2004). One

greenhouse was maintained at ambient CO_2 concentration (approximately 392 µmol mol^{-1} CO_2), and the other at elevated CO_2 (700 µmol mol^{-1} CO_2). Each greenhouse was divided into three modules, thereby providing different temperature values. The middle module was considered as a transition module, and no experimental plants were included. In each greenhouse, the inlet module was maintained at ambient temperature (medium temperature was 18ºC in summer and 14ºC in autumn) and the outlet module at ambient temperature + 4ºC. The CO_2 concentration was monitored continuously using a Guardian Plus (Edinburg Instruments Ltd., Livingston, UK) at the outlet module. Its signal was fed into a proportional integrative differential controller that regulated the opening time (within a 10s cycle) of a solenoid valve that injected CO_2 into both inlet fans. Pots were placed at inlet and outlet modules, and rotated daily in each module to avoid edge effects. The harvest was carried out before flowering, 60 day-old plants were sampled in summer and 67 day-old plants in autumn.

Plant growth parameters

Harvested plants were separated into leaves, stems, roots and nodules. Plant dry matter (DM) was obtained after drying at 60ºC for 48 h and calculated as the sum of all organs' DM.

Gas exchange

Gas exchange parameters were measured in fully expanded young leaves in 60 day-old plants in summer and 67 day-old plants in autumn using a portable gas exchange system GFS-3000 (Walz, Effeltrich, Germany). The gas exchange response to CO_2 was measured from 60 to 1400 µmol mol^{-1} CO_2 at 1400 µmol m^{-2} s^{-1} PPFD provided by LED light. Measurements started at 400 µmol mol^{-1} of CO_2, decreased stepwise to 250, 100, 0 µmol mol^{-1} and restarted at 400 and increased stepwise to 700, 850, 1000 and 1400 µmol mol^{-1}. Net photosynthesis (A_{net}) and leaf conductance (g) were calculated as described by Long and Hallgreen (1985). Net photosynthesis measured either at 400 (A_{400}) and 700 µmol mol^{-1} (A_{700}) or at growth conditions (A_{growth}, ambient ones at 400 µmol mol^{-1} and elevated ones at 700 µmol mol^{-1}) were compared. The A/Ci curve measurements were used to assess the

maximum rate of carboxylation (Vc_{max}), employing the mathematical model developed by Ethier and Livingston (2004) and Sharkey et al. (2007).

N content

Leaf, stem, root and nodule samples were collected corresponding to 60 day-old plants in summer and 67 day-old plants in autumn. Samples were dried at 60ºC for 48 h and analysed for %N of total organic matter (TOM). One milligram of ground sample was used for each determination, and 8 replicates were analysed for each treatment (4 experimental and 2 laboratory replicates). Leaf N was determined using an elemental analyzer (EA1108, Series 1, Carlo Erba Instrumentazione, Milan, Italy). Total N content was calculated as the sum of N content of all organs and expressed as mg of N per plant. Leaf N content was calculated as g m^{-2}.

Leaf total soluble proteins and starch concentration

Leaf total soluble proteins (TSP) and starch concentration were quantified by grinding and filtering 200 mg of leaf fresh weight of 60 and 67 day-old plants in summer and autumn respectively, in a cold mortar using an extraction buffer containing 50 mM K-phosphate (pH 7.5). The extract was filtered and centrifuged at 28,710 g for 15 min at 4ºC. The TSP quantification was performed in supernatant, whereas starch was measured using the pellet as described by Jarvis and Walker (1993). TSP levels were measured using the method of Bradford (1976). All measured parameters are expressed on a per area basis.

Rubisco activity and semi-quantification

Rubisco (E.C. 4.1.1.39) activity was measured by grinding and filtering 250 mg of leaf fresh weight in a cold mortar using an extraction buffer containing 100 mM bicine (pH 7.8), 10 mM $MgCl_2$, 10 mM, 2-β-mercaptoethanol and 2% PVPP. The extract was clarified by centrifugation at 26,850 g for 10 min at 4 ºC. Enzyme activity was determined by measuring the absorbance at 340 nm, as described by Lilley and Walker (1974) using a U-2001 spectrophotometer (Hitachi Instruments, Inc, USA). Rubisco total activity was

calculated after incubating the leaf extract in 10 mM $NaCO_3H$ over 10 min in order to activate all Rubisco protein.

For rubisco semi-quantification, extracts from protein quantification were precipitated using the sodium deoxycholate-trichloroacetic acid protocol described by Peterson (1983). The resulting pellets were air dried and resuspended in Laemmli lysis buffer (Laemmli 1970) and boiled for 10 min to denature proteins. For SDS-PAGE, 4 μg of soluble proteins were prepared and electrophoresis was performed using a 150 g L^{-1} acrylamide separation gel and stained with silver nitrate (Blum et al. 1987). Gel images were scanned and analysed using ImageQuant TL software (GE Amersham Biosciences, UK). The relative proportion of rubisco large (RLS) and small (RSS) subunits was calculated in reference to the abundance value of RLS observed in plants inoculated with 102F78 and growth at ambient temperature and CO_2 concentration.

2.8 RNA isolation, synthesis of cDNA and quantitative real-time RT-PCR.

Total RNA was isolated from alfalfa leaves with a phenol/chloroform extraction method (Kay et al. 1987). Rubisco large and small subunits gene expression (*RbcL* and *RbcS, respectively)* was studied by real-time quantitative PCR using iCycler (Bio-Rad, Hercules, California, USA). cDNAs were obtained from 2.5 μg of total DNase-treated RNA in a 20 μL reaction containing 500 ng of random hexamer primers, 0.5 mM of each dNTP, 10 mM DTT, 40 U of RNase inhibitor, 1x first strand buffer (Invitrogen, Carlsbad, California, USA) and 200 U of Superscript II Reverse Transcriptase (Invitrogen). The primer sets used to amplify *RbcL* were: 5′-GAGTAGCTCTGGAAGCATGTG-3′ as forward and 5′-GACTCCATTTGGTAGCCTCAC-3′ as reverse; to amplify *RbcS* the primers used were forward 5′-TTCGGAGCCACTGATTCTTCTC-3′ and reverse 5′-ACTGCACTTGACGAACATTGTC-3′. Each 25 μL q-PCR reaction contained 1 μL of a 1:10 dilution of the cDNA, 200 nM of the dNTPs, 400 nM of each primer, 3 mM $MgCl_2$, 2.5 μL of 1x SyBR Green (Molecular Probes, Eugene, Oregon, USA), and 0.5 U of Platinum *Taq* DNA polymerase (Invitrogen) in 1x PCR buffer (20 mM Tris–HCl, pH 8.4, 50 mM KCl).

The PCR program consisted of a 4 min incubation at 95 ºC to activate the hot-start recombinant *Taq* DNA polymerase, followed by 30 cycles of 45 s at 94 ºC, 45 s at 69ºC, and 50 s at 72ºC, where the fluorescence signal was measured. The results obtained on the different treatments were standardized according to the β-tubulin gene expression levels, which were analyzed with primer forward 5´-GAAGCAAGCGGTGGAAGATATG-3´ and primer reverse 5´-CCAAATGGACCAGAACGCAAAC-3´, which showed stable expression under the conditions tested in this study.

Real-time PCR experiments were carried out with at least four independent RNA samples, with the threshold cycle (C_T) determined in triplicate. The relative levels of transcription were calculated by using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001). Negative controls without cDNA were used in all PCR reactions. The relative gene expression of rubisco large (*RbcL*) and small (*RbcS*) subunits was calculated in reference to the abundance value of *RbcL* and *RbcS* observed in plants inoculated with *S. meliloti* 102F78 and growth at ambient temperature and CO_2 concentration.

Statistical analysis

Statistical analysis was undertaken with three factor ANOVA (factorial 2 x 2 x 3) (SPSS v.15.0), taking CO_2 as the first factor, temperature as the second factor and *S. meliloti* strain as the third factor. In total, 12 treatments were performed, with four experimental replicates. Significant differences between factors and interactions were calculated at 5%, 1% and 0.1% levels of significance. When differences between treatments were significant according to ANOVA, least significant differences were evaluated post-hoc using the least significant difference test (LSD) ($P \leq 0.05$).

Results

Elevated CO_2 did not enhance A_{growth} in any treatment or season but increased Ci in both seasons (*summer, F= 626.9, P ≤ 0.001; autumn, F= 945.87, P ≤ 0.001*) (Table 1). A_{400} decreased at elevated CO_2 in both seasons (*summer*, *F= 34.35, P ≤ 0.001*; autumn *F= 11.77, P = 0.002*) (Fig. 1). A_{700} also diminished the photosynthesis in both seasons (*summer, F= 16.05, P ≤ 0.001*; *autumn, F= 9.77, P = 0.003*) (Fig. 1).

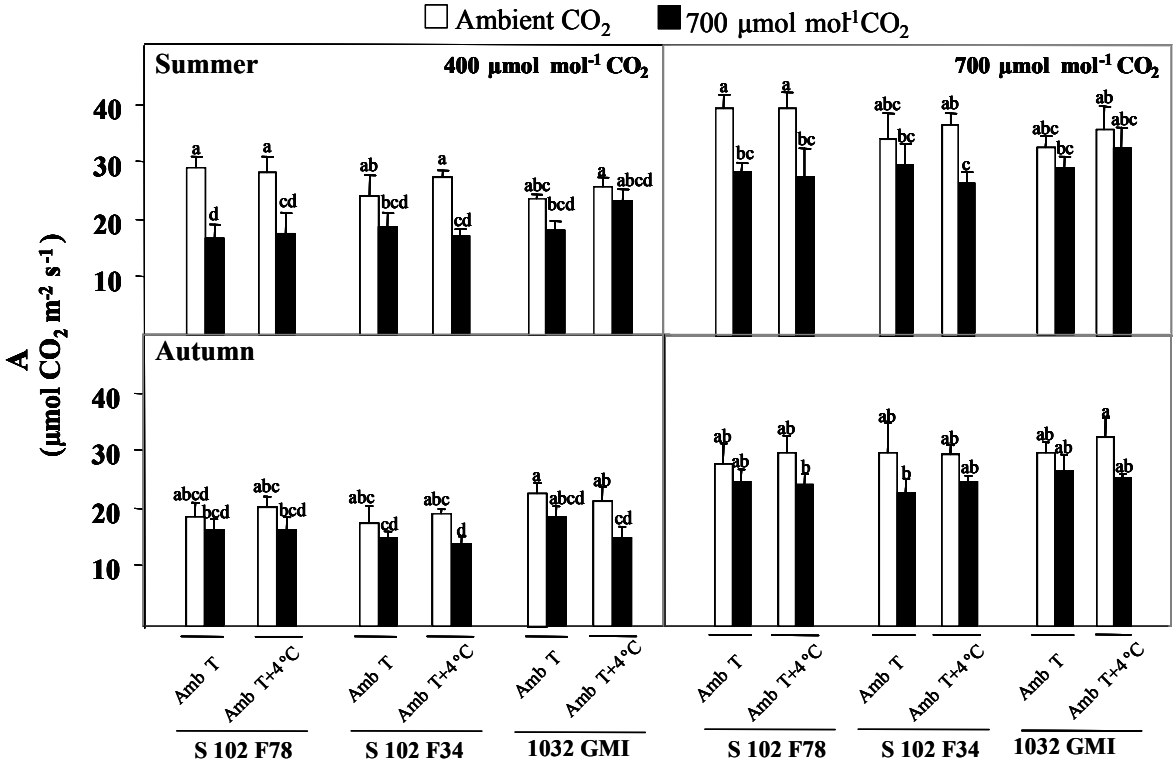

Fig. 1 Effect of CO_2 (ambient, around 400 and elevated, 700 µmol mol^{-1}), temperature (ambient and ambient +4ºC) and *S. meliloti* strain (102F78, 102F34 and 1032GMI) on net photosynthesis (A) measured at 400 (A_{400}) and 700 (A_{700}) µmol mol^{-1} CO_2 in nodulated alfalfa grown in summer and autumn. Bars represent the mean ± SE; n = 4. In each graph, bars with the same letter are not significantly different (P ≤ 0.05) according to the LSD test.

In summer, Vc_{max} increased with elevated temperature under ambient CO_2 concentration but decreased with elevated CO_2 under an ambient + 4ºC temperature regime (F= 18.45, P ≤ 0.001) (Fig. 2). In autumn, Vc_{max} decreased only in 1032GMI at elevated temperature under elevated CO_2 (*F= 4.44, P = 0.019*) (Fig. 2).

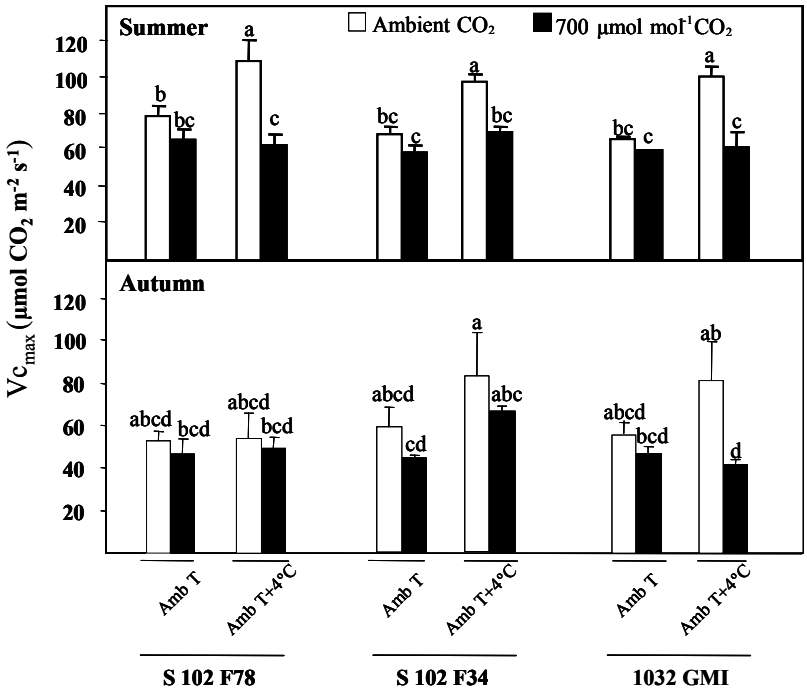

Fig. 2 Effect of CO_2 (ambient around 400 and elevated 700 µmol mol^{-1}), temperature (ambient and ambient +4ºC) and *S. meliloti* strain (102F78, 102F34 and 1032GMI) on maximum rate of rubisco carboxylation (Vc_{max}) (µmol CO_2 m^{-2} s^{-1}) of nodulated alfalfa grown in summer and autumn. Bars represent the mean ± SE; n = 4. Bars with the same letter are not significantly different ($P \leq 0.05$) according to the LSD test.

Total rubisco activity decreased with elevated CO_2 and temperature in plants inoculated with 102F78 or 1032GMI in summer (*F= 5.30, P = 0.011*) (Table 1), whereas, in autumn, the combination of elevated CO_2 and temperature diminished rubisco total activity in plants inoculated with the 102F34 strain (*F= 4.42, P = 0.021*) (Table 1).

In summer, elevated CO_2 and temperature enhanced plant dry matter (DM) (Table 2). Plants inoculated with the 102F78 strain were the most productive under elevated CO_2 and temperature (*F= 14.73, P ≤ 0.001*). In autumn, the combination of elevated CO_2 and temperature enhanced DM, especially with 102F34, which was the most efficient strain in all treatments (Table 2) (*CO_2xTxstrain, F= 11.06, P ≤ 0.001*). In summer, elevated CO_2 reduced leaf N concentration in plants inoculated with 1032GMI, regardless of the temperature regime, and in plants inoculated with 102F78 at elevated temperature (*CO_2xTxstrain, F= 10.91, P ≤ 0.001*). In autumn, leaf N content was not altered by elevated CO_2, temperature or inoculated strain (Table 2).

			Summer			Autumn		
			A_{growth}	Ci	Rubisco total activity	A_{growth}	Ci	Rubisco total activity
102F78	**Amb T**	Ambient CO_2	28.9 ± 2.1 a	272 ± 4.0 bc	31.2 ± 1.68 a	20.8 ± 1.6 a	246 ± 15 c	24.36 ± 2.55 ab
		Elevated CO_2	28.3 ± 1.9 a	500 ± 25 a	18.9 ± 1.44 bc	26.0 ± 2.5 a	533 ±10 ab	24.69 ± 1.77 a
	T +4ºC	Ambient CO_2	27.9 ± 3.2 a	223 ± 45 c	25.8 ± 4.50 a	20.3 ± 2.0 a	219 ± 13 c	15.18 ± 3.39 cd
		Elevated CO_2	27.6 ± 4.7 a	514 ± 6.0 a	19.5 ± 3.63 bc	24.3 ± 1.8 a	496 ± 24 b	19.92 ± 2.50 abc
102F34	**Amb T**	Ambient CO_2	24.2 ± 3.3 a	267 ± 5.0 bc	21.9 ± 2.25 b	24.8 ± 3.0 a	218 ±10 c	25.00 ± 2.50 a
		Elevated CO_2	29.9 ± 3.4 a	505 ± 10 a	20.4 ± 3.0 bc	22.9 ± 2.2 a	522 ± 18 ab	16.40 ± 2.31 cd
	T +4ºC	Ambient CO_2	27.3 ± 1.0 a	273 ± 5.0 bc	18.0 ± 1.21 b	19.9 ± 0.9 a	218 ± 14 c	20.46 ± 1.92 abc
		Elevated CO_2	26.5 ± 2.1 a	520 ± 5.0 a	6.60 ± 1.80 cd	24.8 ± 1.1 a	520 ± 23 ab	6.69 ± 0.84 e
1032GMI	**Amb T**	Ambient CO_2	23.5 ± 0.9 a	286 ± 9.0 b	22.5 ± 3.54 b	24.3 ± 1.8 a	240 ± 12 c	16.62 ± 1.56 cd
		Elevated CO_2	29.1 ± 1.8 a	506 ± 16 a	16.8 ± 1.23 cd	25.1 ± 1.5 a	500 ± 23 b	17.97 ± 2.49 bcd
	T +4ºC	Ambient CO_2	25.6 ± 1.8 a	267 ± 7.0 bc	21.9 ± 1.53 bc	23.1 ± 2.8 a	221 ± 14 c	21.03 ± 0.87 abc
		Elevated CO_2	32.3 ± 4.0 a	542 ± 17 a	13.5 ± 3.03 d	25.1 ± 0.9 a	563 ± 15 a	11.85 ± 2.61 de
		CO_2	ns	***	***	ns	***	**
		T	ns	ns	*	ns	ns	***
		Strain	ns	ns	**	ns	ns	*
		CO_2 x T x strain	ns	ns	ns	ns	ns	ns

Table 1: Effect of CO_2 (ambient, around 400 and elevated, 700 µmol mol^{-1}), temperature (ambient and ambient +4ºC) and *S. meliloti* strain (102F78, 102F34 and 1032GMI) on net photosynthesis at growth CO_2 concentration (A_{growth},) (µmol CO_2 m^{-2} s^{-1}), intercellular CO_2 concentration (Ci) (µmol CO_2 mol^{-1}), rubisco total activity (µmol CO_2 m^{-2} s^{-1}) on alfalfa grown in summer and autumn. Values represent the mean ± SE; n = 4. Statistical analysis was carried out with a three factors Analysis of the Variance (ANOVA), see the results at the bottom of the table. The meaning of symbols used in ANOVA were: *, Significant difference at 5%, ** 1%, *** 0.1%. When significant differences were detected in ANOVA, LSD analysis was applied. Means followed by the same letter are not significantly different ($P \leq 0.05$) according to LSD test parameters.

			Summer		Autumn	
			DM	Leaf N	DM	Leaf N
102F78	Amb T	Ambient CO_2	0.51 ± 0.03 ef	1.4 ± 0.03 bcde	0.59 ± 0.08 ef	1.5 ± 0.10 a
		Elevated CO_2	1.31 ± 0.16 b	1.3 ± 0.04 def	0.93 ± 0.15 cd	1.6 ± 0.16 a
	T +4ºC	Ambient CO_2	1.01 ± 0.14 bcd	1.8 ± 0.01 a	1.46 ± 0.1 b	1.4 ± 0.10 a
		Elevated CO_2	1.70 ± 0.23 a	1.3 ± 0.07 cdef	1.44 ± 0.11 b	1.1 ± 0.21 a
102F34	Amb T	Ambient CO_2	0.48 ± 0.07 ef	1.4 ± 0.06 bcd	0.92 ± 0.11 cd	1.3 ± 0.17 a
		Elevated CO_2	0.69 ± 0.06 cdef	1.5 ± 0.02 bc	1.22 ± 0.05 bc	1.5 ± 0.05 a
	T +4ºC	Ambient CO_2	0.69 ± 0.05 def	1.6 ± 0.07 ab	0.65 ± 0.03 de	1.3 ± 0.22 a
		Elevated CO_2	1.23 ± 0.09 b	1.8 ± 0.20 a	1.99 ± 0.08 a	1.6 ± 0.17 a
1032GMI	Amb T	Ambient CO_2	0.42 ± 0.04 ef	1.4 ± 0.05 bcde	0.36 ± 0.03 f	1.2 ± 0.15 a
		Elevated CO_2	0.78 ± 0.11 cde	1.2 ± 0.04 f	0.61 ± 0.10 ef	1.5 ± 0.11 a
	T +4ºC	Ambient CO_2	0.63 ± 0.06 ef	1.5 ± 0.09 bc	0.64 ± 0.02 def	1.4 ± 0.13 a
		Elevated CO_2	1.04 ± 0.14 bc	1.2 ± 0.07 ef	1.38 ± 0.21 b	1.2 ± 0.03 a
		CO_2	***	**	***	ns
		T	***	**	***	ns
		Strain	***	***	***	ns
		CO_2 x T x strain	***	***	***	ns

Table 2: Effect of CO_2 (ambient, around 400 and elevated, 700 μmol mol^{-1}), temperature (ambient and ambient +4ºC) and *S. meliloti* strains (102F78, 102F34 and 1032GMI) on plant dry matter (DM) (g $plant^{-1}$) and leaf N content (g m^{-2}) on alfalfa grown in summer and autumn. Values represent the mean ± SE; n = 4. Otherwise as for Table 1.

In summer, elevated CO_2 and temperature increased starch content with all strains except for 102F34 at ambient temperature (*F= 23.12, P ≤ 0.001*) (Table 3). In autumn, elevated CO_2 increased starch content with 102F78 at elevated temperature, and in the case of 1032GMI, at ambient temperature (*CO_2xTxstrains, F= 3.81, P = 0.035*) (Table 3). In summer, TSP increased with elevated CO_2 only in plants inoculated with 102F34 (Table 3). In autumn, elevated CO_2 diminished TSP content, except at elevated temperature in plants inoculated with the 1032GMI or 103F34 strains (Table 3).

The expression of *RbcL* was higher in plants inoculated with 102F78 or 1031GMI in summer (*strains, F= 33.57, P ≤ 0.001*) (Fig.3). In that season, elevated CO_2 increased *RbcL* expression with all strains except 102F34 (*CO_2, F = 138.72, P ≤ 0.001*). Nevertheless, temperature decreased *RbcL* expression with 102F34 and 1031GMI (*Txstrains, F= 12.66, P ≤ 0.001*). In autumn, elevated CO_2 and temperature increased *RbcL* expression, while the interaction between elevated CO_2 and temperature decreased it in plants inoculated with 102F78 (*CO_2xTxstrains, F= 29.31, P ≤ 0.001*) (Fig. 3).

			Summer		Autumn	
			Starch	TSP	Starch	TSP
102F78	Amb T	Ambient CO_2	0.52 ± 0.06 de	3.14 ± 0.30 bc	0.29 ± 0.07 c	2.54 ± 0.13 ab
		Elevated CO_2	0.57 ± 0.08 de	3.65 ± 0.23 bc	0.57 ± 0.01 bc	1.69 ± 0.12 d
	T +4ºC	Ambient CO_2	0.41 ± 0.04 e	3.28 ± 0.23 bc	0.45 ± 0.05 bc	2.63 ± 0.19 ab
		Elevated CO_2	1.38 ± 0.13 abc	4.19 ± 0.52 b	1.60 ± 0.25 a	1.89 ± 0.06 cd
102F34	Amb T	Ambient CO_2	0.67 ± 0.12 de	3.40 ± 0.19 bc	0.24 ± 0.06 c	2.94 ± 0.24 a
		Elevated CO_2	1.47 ± 0.06 ab	5.85 ± 0.66 a	0.40 ± 0.03 bc	1.67 ± 0.04 d
	T +4ºC	Ambient CO_2	0.48 ± 0.06 de	3.57 ± 0.29 bc	1.10 ± 0.10 ab	2.31 ± 0.52 bcd
		Elevated CO_2	0.88 ± 0.15 cde	2.88 ± 0.64 c	1.60 ± 0.29 a	2.33 ± 0.18 bcd
1032GMI	Amb T	Ambient CO_2	0.97 ± 0.16 bcd	4.24 ± 0.33 b	0.25 ± 0.03 c	1.82 ± 0.19 d
		Elevated CO_2	1.88 ± 0.51 a	4.31 ± 0.50 b	0.75 ± 0.21 ab	2.25 ± 0.14 bcd
	T +4ºC	Ambient CO_2	0.67 ± 0.15 de	3.60 ± 0.23 bc	0.99 ± 0.15 b	1.96 ± 0.14 cd
		Elevated CO_2	1.40 ± 0.07 ab	4.63 ± 0.88 ab	1.51 ± 0.04 ab	2.48 ± 0.12 abc
		CO_2	*	**	***	*
		T	***	ns	ns	ns
		Strain	ns	ns	**	ns
		CO_2 x T x strain	ns	**	*	ns

Table 3: Effect of CO_2 (ambient, approximately 400 and elevated, 700 µmol mol^{-1}), temperature (ambient and ambient +4ºC) and inoculation with *S. meliloti* strain (102F78, 102F34 and 1032GMI) on starch (g m^{-2}) and total soluble proteins (TSP) (g m^{-2}) in alfalfa grown in summer and autumn. Values represent the mean ± SE; n = 4. Otherwise as for Table 1.

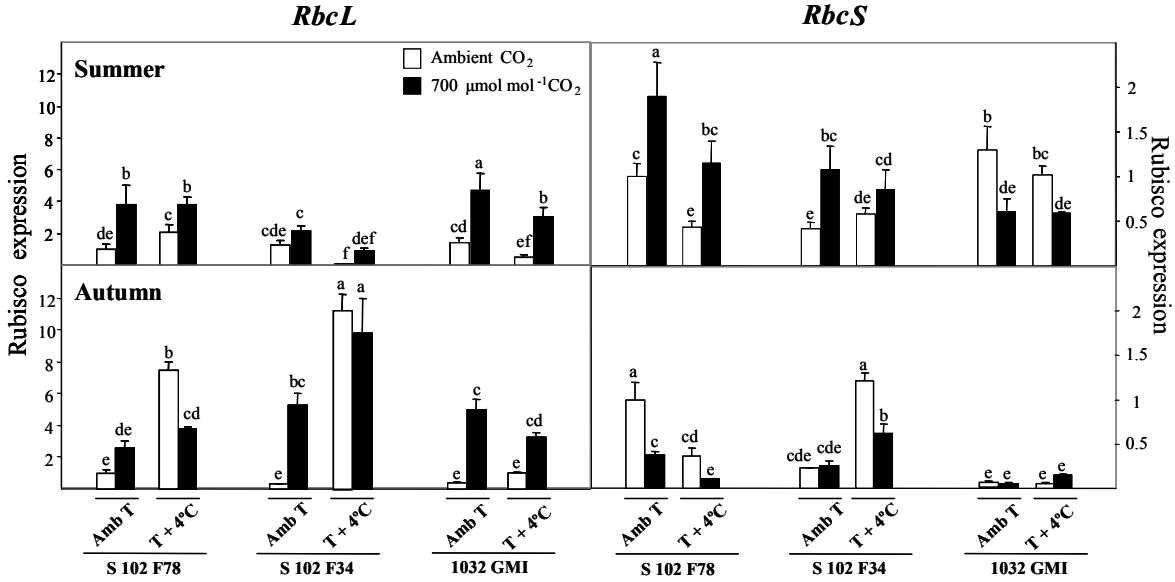

Fig. 3 Effect of CO_2 (ambient, around 400 and elevated, 700 µmol mol^{-1}), temperature (ambient and ambient +4ºC) and *S. meliloti* strain (102F78, 102F34 and 1032GMI) on relative expression of the *RbcL* and *RbcS* genes in alfalfa plants grown in summer and autumn; Bars represent the mean ± SE; n = 4. Bars with the same letter are not significantly different (P ≤ 0.05) according to the LSD test.

In summer, elevated CO_2 enhanced *RbcS* expression except in plants inoculated with 1032GMI (*CO_2xstrains, F= 41.98, P ≤ 0.001*). Nevertheless, in autumn, elevated CO_2 decreased *RbcS* expression in 102F78 inoculated plants, especially in the interaction with elevated temperature (*CO_2xTxstrains, F= 11.14, P ≤ 0.001*) (Fig.3).

In summer, RSS was lower than RLS in all treatments (*F= 344.292, P ≤ 0.001*) (Fig. 4). Elevated CO_2 decreased RLS in all treatments, except in plants grown at elevated temperature and inoculated with 102F34 or 1031GMI. In contrast, RSS only decreased in plants inoculated with 102F78 grown at elevated CO_2 and temperature (Fig. 4) (*CO_2xTxstrains, F= 6.17, P = 0.003*). In autumn, RSS was equal or higher than RLS except for 102F34 and 1032GMI at elevated temperature (*F= 7.9, P = 0.006*). RLS was not affected by elevated CO_2 or temperature. RSS was enhanced by elevated CO_2 at ambient temperature in the 102F78 and 102F34 strains. However, at elevated CO_2 and temperature, the RSS content diminished in all strains (*CO_2xTxstrains F= 3.99, P = 0.02*) (Fig. 4).

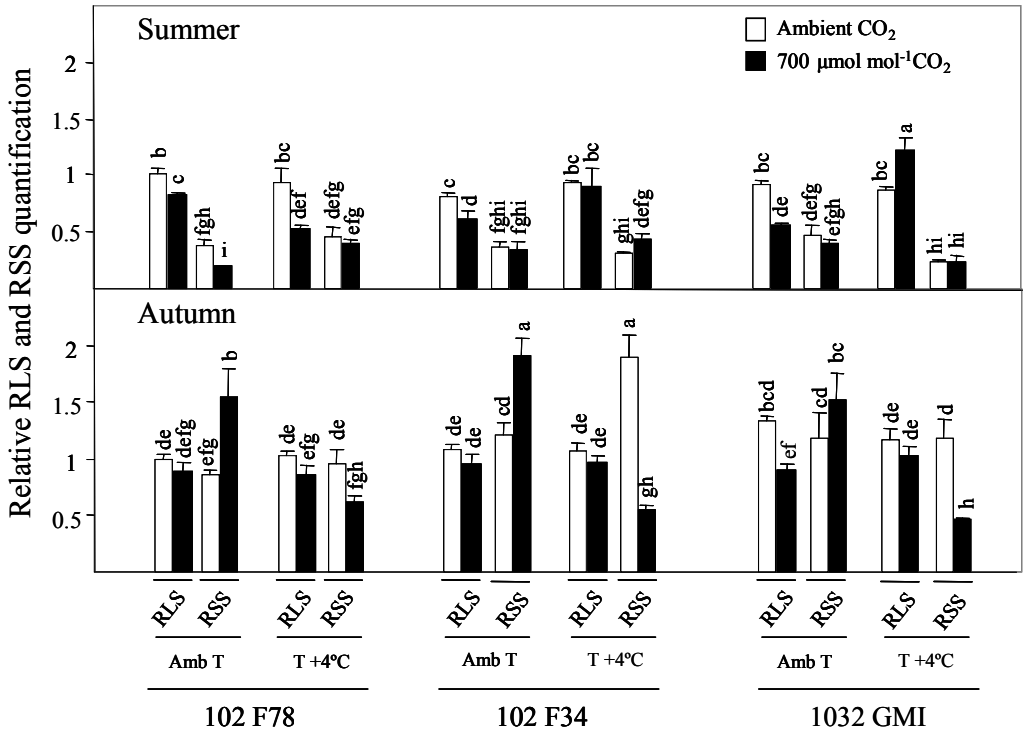

Fig. 4 Effect of CO_2 (ambient, around 400 and elevated, 700 µmol mol^{-1}), temperature (ambient and ambient +4ºC) and *S. meliloti* strain (102F78, 102F34 and 1032GMI) on semi-quantification of the rubisco large (RLS) and small subunits (RSS) in alfalfa plants grown in summer and autumn. The relative proportion of RLS and RSS was calculated in reference to the abundance value of RLS observed in plants inoculated with 102F78 and grown at ambient temperature and CO_2 concentration. Bars represent the mean ± SE; n = 4. Bars with the same letter are not significantly different (P ≤ 0.05) according to the LSD test.

	Summer		Autumn	
	A_{growth}	leaf N	A_{growth}	leaf N
RLS	0.472 **	0.224	0.525 ***	0.224
RSS	0.230	0.575 ***	0.133	0.575 ***
RbcL	0.120	0.208	0.177	0.208
RbcS	0.124	0.131	0.172	0.131

Table 4: Correlation coefficients among A_{growth} and leaf N with rubisco large and small subunit contents (RLS and RSS, respectively) and rubisco large and small subunit gene expression (*RbcL* and *RbcS*, respectively). * significant at $p \leq 0.05$; ** Significant at $p \leq 0.01$; *** Significant at $p \leq 0.001$.

In summer and autumn, A_{growth} was significantly correlated with RLS (*r = 0.472, P = 0.004; r = 0.525, P < 0.001* respectively) (Table 4). Total N content was also correlated with RSS in summer and autumn (r = 0.575, P < 0.001; r = 0.357, P = 0.033).

Summer		A_{growth}	A_{400}	A_{700}	Vc_{max}	Rubisco activity	leaf N	Starch	TSP	RLS	RSS	RbcL	RbcS
102F78	Amb T	*	*	*		*				*	*		
	Amb T +4ºC	*	*	*	*	*	*	*		*			
102F34	Amb T	*						*		*			
	Amb T +4ºC	*	*	*	*	*							
1032GMI	Amb T	*				*	*	*		*			*
	Amb T +4ºC	*			*	*	*	*					*

Autumn		A_{growth}	A_{400}	A_{700}	V_{cmax}	Rubisco activity	leaf N	Starch	TSP	RLS	RSS	RbcL	RbcS
102F78	Amb T	*							*				*
	Amb T +4ºC	*						*	*		*	*	*
102F34	Amb T	*				*			*				
	Amb T +4ºC	*	*			*					*		*
1032GMI	Amb T	*						*		*			
	Amb T +4ºC	*	*		*	*					*		

Table 5: Schematic summary of the effect of CO_2, 700 µmol mol^{-1} CO_2 *versus* ambient CO_2 in all studied parameters in summer and autumn experiments. The symbol (*) indicates that the considered parameter is down-regulated.

Discussion

The literature available about plant responses to elevated CO_2 after long-term exposure is extensive. However, there is no consensus that defines the most suitable parameter to detect photosynthetic down-regulation. The large numbers of papers that have reported photosynthetic down-regulation have used a wide number of techniques and parameters to detect and define it. In the area of gas exchange parameters, A/Ci curves are useful tools that provide a wide range of information, but the time spent collecting the data per measurement is rather high (around 30 minutes per sample point). In contrast, rapid measurements like net photosynthesis measured at growth CO_2 concentration (A_{growth}) are quick parameters for detecting acclimation but they provide less information than A/Ci curves. This study attempts to find the most sensitive physiological and molecular parameters to detect photosynthetic acclimation.

Photosynthetic markers

Photosynthetic down-regulation is defined as the decrease in photosynthetic capacity under elevated CO_2 conditions (Saralabai et al. 1997) and several parameters such as A_{growth}, Vc_{max}, A_{400}, A_{700} and maximum electron transport rate (J_{max}) have been used to study plant photosynthetic response to elevated CO_2 (Ainsworth et al. 2003; Aranjuelo et al. 2008; Erice et al. 2006a; Lee et al. 2001). In both the summer and autumn experiments, A_{growth} remained unchanged despite an elevated CO_2 concentration that indicated photosynthetic acclimation (Aranjuelo et al. 2005a; Sanz-Sáez et al. 2010) (Table 1). In summer, Vc_{max} dropped in elevated CO_2 and temperature treatments in all strains, while in autumn it only decreased in plants inoculated with 1032GMI (Fig. 2). Unaltered Vc_{max} does not necessarily entail the absence of photosynthetic down-regulation; Crous et al. (2010) also showed acclimated plants without significant reduction in Vc_{max}, but decreased A_{growth}.

Others parameters employed to detect photosynthetic down regulation are A_{400} and A_{700}. In summer, similar responses to elevated CO_2 were obtained for A_{400} and A_{700} with significant decreases with 102F78, regardless of temperature regime, and with 102F34 at elevated temperature. Nevertheless, in autumn, A_{400} only showed significant decreases with 102F34 and 1032GMI at elevated temperature, whereas A_{700} was not affected by elevated

CO_2. This result showed that A_{400} is more effective than A_{700} for detecting photosynthetic down-regulation (Ellsworth et al. 2004).

Summer appears as the season when the clearest acclimation occurs to elevated CO_2, according to the results of A_{400} A_{700} and Vc_{max}. Down-regulation was detected in 50% of summer comparisons (Table 5) but only in 16% of those during autumn. A_{growth} has been revealed as the most sensitive photosynthetic parameter for detecting acclimation to elevated CO_2, followed by A_{400}.

Biochemical and molecular markers

Rubisco total activity is an *in-vitro* parameter that is well correlated with CO_2 assimilation (Caemmerer and Edmondson 1986). In summer, rubisco activity decreased in 83% of cases at elevated CO_2 (Table 5). Nevertheless, in autumn, the elevated CO_2 only decreased it in 50% of the comparisons (Table 5), confirming the greater acclimation under warmer temperatures (summer versus autumn). This result agrees with Galmés et al. (2006) who considered rubisco *in-vitro* activity as a more reliable parameter than Vc_{max}, particularly under high temperature.

Some authors have associated the photosynthetic down-regulation with decreases in leaf N in forbs, grasses and trees (Crous et al. 2010; Ellsworth et al. 2004). In our summer experiment, plants grown at elevated CO_2 and temperature decreased leaf N; however, in autumn, with less marked down-regulation, this parameter remained unchanged (Table 2). The greater decrease in leaf N in summer could be due to the negative effect of high temperatures decreasing N_2 fixation and thus N availability (Sanz-Sáez et al. unpublished data, Capítulo 3). However, in autumn, the mild temperatures would allow a better N_2 fixation (Sanz-Sáez et al. unpublished data, Capítulo 3) remaining leaf N unchanged, and thus resulted in a less severe down-regulation. The decrease in leaf N, was accompanied by a generally enhanced summer starch content (Table 3). It has been already stated that under elevated CO_2, the decrease in leaf N is a consequence of dilution by carbohydrate accumulation, the formation of leaf structural material, and increases in plant internal demands for N (Ellsworth et al. 2004). In a low N availability situation as observed in the summer experiment, elevated CO_2 may partition resources away from leaves and, through

increased production, result in sequestration of nutrients (like N) into organic matter causing deficiencies that indirectly result in decreased photosynthetic capacity (Ainsworth et al. 2003; Rogers and Ellsworth 2002). Our study revealed that the leaf N content was not directly associated with down-regulation in all treatments; however, in treatments in which leaf N decreased under elevated temperature, the photosynthetic acclimation was also associated with reduced Vc_{max} (Table 5).

Geiger et al. (1999) reported that leaves grown under elevated CO_2 concentrations show higher carbohydrate content than those grown under ambient CO_2. In our study, all plants grown at elevated CO_2 showed a slight increase in leaf starch content at ambient temperature, but the starch accumulation was more significant at elevated temperature (Table 3). It is generally assumed that the enhancement of non-structural carbohydrates and the inhibition of the expression of genes that encode for different photosynthetic apparatus compounds are suppressed through a possible increase of hexose cycling within the leaf, resulting in decreased photosynthetic capacity and a notable decrease in the amount of rubisco (Drake et al. 1997; Moore et al. 1999). As mentioned above, when plants are N limited, down-regulation has been related to reallocation of N away from the photosynthetic apparatus (Aranjuelo et al. 2005b) or from particular photosynthetic enzymes (Moore *et al.,* 1999; Rogers and Ellsworth, 2002). In this context, in the summer experiment, TSP generally remained unaffected at elevated CO_2 (Table 3); however, a selective decrease in RLS content was detected (Fig.4). This could be due to a selective drop in rubisco concentration under elevated CO_2 (Aranjuelo et al. 2009). It is considered that due to the relative quantity of rubisco large (RLS) and small subunits (RSS) and the lowest specificity of RSS (Jordan and Ogren 1981), rubisco enzymes lack RSS, which may suggest that RSS contribute substantially to the differences in kinetic properties of rubisco (Andersson and Blacklund 2008). In summer, RLS showed significant decreases in 66% of CO_2 comparisons, whereas RSS only decreased in 16% (Table 5). In this season a strong correlation between A_{growth} and RLS has been found (Table 4). Nevertheless, in autumn, RSS was specially decreased by a combination of elevated CO_2 and temperature (Fig. 4). This decrease in both rubisco subunits could be due to N leakage (Ainsworth et al. 2003) away from the photosynthetic apparatus, caused by higher DM production (Table 5). This drop in RLS and RSS and the associated reduced rubisco activity may be responsible for photosynthetic down-regulation (Aranjuelo et al. 2005b; Erice et al. 2006b; Urban 2003). In

summer, expression corresponding to the *RbcL* and *RbcS* genes that encode the RLS and RSS subunits of rubisco expression was generally increased at elevated CO_2, depending on the inoculated *S. meliloti* strain, as was also reported by Bertrand et al. (2007) (Fig.3). Despite the general decrease in rubisco protein under elevated CO_2, *RbcL* was enhanced in autumn (Fig.3; Table 5). A_{growth} was directly related to rubisco content but was not coordinated with rubisco mRNA expression (Table 4), contrary to statements for some species like pea, soybean, wheat and tomato (Majeau and Coleman 1996; Moore et al. 1998; Webber et al. 1994). This divergence between rubisco content and expression could be due to a temporal control of photosynthetic gene expression that can oscillate in a circadian way, as pointed out by Moore et al. (1999).

Conclusion

In this study, parameters to detect photosynthetic down-regulation were analyzed. All plants grown at elevated CO_2 presented down-regulation in both seasons, especially when combined with high temperatures. This could be due to a low N availability caused by a decrease in N_2 fixation induced by higher summer temperatures. The most sensitive photosynthetic parameters for detecting acclimation were A_{growth} and A_{400}. Within biochemical parameters, rubisco *in-vitro* activity was suitable and even more sensitive than Vc_{max}. The decrease in leaf N was useful for detecting severe acclimation in summer. Elevated CO_2 did not decrease leaf TSP but affected specifically rubisco protein (RLS and RSS) resulting in decreased rubisco activity *in-vitro* and hence reduced photosynthesis rates. Opposite to observations in other species, rubisco content and expression in alfalfa were not directly correlated, which may be due to a temporal (circadian) control of both processes.

Acknowledgements

This work was supported by Ministerio de Ciencia e Innovación (MICINN BFU2008-01405), Fundación Universitaria de Navarra (PIUNA-2008) and Fundación Caja Navarra. A. Sanz-Sáez was the recipient of a research grant from Asociación de Amigos de la Universidad de Navarra. Iker Aranjuelo was the recipient of a Ramón y Cajal research

contract (Ministerio de Ciencia e Innovación). The assistance of A. Urdiain, M. Oyarzun, L. González and J. Paz is also appreciated.

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CAPÍTULO 5

Daily variation in carbon exchange, rubisco content and gene expression in nodulated alfalfa under ambient and elevated CO_2

Variación diaria del intercambio gaseoso, contenido y expresión génica de la rubisco en alfalfa nodulada crecida en CO_2 ambiente y elevado

Enviado a *Plant Physiology*

Daily variation in carbon exchange, rubisco content and gene expression in nodulated alfalfa under ambient and elevated CO_2

Resumen

El crecimiento prolongado de muchas plantas en condiciones de CO_2 elevado suele resultar en descensos de la actividad fotosintética debidos a una caída en el contenido o actividad de la enzima rubisco. A pesar del gran número de publicaciones a este respecto, se han descrito muchas discrepancias entre el contenido y la expresión génica de esta enzima en condiciones de CO_2 elevado. El objetivo de este trabajo fue estudiar la variación diaria de la fotosíntesis, así como su relación con el contenido y expresión de la rubisco en alfalfa crecida en CO_2 ambiente y elevado. Para alcanzar este objetivo, se realizaron medidas fisiológicas y recolección de material vegetal cada 4 horas (de 9 am a 5 am) durante tres días consecutivos para análisis bioquímicos. El CO_2 elevado no afectó el intercambio neto de carbono (NCE) durante el día o la noche, excepto a la 1 pm, en el que su valor descendió un 50% con respecto al de las plantas crecidas en CO_2 ambiente lo cual es indicativo de una aclimatación de la fotosíntesis. Esta aclimatación fotosintética también se hizo evidente por el menor contenido de subunidad grande (RLS) y pequeña (RSS) de rubisco. A lo largo del día, la RSS fue la subunidad limitante en los dos tratamientos de CO_2. RLS solo descendió significativamente su contenido a la 1 pm. Este descenso de las dos subunidades de rubisco a la 1 pm se asoció con la gran caída de NCE a la misma hora. Por otro lado, el contenido de rubisco estuvo fuertemente correlacionado con la fotosíntesis, no teniendo correlación con la expresión génica de esta enzima. En condiciones de CO_2 elevado, la expresión génica de la subunidad grande (*RbcL*) y pequeña (*RbcS*) descendió por la noche (9 pm) posiblemente debido a una acumulación de almidón y azúcares solubles totales, que produjo la caída en el contenido de RLS y RSS al medio día (1 pm). Estos resultados podrían confirmar la existencia de un retraso de larga duración entre la expresión génica y la síntesis de la rubisco en alfalfa.

Summary

Growth of many plant species at elevated CO_2 in log-term experiments often results in decreased photosynthetic capacity due to a drop in rubisco content and/or activity. This process is referred to as photosynthetic down-regulation. Despite the large number of publications, discrepancies between rubisco protein content and gene expression have been reported. One of the possible explanations could be that measurements have been conducted at different times of the day. Therefore, the aim of the present work was to study daily variation in photosynthesis, as well as the relationship between rubisco content and gene expression in alfalfa grown at ambient or elevated CO_2. Physiological measurements and harvesting for biochemical and molecular analysis were performed every four hours from 9 am to 5 am (GMT) (6 times per day) during three consecutive days (considering each day as a repetition). Our study showed that net carbon exchange (NCE) was unaffected by elevated CO_2 throughout the day and night, excepting at 1 pm, where its values decreased to 50% of the corresponding ambient CO_2 treatment, showing photosynthetic down-regulation or acclimation. This acclimation was also evidenced by the lower rubisco content (measured as both large, RLS, and small, RSS, subunits) of those plants. Throughout the day, RSS was the limiting factor of photosynthesis under both CO_2 conditions. RLS only decreased under elevated CO_2 at 1 pm. This decline in rubisco subunits at 1 pm was associated to the greater decrease of NCE at the same time. Daily variations in rubisco protein content were significantly correlated with photosynthesis, but not with rubisco gene expression. Under elevated CO_2 rubisco large (*RbcL*) and small (*RbcS*) gene expression dropped at night (9 p.m.) possibly due to starch and total soluble sugars accumulation at that time, inducing decreased RLS and RSS content at noon (1 pm). Results could confirm the existence of a long duration lag between rubisco gene expression and protein synthesis in alfalfa.

Key words: Carbon dioxide, *Medicago sativa* (alfalfa), Photosynthesis, *RbcL*, *RbcS*.

Abbreviations: C_i, intercellular CO_2; IPCC, Intergovernmental Panel on Climate Change; NCE_{growth}, net carbon exchange measured under growth CO_2 conditions; NCE_{350}, net carbon exchange measured at 350 μmol mol^{-1} CO_2; NCE_{700}, net carbon exchange measured at 700 μmol mol^{-1} CO_2; RLS, rubisco large subunit content; RSS rubisco small subunit content; *RbcL*, rubisco large subunit expression; *RbcS*, rubisco small subunit expression; TGG, temperature gradient greenhouse; TSS, total soluble sugars.

Introduction

The mean concentration of CO_2 in Earth's atmosphere is currently 378 μmol mol^{-1}. It has been predicted that by 2100, plants will grow in an atmosphere with approximately 700 μmol mol^{-1} CO_2 (IPCC, 2007). Growth of many plant species at elevated CO_2 in log-term experiments often results in decreased photosynthetic capacity due to reduced rubisco content and/or activity (Aranjuelo *et al.,* 2005b; Ellsworth *et al.,* 2004; Moore *et al.,* 1998, 1999), This process is often known as photosynthetic down-regulation (Saralabai *et al.,* 1997). The magnitude of this phenomenon can vary between different species, but also among different cultivars and daily growth conditions (Campbell, 1997; Kalina and Ceulemans, 1997; Moore *et al.,* 1998).

Structurally, rubisco from higher plants is composed of eight large subunits (RLS, 56 kD each) encoded by the chloroplast genome (*RbcL*), and eight small subunits (RSS, 14 kD each) encoded by the nuclear genome (*RbcS*) (Pilgrim and McClung, 1993). Rubisco is activated by light (Campbell and Ogren, 1990a, b) and it has been demonstrated that activity is higher in extracts of leaves collected before dawn producing a daily rhythm (Servaites, 1985). Rubisco activity is finally controlled by its content and rubisco activase (Portis, 1992). The control of rubisco content involves a number of processes, including transcription, post-transcription (mRNA stability), translation and post-translation events (protein turnover) (Wanner and Gruissem, 1991; Moore *et al.,* 1999). Moore *et al.* (1998) found that elevated CO_2 affected the relative expression and content of rubisco in different ways depending on plant species. For example in pea, *RbcS* and *RbcL* expression and rubisco content remain stable under elevated CO_2; however, in *Arabidopsis* these parameters decreased with elevated CO_2 (Moore *et al.,* 1998). One manifestation of such variations is the diurnal or circadian control of rubisco gene expression (Moore *et al.,* 1999), which could cause a lag between gene expression and protein content.

Inhibition of photosynthesis in long-term growth at high CO_2 has been associated with leaf carbohydrate accumulation and N availability limitations (Aranjuelo *et al.,* 2005b; Erice *et al.,* 2006a, b; Neals and Incoll, 1968; Sanz-Sáez *et al.,* 2010). Furthermore, carbohydrates are known to modulate the expression of many photosynthetic genes (Jang

and Sheen 1994; Moore *et al.* 1998). End-product inhibition has been related to increased content of hexoses, probably derived from sucrose hydrolysis (Krapp *et al.,* 1993).

In this situation of limited photosynthesis, some authors argued that species capable of symbiotic N_2 fixation often show a larger stimulation of photosynthesis and thus higher growth under elevated CO_2 than plants that do not fix N_2 (Ainsworth *et al.,* 2002; Lee *et al.,* 2003). It has been suggested by Bertrand *et al.* (2007) that inoculation of *Medicago sativa* with a high N_2-fixing *Sinorhizobium meliloti* strain avoided photosynthetic down-regulation. However, numerous studies (Aranjuelo *et al*., 2005a, b; Erice *et al*., 2006a, b) performed on alfalfa grown in temperature gradient greenhouses (TGG) under elevated CO_2 and temperature, showed photosynthetic down-regulation with increases in non-structural leaf carbohydrates. Moreover, no studies have been conducted in down-regulated alfalfa to determine the effect of elevated CO_2 on daily photosynthesis and rubisco content and expression.

Therefore, the objectives of the current work were: firstly, to analyze daily variations in rubisco protein content and expression; secondly, to verify if photosynthetic down-regulation is caused by decreases in rubisco content (RLS and RSS subunit) and/or expression (*RbcL* and *RbcS*); thirdly, to test if rubisco content (RLS and RSS) is correlated with expression (*RbcL* and *RbcS*) or, contrariwise, if a lag exists between rubisco expression and content.

Material and Methods

Plant material

Alfalfa (*Medicago sativa* L. cv. Aragón) seeds were sterilized in a solution of $HgCl_2$ (0.1% w/v) and germinated in Petri dishes. One week later, seedlings were planted in pots (20 plants per pot) containing a mixture of perlite–vermiculite (2/1; v/v). Pots with a capacity of 13 L were used to prevent the plants from becoming pot-bound. Plants were grown in two greenhouses and irrigated alternately with Evans N-free solution (Evans, 1974) and distilled water to avoid salt accumulation in the substrate during the experiment. At planting, seedlings were inoculated four times with *Sinorhizobium meliloti* strain 102F78 (Nitragin Co., Milwaukee, WI, USA).

Experimental design

Alfalfa seedlings (9 replicates per treatment) were placed into two greenhouses placed at the Pamplona Campus of the University of Navarra (42.80N, 1.66W; Spain). CO_2 concentration, temperature, relative humidity and solar radiation levels inside the greenhouses were continuously monitored and controlled by a computerized system (Fig. 1). Plants were grown during autumn at ambient temperature (mean experimental temperature was 18ºC) and two CO_2 regimes (ambient CO_2, approximately 400 μmol mol^{-1}, and elevated, 700 μmol mol^{-1}). The CO_2 concentration was continuously monitored using a Guardian Plus gas monitor (Edinburgh Instruments Ltd., Livingston, UK). Its signal was fed into a proportional integrative differential controller that regulated the opening time (within a 10 s cycle) of a solenoid valve that injected CO_2 into both inlet fans. Physiological measurements and harvesting for biochemical and molecular analysis were done every four hours (9 am, 1 pm, 5 pm, 9 pm, 1 am and 5 am; Greenwich Mean Time, GMT), 6 times per day, over three days (considering each day as a repetition). Immediately after gas exchange measurements, leaves were frozen in liquid N_2 for biochemical and molecular analysis and stored at -80ºC.

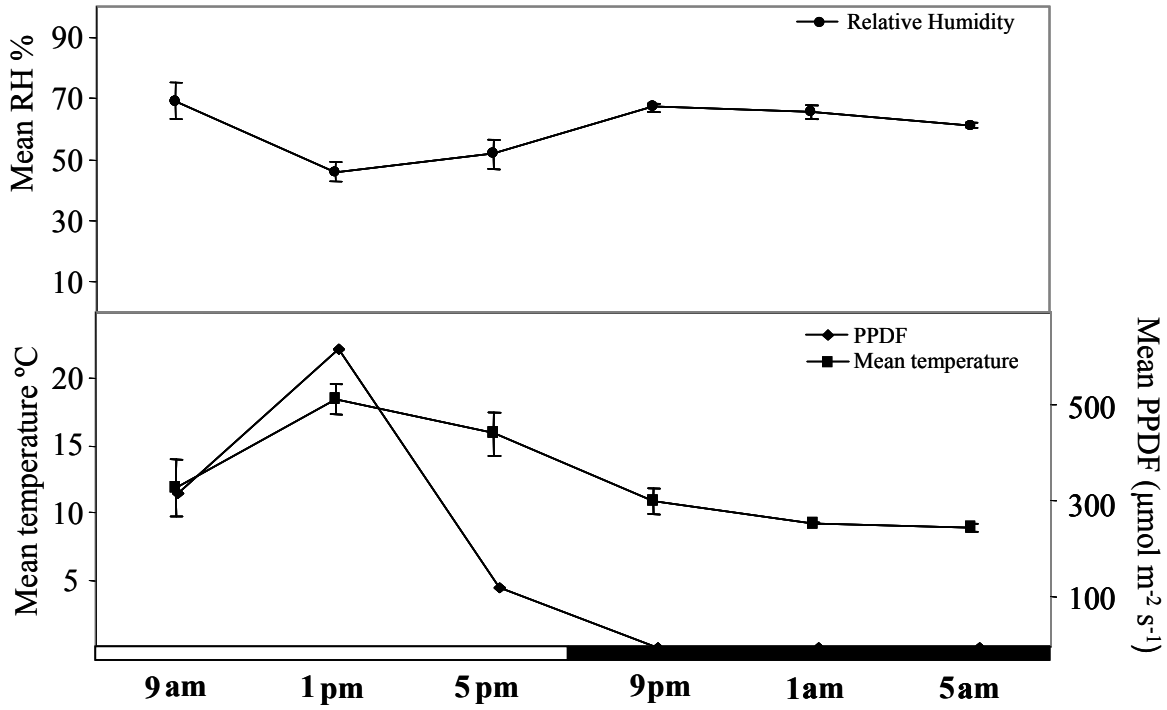

Figure 1: Mean relative humidity (RH), temperature and photosynthetic photon flux density (PPDF) during all day sampling.

Gas exchange parameters

Gas exchange parameters were measured in fully expanded young leaves (9 replicates per treatment), corresponding to 67-69 day old plants using a GFS-3000 portable gas exchange system (Walz, Effeltrich, Germany). The gas exchange response to CO_2 was determined as net carbon exchange (NCE) measured either at 400 (NCE_{400}) and 700 µmol mol^{-1} CO_2 (NCE_{700}) or at growth conditions (NCE_{growth}, ambient ones at 400 µmol mol^{-1} and elevated ones at 700 µmol mol^{-1} CO_2). During the day, the gas exchange system was maintained at 20ºC and 1400 µmol m^{-2} s^{-1} PPFD provided by LED light. However, during the night measurements, temperature was maintained at 10ºC and the light was switched off. Intercellular CO_2 concentrations (Ci) were calculated as described by Long and Hallgreen (1985).

Biochemical measurements

Leaf total soluble sugars (TSS) and starch concentrations were quantified by grinding and filtering 200 mg of leaf fresh weight from leaves (9 replicates per treatment)

in a cold mortar using an extraction buffer containing 50 mM K-phosphate (pH 7.5). The extract was filtered and centrifuged at 28,710 g for 15 min at 4ºC. The TSS quantification was performed in the supernatant as described by Yenm and Willis (1954), whereas starch was measured using the pellet as described by Jarvis and Walker (1993). Total soluble protein (TSP) levels were measured using the method of Bradford (1976).

Since reduction in specific leaf area (SLA) by elevated CO_2 could underestimate the physiological and biochemical values when represented per mass unit, all measured parameters are expressed on a per area basis (Sanz-Sáez *et al.,* 2010).

RNA isolation, synthesis of cDNA and Quantitative real-time RT-PCR

Total RNA was isolated from alfalfa leaves with a phenol/chloroform extraction method (Kay *et al.,* 1987). Rubisco large and small subunit gene expression (*RbcL* and *RbcS*, respectively) was studied by real-time quantitative PCR using iCycler (Bio-Rad, Hercules, California, USA). cDNAs were obtained from 2.5 μg of total DNase-treated RNA in a 20 μL reaction buffer containing 500 ng random hexamer primer, 0.5 mM of each dNTP, 10 mM of DTT, 40 U of RNase inhibitor, 1x first strand buffer (Invitrogen, Carlsbad, California, USA) and 200 U of Superscript II Reverse Transcriptase (Invitrogen). The primers sets used to amplify *RbcL* were: 5´-GAGTAGCTCTGGAAGCATGTG-3´ as the forward and 5´-GACTCCATTTGGTAGCCTCAC-3´ as the reverse primers; to amplify *RbcS* the primers used were 5´-TTCGGAGCCACTGATTCTTCTC-3´ forward and 5´-ACTGCACTTGACGAACATTGTC-3´ reverse. Each 25 μL q-PCR reaction contained 1 μL of a 1:10 dilution of the cDNA, 200 nM dNTPs, 400 nM of each primer, 3 mM $MgCl_2$, 2.5 μL of 1x SyBR Green (Molecular Probes, Eugene, Oregon, USA), and 0.5 U Platinum *Taq* DNA polymerase (Invitrogen) in 1x PCR buffer (20 mM Tris–HCl, pH 8.4, 50 mM KCl).

The PCR program consisted in a 4 min incubation at 95 ºC to activate the hot-start recombinant *Taq* DNA polymerase, followed by 30 cycles of 45 s at 94 ºC, 45 s at 69 ºC, and 50 s at 72ºC, where the fluorescence signal was measured. The results obtained for the different treatments were standardized according to the β-tubulin gene expression levels, which were analyzed with the forward primer 5´-GAAGCAAGCGGTGGAAGATATG-3´

and the reverse primer 5´-CCAAATGGACCAGAACGCAAAC-3´, showing stable expression under the conditions tested in this study.

Real-time PCR experiments were carried out with at least four independent RNA samples, with the threshold cycle (C_T) determined in triplicate. The relative levels of transcription were calculated by using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). Negative controls without cDNA were used in all PCR reactions. The relative gene expression of rubisco large (*RbcL*) and small (*RbcS*) subunits was calculated in reference to the abundance value of *RbcL* and *RbcS* observed in plants grown at ambient CO_2 concentration and harvested at 9 am.

Rubisco semi-quantification

The extract from protein quantification was 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, 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 relative proportion of rubisco large (RLS) and small (RSS) subunits was calculated in reference the abundance value of RLS observed in plants inoculated with 102F78 and grown at ambient temperature and CO_2 concentration.

Statistical analysis

Statistical analysis was carried out with two factor analysis of variance (ANOVA) (factorial 2 x 6) (SPSS v.15.0). Taking CO_2 as the first factor (ambient CO_2, around 400 µmol mol^{-1}; and elevated, 700 µmol mol^{-1}) and sampling time as the second factor (9 am, 1 pm, 5 pm, 9 pm, 1 am, 5 am). Twelve treatments in total were performed, with four experimental replicates per treatment. Significant differences between factors and interactions were calculated at 5%, 1% and 0.1% levels of significance. When differences between treatments (CO_2 and/or measurement time) were significant according to the

ANOVA analysis, significant differences were evaluated using the least significant difference test (LSD) ($P < 0.05$).

Results

Elevated CO_2 only decreased plant net carbon exchange at growth CO_2 conditions (NCE_{growth}) at 1 pm (CO_2 x time, *F= 8.44, P ≤ 0.001*) (Fig. 2A). Intercellular CO_2 concentration (Ci) enhanced by elevated CO_2 at all times (CO_2, *F= 204.06, P ≤ 0.001*) (Fig. 2B). During night samplings (9 pm, 1 am and 5 am), Ci was significantly higher than during day samplings especially at elevated CO_2 (9 am, 1 pm and 5 pm) (Fig. 2B).

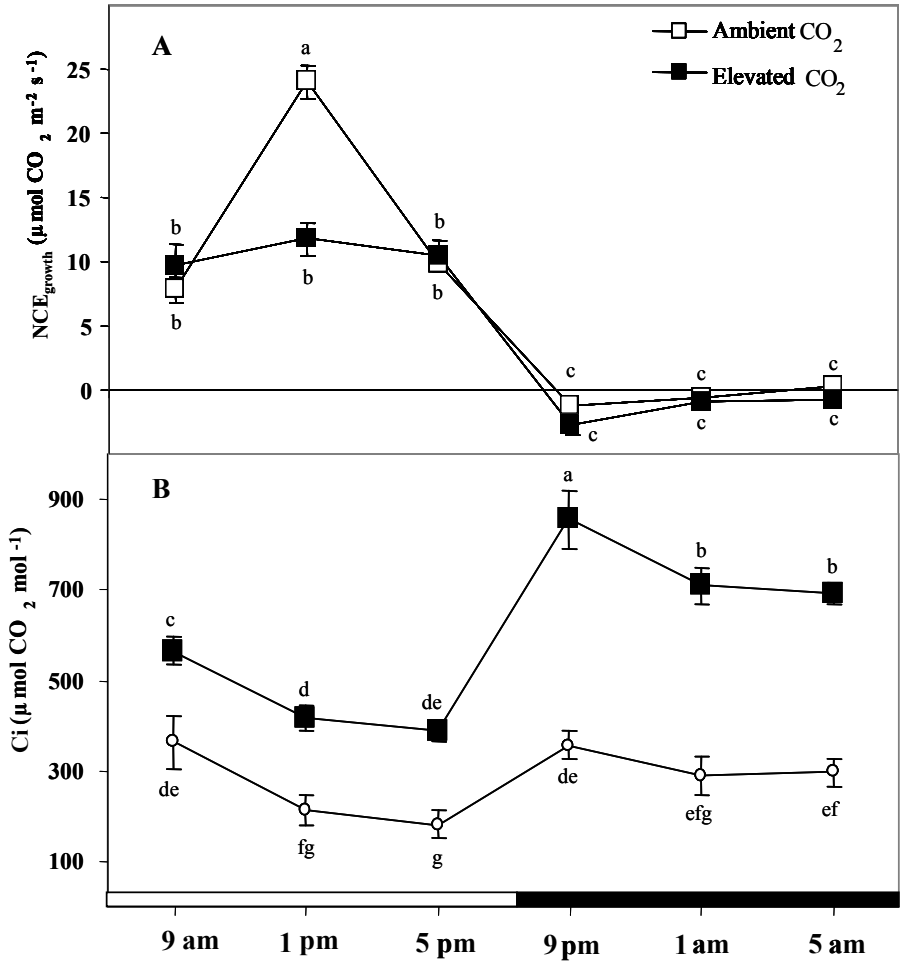

Figure 2: Effect of CO_2 (ambient, approximately 400 µmol mol^{-1} and elevated, 700 µmol mol^{-1}) throughout the day on net carbon exchange measured at growth CO_2 concentration (NCE_{growth}) (A), and intercellular CO_2 concentration measured at growth CO_2 concentration (Ci_{growth}) (B). Bars represent the mean ± SE; n= 12. Bars with the same letter are not significantly different (P≤ 0.05) according to the LSD test.

NCE measured at 400 µmol mol^{-1} CO_2 (NCE_{400}) was affected by sampling time, but not by elevated CO_2 (time, *F= 116.9, P ≤ 0.001*) (Fig. 3A). Elevated CO_2 decreased NCE

measured under 700 µmol mol^{-1} CO_2 (NCE_{700}) at 1 pm and 5 pm (CO_2 x time, *F= 4.47, P = 0.002*) (Fig. 3B).

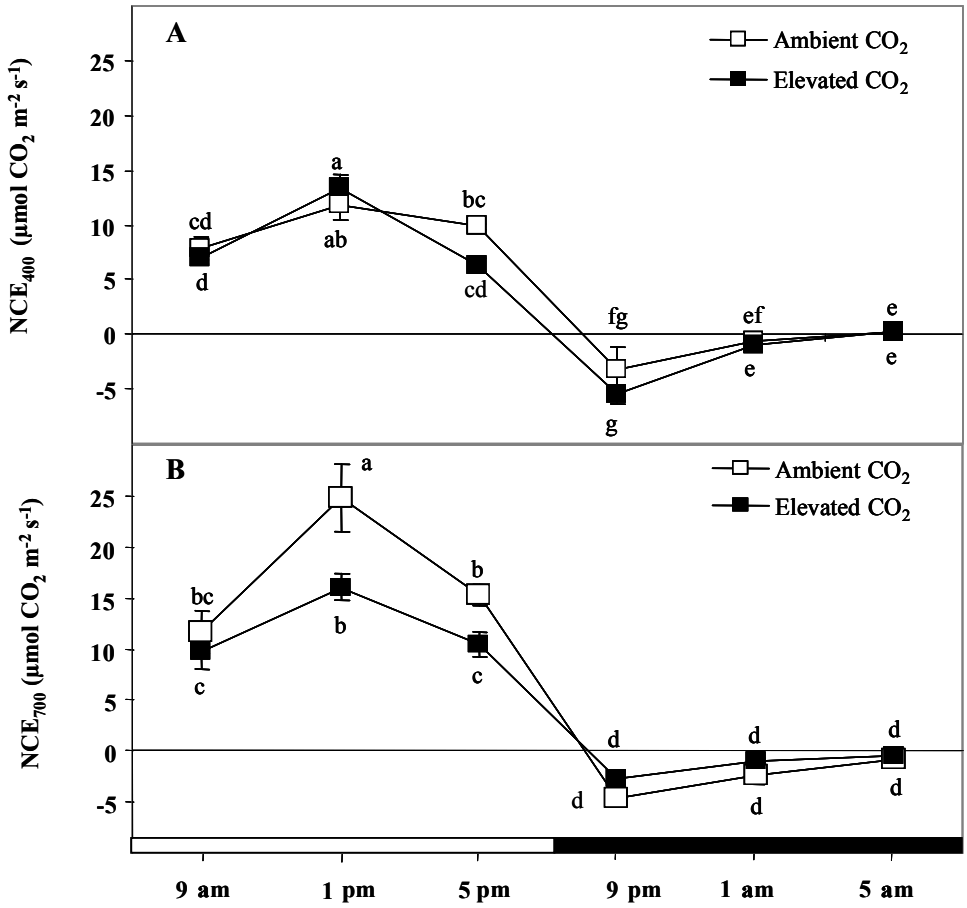

Figure 3: Effect of CO_2 (ambient, approximately 400 µmol mol^{-1} and elevated, 700 µmol mol^{-1}) throughout the day on net carbon exchange measured at 400 µmol mol^{-1} (NCE_{400}) (A), and at 700 µmol mol^{-1} (NCE_{700}) (B). Bars represent the mean ± SE; n= 12. Bars with the same letter are not significantly different (P> 0.05) according to the LSD test.

Rubisco large subunit (RLS) content was always higher than rubisco small subunit content (RSS) (*F= 377.15, P ≤ 0.001*) (Fig. 4). Elevated CO_2 decreased RLS significantly at 1 pm (Fig. 4). Whereas RSS decreased under elevated CO_2 throughout the whole day (CO_2 x time, *F= 7.05, P ≤ 0.001*) except at 9 am, 9 pm, and 5 am.

Rubisco large subunit gene expression (*RbcL*) was enhanced by elevated CO_2 at 9 am, but decreased at 9 pm (Fig.5) (CO_2 x time, *F= 51.21, P ≤ 0.001*). Rubisco small subunit gene expression (*RbcS*) was enhanced by elevated CO_2 in the 9 am, 1 pm, 5 pm and 5 am samplings, whereas it was decreased at 9 pm and 1 am (Fig.5) (CO_2 x time, *F = 34.29, P ≤ 0.001*).

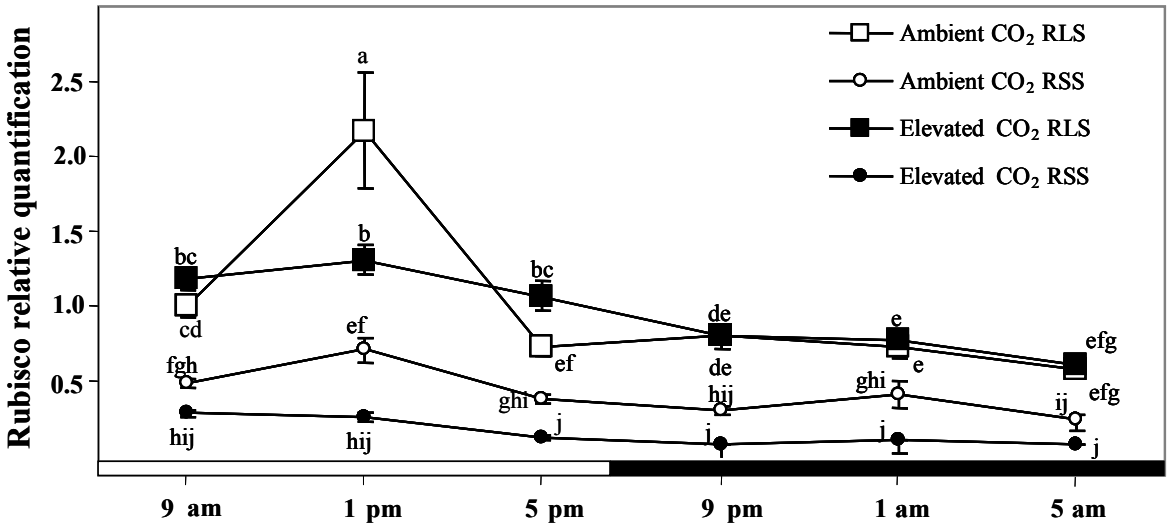

Figure 4: Effect of CO_2 (ambient, approximately 400 µmol mol^{-1} and elevated, 700 µmol mol^{-1}) throughout the day on rubisco large (RLS) and small (RSS) subunit relative quantification. The relative proportion of rubisco large (RLS) and small (RSS) subunits was calculated with respect to the ambient CO_2 RLS content at 9 a.m. Bars represent the mean ± SE; n= 12. Bars with the same letter are not significantly different (P≤ 0.05) according to the LSD test.

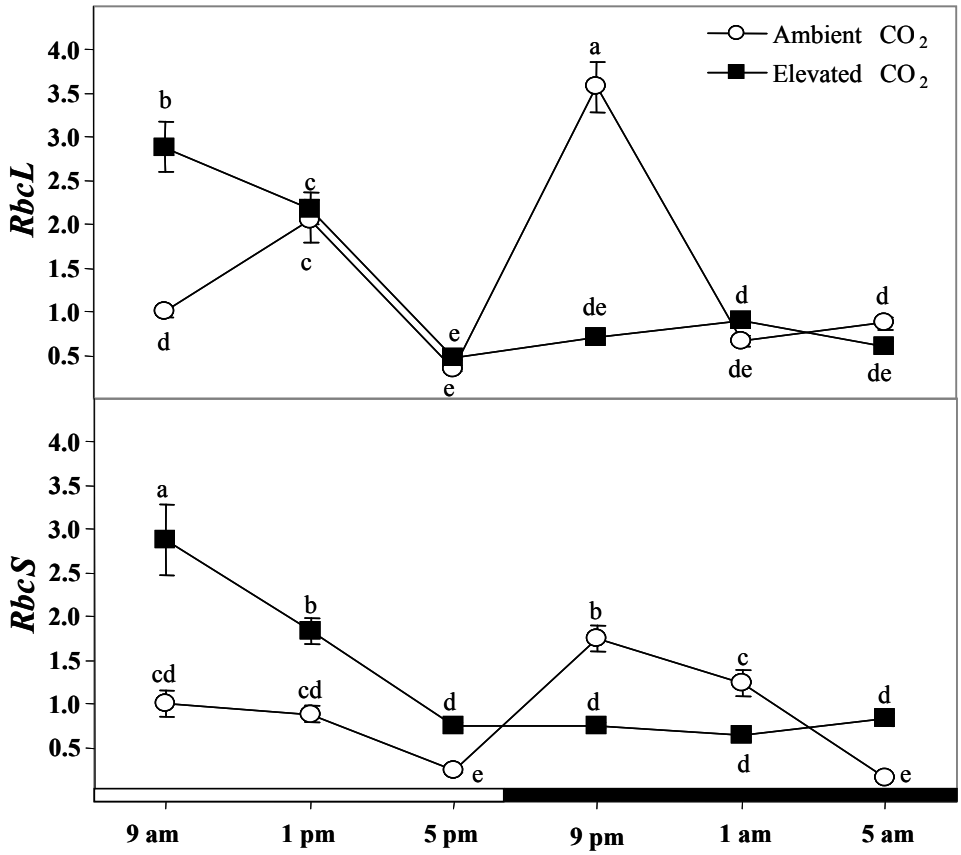

Figure 5: Effect of CO_2 (ambient, approximately 400 µmol mol^{-1} and elevated, 700 µmol mol^{-1}) throughout the day on large (*RbcL*) and small (*RbcS*) rubisco subunit encoding gene expression. Bars represent the mean ± SE; n= 12. Bars with the same letter are not significantly different (P≤ 0.05) according to the LSD test.

Leaf total soluble sugars (TSS) increased under elevated CO_2 except at 1 pm where no significant differences were detected. Under elevated CO_2, TSS increased throughout the day reaching the maximum value at 9 pm (CO_2, *F= 73.1, P ≤ 0.001*). At ambient CO_2, TSS were only enhanced at 1 pm (time, *F= 3.59, P = 0.004*) (Fig. 6A). Elevated CO_2 also increased starch content for most time points (CO_2, *F= 25.37, P ≤ 0.001*) (Fig. 6B).

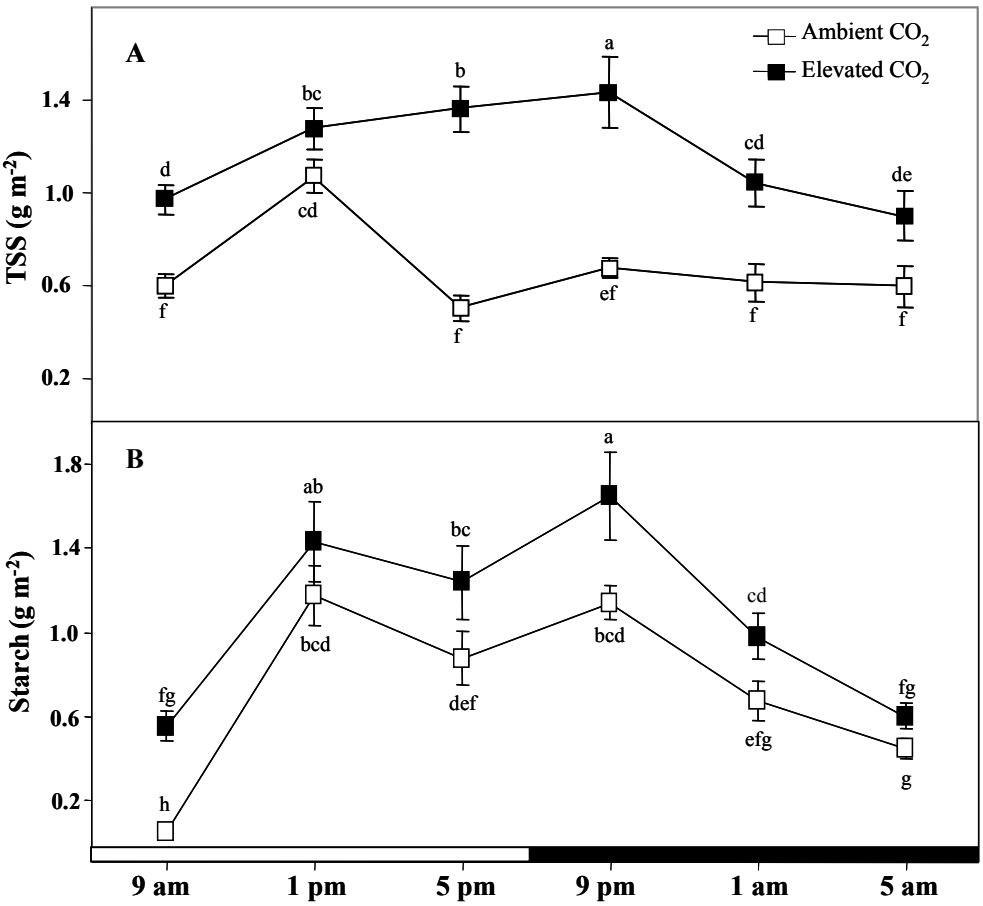

Figure 6: Effect of CO_2 (ambient, approximately 400 µmol mol^{-1} and elevated, 700 µmol mol^{-1}) throughout the day on leaf total soluble sugar content (TSS, g m^{-2}) (A) and starch content (g m^{-2}) (B). Bars represent the mean ± SE; n= 12. Bars with the same letter are not significantly different (P> 0.05) according to the LSD test.

NCE_{growth} was significantly correlated with RLS (*P ≤ 0.001; r = 0.459*) (Table 1). The correlation between NCE_{growth} and RSS was also significant (*P = 0.002, r = 0.389*), with the NCE_{growth}-RLS correlation being stronger than NCE_{growth}-RSS (Table 1). The correlation between RLS and *RbcL* was not significant (Table 1). Starch and TSS content was not correlated with *RbcL* or *RbcS* gene expression (Table 1).

Correlations	P	Pearson R
NCE_{growth} vs RLS	<0.001	0.459
NCE_{growth} vs RSS	0.002	0.389
RbcL vs RLS	0.138	0.153
RbcS vs RSS	0.1	0.170
RbcL vs TSS	0.955	0.006
RbcS vs TSS	0.286	0.111
RbcL vs starch	0.326	0.107
RbcS vs starch	0.908	0.013

Table 1: Interaction between parameters: net carbon exchange measured at growth CO_2 concentration (NCE_{growth}), rubisco large subunit (RLS) and rubisco small subunit (RSS) relative quantification, rubisco large (*RbcL*) and small (*RbcS*) subunit gene expression, total soluble sugars (TSS) and starch content.

Discussion

Elevated CO_2 exposure decreased up to 50% of the net carbon exchange measured under growth CO_2 (NCE_{growth}) at 1 pm. However, net carbon exchange remained unchanged at 9 am and 5 pm, revealing photosynthetic down-regulation (Fig. 2A) (Aranjuelo *et al.,* 2005a; Sanz-Sáez *et al.,* 2010). At 1 pm, higher temperature and light could affect photosynthetic apparatus (Fig. 1), causing a drop in NCE_{growth} at elevated CO_2 as a consequence of the strong acclimation at that time. This behaviour is also supported by the fall in NCE measured under 700 μmol mol^{-1} CO_2 (NCE_{700}) at 1 pm (Fig. 3B). These data suggest that plants grown at elevated CO_2 showed photosynthetic down-regulation throughout the day, being more marked at 1 pm. As previously shown in alfalfa, the observed acclimation was not caused by lower intercellular CO_2 concentration (Ci). Plants grown at elevated CO_2 showed higher Ci than those grown at ambient CO_2 (Aranjuelo *et al*., 2005b, Erice *et al.,* 2006b) (Fig. 2B).

As observed for CO_2 exchange during light-samplings, dark respiration at night was not modified by elevated CO_2 (den Hertog *et al.,* 1993) (Fig. 2A). Wang and Curtis (2002) showed increases in the relative growth rate with enhanced dark respiration. This could explain that after long-term exposure to elevated CO_2, the relative growth rate decreases, and thus dark respiration remains unaffected (Wang and Curtis, 2002). Nevertheless, when young plants are first exposed to high CO_2 their growth rate increases, which leads to an increase in respiration (Thomas and Griffin, 1994).

Photosynthesis down-regulation is usually attributable to a reduced carboxylation activity (Aranjuelo *et al*., 2005*b*; Erice *et al*., 2006b) and/or a reduced amount of rubisco at elevated CO_2 (Aranjuelo *et al*., 2005b; Urban, 2003). It is considered that due to the relative quantity of RLS and RSS, and the lower specificity and content of RSS (Jordan and Ogren, 1981), changes in photosynthesis result from alterations in RSS (Andersson and Blacklund, 2008). In this study, at ambient CO_2, RLS doubled RSS content at all times, confirming that RSS is the subunit that may limit rubisco carboxylation capacity (Fig. 4). Under elevated CO_2 this proportion was also maintained. Moreover, RSS at elevated CO_2 dropped mainly at 1 pm (63.3% drop) (Fig. 4). RLS also declined by elevated CO_2 at 1 pm, confirming the higher acclimation observed at this time. Decreases in rubisco content at elevated CO_2 were

usually related to drops in leaf N content (Aranjuelo *et al.,* 2005b). These diminutions in leaf N under elevated CO_2 are generally a consequence of dilution by carbohydrate accumulation (Fig. 6) (Sanz-Sáez *et al.,* 2010) or increases in plant internal demands for N (Ellsworth *et al*., 2004). Under this low N availability situation, elevated CO_2 may partition resources away from leaves and, through increased production, sequester nutrients (like N) into organic matter causing deficiencies which indirectly cause decreased photosynthetic capacity (Ainsworth *et al.* 2003; Rogers and Ellsworth, 2002). In the present study, RLS and RSS were highly correlated with NCE_{growth} at any CO_2 concentration or time of measurement (Table 1). Therefore, the decrease in photosynthesis was directly related to decreases in rubisco protein (RLS and RSS) (Aranjuelo *et al*., 2005b; Urban, 2003). However, photosynthesis acclimation through rubisco deactivation (Erice *et al*., 2006b) cannot be discarded, especially at times with no decreased RLS or RSS (5 am, 9 am and 9 pm).

In some species including legumes like pea, rubisco protein content has been described as coordinated with *RbcL* and *RbcS* mRNA levels (Majeau and Coleman, 1996; Webber *et al.,* 1994). Moore *et al.* (1998) found that long-term growth at elevated CO_2 affects the relative rubisco protein content and mRNA levels in different ways in several plant species. Bertrand *et al.* (2007) showed that nodulated alfalfa grown at elevated CO_2 leads to photosynthetic acclimation with slightly increased *RbcS* mRNA. This result also shows that *RbcL* and *RbcS* do not correlate with RLS and RSS, and nor do they correlate with NCE (Fig. 2A; Table 1). Plants grown at ambient CO_2 presented a maximum *RbcS* and *RbcL* gene expression at night (Fig. 5) suggesting a lag with RSS and RLS translation, possibly due to a circadian control (Giuliano *et al.,* 1988) and/or post-transcriptional regulation (Kay and Millar 1992, Pilgrim and McClung 1993). For that reason, the maximum contents of RLS and RSS at 1 pm in plants grown at ambient CO_2 may be caused by mRNA expression at 9 pm (Moore *et al.,* 1999) (Fig. 4 and Fig. 5). Nevertheless, at elevated CO_2 the maximum expression of both rubisco genes occurred in the morning (9 am), but without translation into more rubisco content or activity at any time. Moreover, expression of *RbcL* and *RbcS* was significantly decreased at 9 pm by elevated CO_2 (3 and 2 fold, respectively) (Fig. 4). This caused a later decrease in both subunits at 1 pm (Fig. 4), leading to the strong down-regulation showed at that time (Fig. 2A).

At ambient CO_2, total soluble sugars (TSS) showed a peak at 1 pm possibly due to the high photosynthesis rate at this time (Fig. 1A) (Fig. 6A). Throughout the day, elevated CO_2 increased TSS in reference to ambient CO_2 (Fig. 6A), except at 1 pm. The highest TSS value was reached at the end of the afternoon (9 pm) and was followed by a period of degradation/utilization of carbohydrates at night (Ainsworth and Bush, 2011). This 2 fold TSS increase by elevated CO_2 could be responsible for *RbcL* and *RbcS* gene repression at this time (Krapp *et al.,* 1993). Similar results have been reported in *Arabidopsis,* which accumulates starch in the light period in order to support metabolism, assimilate export, and growth during the dark periods, at the end of which starch levels are minimal (Ainsworth and Bush, 2011).

Conclusions

In summary, maximum levels of NCE were observed at noon measurements, showing a strong photosynthetic down-regulation in plants grown at elevated CO_2. The acclimation of alfalfa grown at elevated CO_2 was a consequence of decreased content of both rubisco subunits (RLS and RSS), but especially RSS. However, variations in *RbcL* and *RbcS* gene expression were not correlated with NCE and hence, with photosynthetic down-regulation under elevated CO_2. Moreover, there was no correlation between rubisco content and gene expression of both subunits. Only alfalfa grown under ambient CO_2 concentration showed a rubisco expression peak in the early night followed by a high rubisco content value at noon, suggesting the existence of a long duration lag between gene expression and protein synthesis. In contrast, rubisco expression under elevated CO_2 was enhanced in the early morning but without translation to rubisco protein content at any time, suggesting that mRNA could be degraded without protein translation.

Acknowledgements

This work was supported by Ministerio de Ciencia e Innovación (MICINN BFU2008-01405), Fundación Universitaria de Navarra (PIUNA-2008) and Fundación Caja Navarra. A. Sanz-Sáez was the recipient of a research grant from Asociación de Amigos de la Universidad de Navarra. Iker Aranjuelo was the recipient of a Ramón y Cajal research

contract (Ministerio de Ciencia e Innovación). The assistance of A. Urdiain, M. Oyarzun and L. González is also appreciated.

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CAPÍTULO 6

Alfalfa yield, forage quality and digestibility under future climate change scenarios varies with *Sinorhizobium meliloti* strain

La producción de alfalfa, y la calidad y digestibilidad del forraje en futuros escenarios de cambio climático varía con la cepa de *Sinorhizobium meliloti* inoculada

Alfalfa yield, forage quality and digestibility under future climate change scenarios varies with *Sinorhizobium meliloti* strain

Resumen

De acuerdo con las predicciones del IPCC para finales de este siglo la concentración atmosférica de CO_2 será cercana a 700 μmol mol^{-1} y el calentamiento global podría aumentar la temperatura media del planeta en 4ºC. El CO_2 elevado podría disminuir la calidad y digestibilidad del forraje de la alfalfa, descendiendo el contenido de proteína cruda y aumentando el de fibras. El objetivo del presente estudio ha sido analizar el efecto del CO_2 y la temperatura elevados y la inoculación con diferentes cepas de *Sinorhizobium meliloti* en la producción, calidad y digestibilidad *in-vitro* de la materia seca de la parte aérea de la alfalfa. Este objetivo pretende contribuir a la selección de cepas de *S. meliloti* que sean capaces de obtener una alta producción y calidad en el forraje de alfalfa en las futuras condiciones de cambio climático. La producción de la parte aérea en condiciones de CO_2 y temperatura elevados fue diferente dependiendo de la cepa de *S. meliloti* inoculada, siendo las plantas inoculadas con la cepa 102F34 las más productivas, seguidas por la 102F78 y la 1032GMI. Las plantas inoculadas con la cepa 102F34 no aumentaron el contenido de fibra detergente neutro o ácido en condiciones de CO_2 o temperatura elevados, y por lo tanto la digestibilidad *in-vitro* de la materia seca tampoco se vió afectada. La proteína cruda, indicador de la calidad del forraje se correlacionó negativamente con la producción de parte aérea. Las plantas inoculadas con la cepa 102F78 mostraron una producción de parte aérea similar a las inoculadas con la cepa 102F34, pero con un mayor contenido en proteína cruda en condiciones de CO_2 y temperatura elevados. En estas condiciones de cambio climático las plantas inoculadas con la cepa 102F78 produjeron un forraje de mayor calidad. Sin embargo, la mayor digestibilidad de las plantas inoculadas con la cepa 102F34 en condiciones de CO_2 y temperatura elevados las hacen las más adecuadas para su crecimiento en las futuras condiciones de cambio climático.

Abstract

According to the predictions of the IPCC, at the end of the present century CO_2 concentration may be around 700 μmol mol^{-1} and the resulting mean global warming may lead to a temperature increase of 4ºC. Elevated CO_2 may decrease alfalfa forage quality and *in-vitro* digestibility through crude protein drop and fibre content enhancement. The aim of the present study was to analyze the effect of elevated CO_2, elevated temperature and *Sinorhizobium meliloti* strains on alfalfa yield, forage quality and *in-vitro* dry matter digestibility. This objective is in line with the selection of *S. meliloti* strains in order to maintain high forage yield and quality under future climate conditions. Shoot dry matter under elevated CO_2 and temperature was different depending on *S.meliloti* strain, being 102F34 inoculated plants the most productive, followed by 102F78, and then 1032GMI. Plants inoculated with 102F34 strain did not enhance neutral or acid detergent fibre under elevated CO_2 or temperature and hence, *in-vitro* dry matter digestibility was unaffected. Crude protein content, indicator of forage quality, was negatively related to shoot yield. Plants inoculated with 102F78 showed similar shoot yield than 102F34 ones, but higher crude protein content at elevated CO_2 and temperature. In this climate change conditions, 102F78 inoculated plants produced more quality forage. However, the higher digestibility of plants inoculated with 102F34 strain under any CO_2 or temperature conditions, makes them more adequate for growing under climate change scenario conditions.

Key words: Carbon dioxide, *Medicago sativa* (alfalfa), *Sinorhizobium meliloti*, forage quality, *in-vitro* dry matter digestibility.

Introduction

The United Nations Population Division (UNPD, 2010) expects a further growth of the population from close to 7 billions today to 9.3 billions in 2050, and a possible stabilization around 10 billions by the end of the century. To sustain this population growth, over the next 30 years it would be necessary to produce throughout the world more foodstuffs than over the whole of the last 10 000 years (Kern, 2002a). In order to ensure sufficient food supplies for the world population on a sustainable basis, yields must be increased with the same cultivable surface (Kern, 2002b). This rise of human population is followed by an increase in atmospheric CO_2 concentration (Krausmann et al., 2009). Nowadays the CO_2 concentration is around 389 μmol mol^{-1} and currently increases at 1.9 μmol mol^{-1} per year on average (Intergovernmental Panel on Climate Change; IPCC, 2007). According to the predictions of the IPCC, at the end of the present century this concentration may be around 700 μmol mol^{-1}. By the end of the present century the global warming resulting from rising CO_2 may lead to a temperature increase of 4ºC (IPCC, 2007).

Many studies have shown that photosynthesis is reduced after long-term elevated CO_2 exposure causing a reduction in potential growth (Ainsworth and Rogers, 2007). This phenomenon is known as photosynthetic down-regulation (Saralabai et al., 1997). It has been widely reported that other environmental variables such as temperature, soil N and water content, atmospheric humidity, and solar radiation (PPDF intensity), may interact with the CO_2 effect on plant yield (Ainsworth and Long, 2005; Aranjuelo et al., 2005a, b). Previous studies have shown that the combination of elevated CO_2 and temperature (ambient temperature +4ºC) increased yield (Aranjuelo et al., 2005a, 2005b; Erice et al., 2006ab, 2007; Rawson, 1992; Rawson et al., 1995).

Within environmental parameters, N availability is a critical factor, limiting plant growth and increasing the response to elevated CO_2 conditions. At elevated CO_2 concentration, a low soil N supply could limit photosynthesis (Luo et al., 2004; Peterson et al., 1999; Sanz-Sáez et al., 2010). Decreased leaf N content could be the consequence of the accumulation of carbohydrates, leaf structural material and the increase in plant internal demands for N (Ellsworth et al., 2004). Furthermore, the low leaf N availability in elevated

CO_2 conditions causes the immobilisation of C, leading to carbohydrate accumulation and thus enhancing photosynthetic down-regulation and yield limitation (Sanz-Sáez et al., 2010).

Nitrogen content is usually correlated with forage quality (Owensby et al., 1996). Reduction in shoot N content under elevated CO_2 leads to a reduction in forage quality determined as crude protein drops (Campbell et al., 2000; Owensby et al., 1996; Wand et al., 1999). Moreover, Morgan et al. (2004) showed that different grassland forages reduced its in-vitro dry matter digestibility (IVDMD) under elevated CO_2 exposure caused by an enhancement of fibres concentration (Owensby et al., 1996). Fibre content, often measured as acid detergent fibre (ADF) and neutral detergent fibre (NDF), can limit digestibility, palatability and intake of grass forages (Van Soest, 1994). Neutral detergent fibre includes cellulose, hemicellulose and lignin as wall components, whereas the ADF fraction is composed mainly of cellulose and lignin without hemicellulose (Van Soest, 1994). It is known that elevated CO_2 induces the synthesis of secondary phenolic compounds, mainly lignin, usually associated with decreases in IVDMD (Gifford, 2000). Nevertheless this response is not unequivocal; Picon-Cochard et al. (2004) observed increased water soluble sugar concentration and increased IVDMD of bulk forage from a semi-natural grassland community exposed to high CO_2 concentration.

Alfalfa (*Medicago sativa* L.) is an important forage crop due to ecological and economical reasons. It is distributed along the world and in comparison to most common field crops is highly persistent (Bagavathiannan and Van Acker, 2009). Alfalfa establishes symbiotic relationship with a N_2-fixing bacterium (*Sinorhizobium meliloti*) providing an extra source of N for the plant and soil, improving soil structure and increasing soil organic matter (Bourgeois, 1990). Alfalfa, as N_2-fixing plant, may grow without N fertilizers application, avoiding N compounds lixiviation and contamination. N_2-fixing legumes often show larger stimulation of growth and photosynthetic rate under elevated CO_2 than species without this capability (Lüscher et al., 1998). In this symbiosis, atmospheric N_2 is fixed in exchange for plant carbohydrates (Bergersen, 1969). To this respect, increases in nodule C availability under elevated CO_2 may enhance nodule activity providing more N for plant growth thus avoiding photosynthetic acclimation (Vance and Heichel, 1991). In previous studies, the application of N compounds avoided acclimation and increased yield of alfalfa

(Sanz-Sáez et al., 2010). Furthermore, the inoculation with high efficient N_2-fixing *Sinorhizobium* strain may lead to the avoidance of photosynthetic acclimation and to the enhancement of potential production (Bertrand et al., 2007b). Moreover, Bertrand et al. (2007a) showed that the interaction between *S. meliloti* strain and alfalfa genotype alters plant responses to different ambient variables like freeze tolerance at elevated CO_2.

Therefore, the aim of the present study was to analyze the effect of elevated CO_2, elevated temperature and *S. meliloti* strain on alfalfa yield, forage quality and *in-vitro* dry matter digestibility. This objective is in line with the selection of *S. meliloti* strains in order to maintain high forage yield and quality in future climate conditions.

Materials and Methods

Biological material

Alfalfa (*Medicago sativa* L. cv. Aragón) seeds were sterilised in a solution of $HgCl_2$ (0.1% w/v) and germinated in Petri dishes. One week later, seedlings were planted in pots (20 plants per pot) containing a mixture of perlite–vermiculite (2/1; v/v). The pots were used to allow the growth of plants without N and without natural *Sinorhizobium* contamination. Pots with a capacity of 13 L were used to avoid the possibility of becoming pot-bound. At planting, seedlings were divided into three groups and inoculated with *S. meliloti* strains: 102F78, 102F34 (Nitragin Co., Milwaukee, WI), or 1032GMI (Biotechnology Department, Polytechnic University of Madrid, Spain). In previous studies conducted with 102F78, the tolerance of this strain to elevated temperatures has been demonstrated (Aranjuelo et al. 2005 a, b; Erice et al. 2006 a, b). In contrast, less information is available about 102F34 and 1032GMI, although previous studies have shown that 102F34 was more efficient than 1032GMI (Muro, 2009).

Experimental design and growth conditions

Plants were grown from September 20th to November 20th 2009 in two temperature gradient greenhouses (TGG) and irrigated alternately with Evans N-free solution (Evans, 1974) and distilled water to avoid salt accumulation in the substrate during the experiment. TGG were placed at the University of Navarra campus (42.80N, 1.66W; Pamplona, Spain). The design of the TGG was similar to that described by Aranjuelo et al. (2005a) and Erice et al. (2007) based on Rawson et al. (1995). CO_2 concentration, temperature, relative humidity and solar radiation levels inside and outside the greenhouses were continuously monitored and controlled by a computerised system. Plants were divided into twelve treatments comprising a combination of two CO_2 levels (ambient, around 380 and elevated, 700 μmol mol^{-1}), two temperature regimens (ambient and ambient + 4ºC) and three *S. meliloti* strains (102F78, 102F34 and 1032GMI). One greenhouse was maintained at ambient CO_2 concentration level (around 380 μmol mol^{-1} CO_2), and the other was maintained at elevated CO_2 (700 μmol mol^{-1} CO_2). Each greenhouse was divided into three modules, thereby providing different temperature values. The middle module was

considered as a transition module, and no experimental plants were included. In each greenhouse the inlet module was maintained at ambient temperature (mean temperature was 18ºC) and the outlet module at ambient temperature + 4ºC. The CO_2 concentration was continuously monitored using a Guardian Plus (Edinburgh Instruments Ltd., Livingston, UK) at the outlet module. The monitor's signal was fed into a proportional integrative differential controller that regulated the opening time (within a 10 s cycle) of a solenoid valve that injected CO_2 into both inlet fans. Pots were placed at inlet and outlet modules, and rotated daily in each module to avoid edge effects. The harvest was carried out at the beginning of flowering, corresponding to 67 day-old plants.

Plant growth parameters

Harvested plants were separated into leaves, stems, roots and nodules. Shoot dry matter (SDM) was obtained after drying at 85ºC for 48 h. The root/shoot ratio (R/S) was calculated as root DM including nodules divided by the sum of leaf and stem DM. The stem/leaf ratio (S/L) was calculated as stem DM divided by the leaf DM.

Fibres content and in-vitro dry matter digestibility

Shoot samples were dried at 60°C in an oven with ventilation and later ground to a size of 1 mm. The samples for neutral detergent fibre (NDF), acid detergent fibre (ADF), lignin, cellulose and hemicellulose content were performed by Near Infrared Reflectance Spectroscopy (NIRS). Each sample was analysed using near-infrared reflectance spectroscopy (NIRS) to determine CP concentration and enzymatic pepsin-cellulase DM digestibility. Near infra-red spectra were collected with a monochromator FOSSNIRSystems 6500, Silver Spring, MD, USA) which scans the spectral range of 400–2500 nm. Modified partial least square (MPLS) calibration equations were developed using X samples which were selected from the total spectra population collected (Shenk and Westerhaus, 1991). The calibration set was analysed for CP concentration (using Kjeldahl N · 6.25) and pepsin cellulase DM digestibility (Aufrère and Demarquilly, 1989) of herbage. All spectra and reference data were recorded and managed with the software WINISI Version 1.5 (Infrasoft International, Port Matilda, PA, USA).

Nitrogen and crude protein content

Leaf and stem samples from 67 day-old plants were collected. Samples were dried at 60ºC for 48 h and analysed for %N of total organic matter. One milligram of ground sample was used for each determination, and 8 replicates were analysed for each treatment (4 experimental and 2 laboratory replicates). Leaf and stem N was determined using an elemental analyser (EA1108, Series 1, Carlo Erba Instrumentazione, Milan, Italy). Nitrogen content was calculated as mg g SDM^{-1}. Crude protein (CP) content was calculated as nitrogen content x 6.5 (Licitra et al., 1996). The crude protein/shoot dry matter ratio (CP/SDM) was calculated as CP content (mg g DM^{-1}) divided by SDM (g DM $plant^{-1}$), and is indicator of quality versus production.

Non Structural Carbohydrates

Non structural carbohydrates (NSC) were calculated as the sum of total soluble sugars and starch content. Leaf total soluble sugars (TSS) and starch contents were quantified by grinding and filtering 200 mg of leaf fresh weight from leaves (4 replicates per treatment) in a cold mortar using an extraction buffer containing 50 mM K-phosphate (pH 7.5). The extract was filtered and centrifuged at 28,710 g for 15 min at 4ºC. The TSS quantification was performed in the supernatant as described by Yemm and Willis (1954), whereas starch was measured using the pellet as described by Jarvis and Walker (1993).

Statistical analysis

Statistical analysis was carried out using three-factor ANOVA (factorial 2 x 2 x 3) (SPSS v.15.0). Taking CO_2 concentration as the first factor (ambient CO_2, around 380 µmol mol^{-1}; or elevated, 700 µmol mol^{-1}), temperature as the second factor (ambient T or ambient T +4ºC) and the *S. meliloti* strain as the third factor (102F78, 102F34 or 1031GMI). Significant differences between factors and interactions were calculated at 5%, level of significance. When differences between treatments (CO_2 and/or T and/or strain and/or interactions) were significant according to the ANOVA analysis, least significant differences were evaluated using the least significant different test (LSD) ($P \leq 0.05$).

Results

The combination of elevated CO_2 and temperature enhanced shoot dry matter (SDM) regardless inoculated strain (Fig. 1A) (*CO_2xT, F= 9.46, P = 0.004*). Plants inoculated with 102F34 strain were the most productive (0.77 ± 0.08 g DM plant^{-1}), followed by those inoculated with 102F78 (0.66 ± 0.08 g DM plant^{-1}) and 1032GMI (0.47 ± 0.1 g DM plant^{-1}) (Fig. 1A) (*strain, F= 10.98, P = 0.001*). The root/shoot ratio (R/S) of plants inoculated with 102F34 and 1032GMI was not modified by elevated CO_2 or temperature (Fig. 1B). However, the inoculation with 102F78 increased R/S under elevated CO_2 (Fig. 1B) (*CO_2xTxstrain, F= 5.12, P = 0.011*). Stem/leaf ratio (S/L) remained unchanged under any treatment (Fig. 1C).

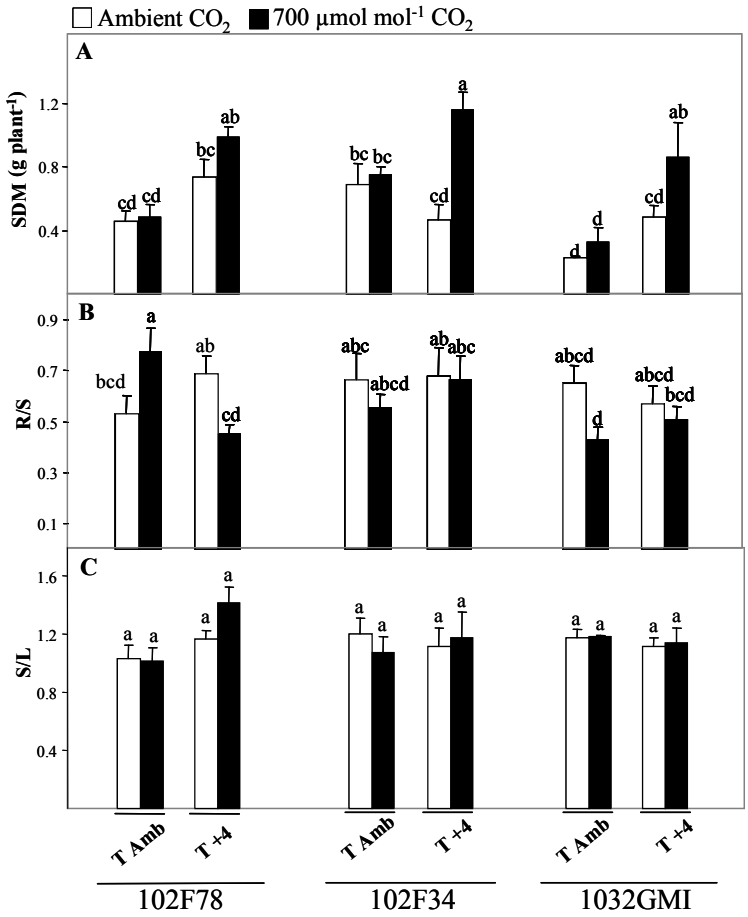

Figure 1: Effect of CO_2 (ambient, around 400 or elevated, 700 µmol mol^{-1}), temperature (ambient or ambient +4ºC) and *S. meliloti* strain (102F78, 102F34 and 1032GMI) on shoot dry matter (A) (SDM, g DM plant $^{-1}$), root/shoot (R/S) (B) and stem/leaf (S/L) (C) ratio in nodulated alfalfa. Bars represent the mean ± SE; n = 4. In each graph, bars with the same letter are not significantly different (P ≤ 0.05) according to the LSD test.

Elevated temperature increased neutral detergent fibre (NDF) content, regardless inoculated strain at ambient CO_2 (Fig 2. A) (*T, F= 43.91, P ≤ 0.001*). Nevertheless, elevated CO_2 effect was dependant on inoculated strain (*CO_2xStrain, F= 4.96, P = 0.015*). Only in plants inoculated with 102F78, elevated CO_2 increased both NDF and ADF contents (Fig. 2A, 2B) (*CO_2xstrain, F= 11.55, P ≤ 0.001; Txstrain, F= 4.22, P = 0.025*). In 102F34 inoculated plants, ADF content remained unchanged under elevated CO_2 or temperature, whereas increased by elevated temperature in 1032GMI plants (Fig. 2B). *In-vitro* dry matter digestibility (IVDMD) was no affected by elevated CO_2; however, elevated temperature decreased IVDMD in 102F78 and 1032GMI plants (Fig. 2C) (*T, F= 17.37, P ≤ 0.001*). The mean IVDMD values showed that 102F34 inoculated plants were the most digestible (77.1% ± 1.1), followed by 1032GMI (76.3 ± 1.4) and 102F78 (73.6 ± 1.8) (Fig. 2C) (*strain, F= 3.83, P = 0.031*).

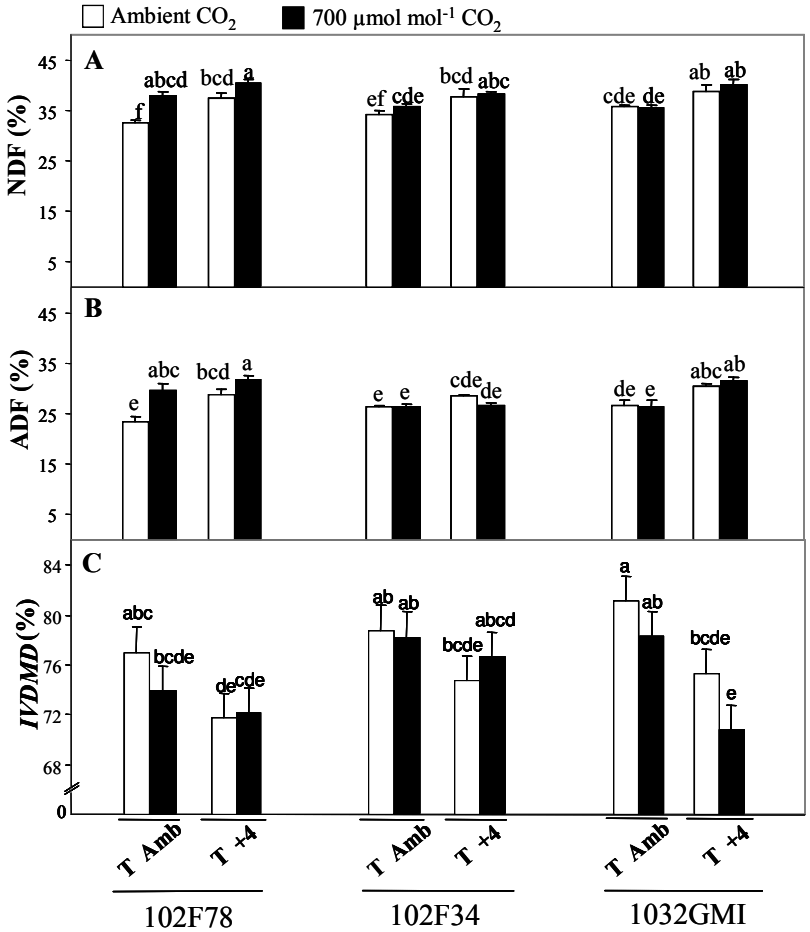

Figure 2 Effect of CO_2 (ambient, around 400 or elevated, 700 µmol mol^{-1}), temperature (ambient or ambient +4ºC) and *S. meliloti* strain (102F78, 102F34 and 1032GMI) on neutral detergent fibre content (A) (NDF, percentage of DM), acid detergent fibre content (B) (ADF, percentage of DM) and *in-vitro* dry matter digestibility (IVDMD) (C) (percentage of DM). Bars represent the mean ± SE; n = 4. In each graph, bars with the same letter are not significantly different (P ≤ 0.05) according to the LSD test.

Hemicellulose content only showed a significant increase by elevated CO_2 in plants inoculated with 102F34 grown under elevated temperature (*CO_2xstrain, F=8.1, P = 0.001*) (Fig. 3A). In 102F78 inoculated plants, cellulose content increased by elevated CO_2, temperature and the interaction of both factors (Fig. 3B) (*CO_2xTxstrain*, *F= 9.68, P ≤ 0.001*). In plants inoculated with 102F34, cellulose content remained unchanged, however, in 1032 GMI plants, elevated temperature increased cellulose content (Fig. 3B). Lignin content increased by elevated CO_2, elevated temperature and by the combination of both treatments in 102F78 inoculated plants, (Fig. 3C) (*CO_2xTxstrain, F= 9.48, P = 0.004*). In plants inoculated with 1032GMI lignin content only increased by elevated temperature (Fig. 3C) (*T, F= 32.89, P ≤ 0.001*).

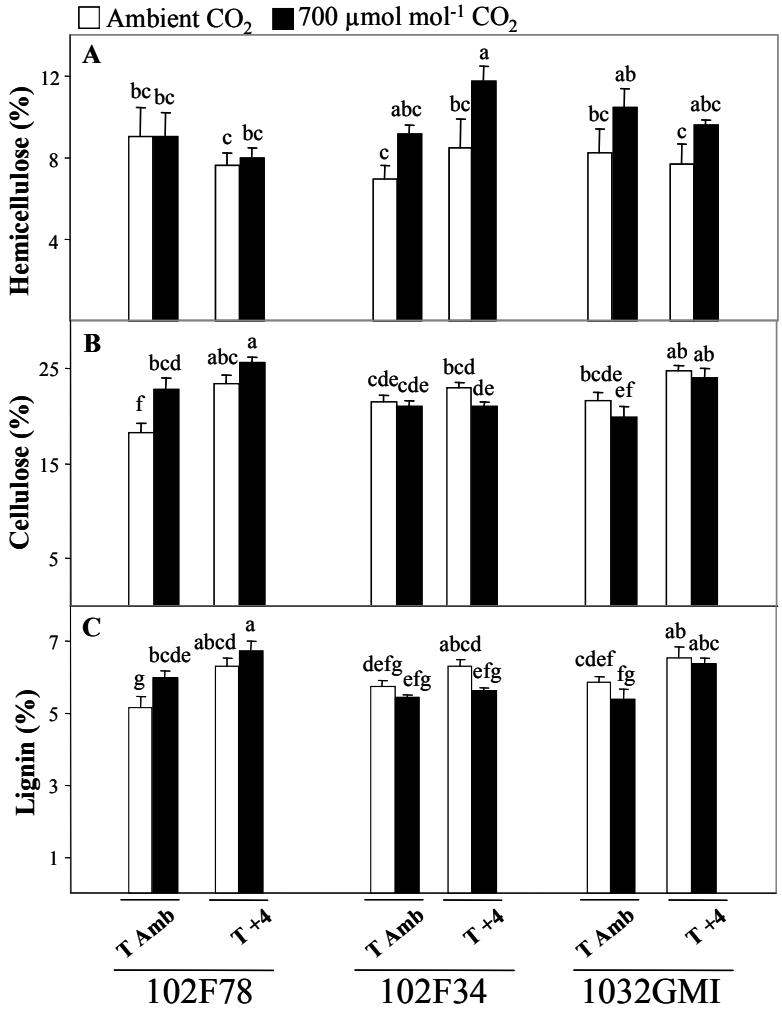

Figure 3: Effect of CO_2 (ambient, around 400 or elevated, 700 µmol mol^{-1}), temperature (ambient or ambient +4ºC) and *S. meliloti* strain (102F78, 102F34 and 1032GMI) on hemicellulose (A) (percentage of DM), cellulose (B) (percentage of DM) and lignin content (C) (percentage of DM). Bars represent the mean ± SE; n = 4. In each graph, bars with the same letter are not significantly different (P ≤ 0.05) according to the LSD test.

Crude protein (CP) content (Fig. 4A) decreased significantly at elevated CO_2 and temperature in 102F78 plants (*CO_2xTxstrain, F= 55.33, P ≤ 0.001*). 102F34 plants grown at ambient CO_2 and elevated temperature, showed higher CP content than ambient ones; however, when plants were exposed to elevated CO_2 and temperature, CP content decreased (Fig. 4A). In 1032GMI inoculated plants, CP decreased with elevated CO_2, high temperature and their combination. Elevated CO_2 and temperature decreased CP/SDM ratio. Crude protein/shoot DM ratio (CP/SDM) decreased in 102F78 inoculated plants at elevated temperature. In plants inoculated with 102F34 strain grown at elevated CO_2 and temperature CP/SDM decreased respect to its ambient CO_2 control (Fig. 4B) (*CO_2xTxstrain, F= 19.95, P ≤ 0.001*). The high CP/SDM obtained in 1032GMI plants grown at ambient CO_2 and temperature, was caused by the lowest SDM. Plants inoculated with 1032GMI accumulated more non structural carbohydrates (NSC) than those inoculated with the other strains (Fig. 4C) (*strain, F= 5.94, P = 0.006*).

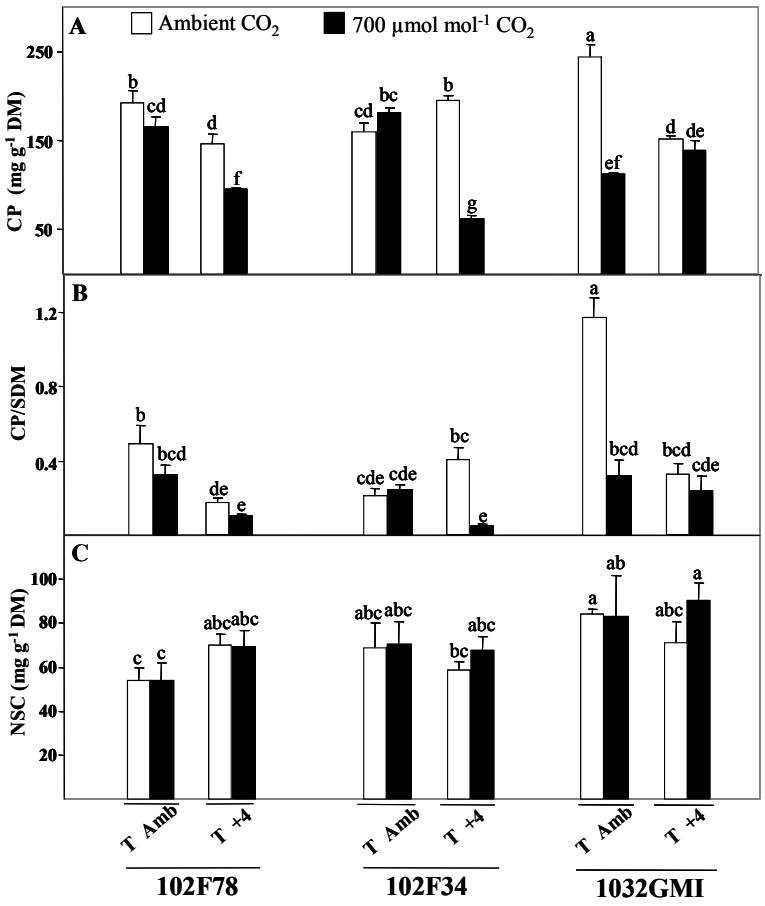

Figure 4: Effect of CO_2 (ambient, around 400 or elevated, 700 µmol mol^{-1}), temperature (ambient or ambient +4ºC) and *S. meliloti* strain (102F78, 102F34 and 1032GMI) on crude protein (A) (CP, mg g DM^{-1}), Crude Protein/Shoot DM ratio (B) (CP/SDM) and non structural carbohydrates content (C) (NSC, mg g DM^{-1}) Bars represent the mean ± SE; n = 4. In each graph, bars with the same letter are not significantly different (P ≤ 0.05) according to the LSD test.

Discussion

In this study, elevated CO_2 did not enhance shoot dry matter (SDM) at ambient temperature, possibly due to mild temperatures of Autumn (Fig. 1A). However, the combination of elevated CO_2 and higher temperature (ambient + 4 ºC) enhanced the vegetative growth. This is consistent with previous works which reported that stimulation of growth by elevated CO_2 is usually greater under higher temperatures (Aranjuelo et al., 2005a, 2006; Erice et al., 2006b; McKee and Woodward, 1994). The most productive *alfalfa-Sinorhizobium* combination in terms of SDM was the inoculated with 102F34 strain (40.5 % of total SDM obtained in all treatments) (Fig. 1A) followed by 102F78 (34.7 %) and then by 1032GMI (24.8 %). Differences in SDM production could be linked to differences in the C cost of N_2 fixation (Twary and Heichel, 1991) and/or differences in N_2 fixation rate under elevated CO_2 (Bertrand et al., 2007b). In this sense, a higher C consumption decreases the amount of photosynthates available for plant growth (Bertrand et al., 2007b); Saari and Ludden (1986) reported that the C cost of N_2 fixation is mainly due to the respiratory rate. In order to ensure the perennity of the alfalfa, roots as source organ during regrowth may be altered under high CO_2 concentration (Erice et al., 2006b). The root/shoot ratio (R/S) can be affected differently by elevated CO_2 (Koch et al., 1986; Patterson and Flint, 1980; Rogers et al., 1992, 1996), but the cause of this behaviour remains unclear (Erice et al., 2007). In the present study R/S decreased at elevated CO_2, due to increased SDM (Fig. 1B), confirming that shoot biomass of alfalfa is more sensitive to CO_2 than root biomass (Erice et al., 2007).

A parameter that could affect alfalfa quality as feed, is the stem/leaf ratio (S/L). Early flowering harvest is recommended to provide forage with high to medium nutrient concentrations (Lamb et al., 2003). When alfalfa is harvested at mid-late flowering has greater stem than leaf yield (with a less nutritive value), while in the early flowering, leaf and stem yields are nearly the same (Sheaffer et al., 2000). In present study, S/L was around one, with no modifications by elevated CO_2 or temperature (Fig. 1C) as also showed Aranjuelo et al. (2005a). Therefore, the proportion between leaves and stems did not affect digestibility and feed quality under the climate change conditions described in the present work.

Under elevated CO_2 only plants inoculated with 102F78 showed increased NDF and ADF but without changes in IVDMD. Contrariwise, higher temperature decreased IVDMD. It is probably associated to NDF and ADF increases resulting from lignin accumulation. Despite 102F34 also increased NDF, ADF was not affected by higher temperature and IVDMD was not decreased. To this respect, we can point that plants inoculated with 102F34 reached the best forage quality for animal consumption in terms of fibre content and digestibility. Bertrand et al. (2007b) also observed a different response of NDF and ADF content to elevated CO_2 in alfalfa depending on *S. meliloti* strain (Fig. 2A, 2B). Available literature about the effect of high temperature in forage quality is scarce. Thorvaldsson (1992) and Thorvaldsson et al. (2007) showed decreased digestibility and increased NDF content at high temperatures, while Bertrand et al. (2008) found that NDF content remained unchanged under elevated temperatures.

Hemicellulose and cellulose of the fibrous fraction (NDF and ADF) are potentially digestibles, but the rate of digestibility is dependant on lignin content and encrustation, the availability of N in the rumen and the rate of particle size breakdown (Van Soest, 1994). Lignin is virtually indigestible by the ruminants (Milchunas et al., 2005; Powell et al., 2003), and the increase in this compound under elevated CO_2 could be associated with quality losses. Cellulose enhanced with elevated CO_2 and temperature in plants inoculated with 102F78 strain, while hemicellulose did no change (Fig. 3A, 3B). However, this was not translated into higher IVDMD levels, probably due to the enhancement of lignin. Elevated CO_2 and temperature increased hemicellulose content in plants inoculated with 102F34, and did not modify lignin values (Fig. 3). This increase in digestible compounds (hemicellulose) over the indigestible ones (lignin) (Fig. 3A, 3C) may cause that the IVDMD remain stable under CO_2 and temperature with 102F34 strain (Fig. 2C). In the case of 1032GMI inoculated plants, elevated temperature increased lignin under ambient CO_2 and consequently IVDMD decreased. But the great IVDMD drop by the combination of elevated CO_2 and temperature was not related to lignin increase and it may be caused by increased lignin incrustation with other fibres (Van Soest, 1994).

The soluble protein in forage is degraded to ammonia in the rumen and excreted as urea; however, crude protein (CP) is highly digestible and non-degradable protein that passes through the rumen and can be efficiently utilized in the lower digestive tract (Casler,

2001). Moreover, it is considered as one of the most important indicators of forage quality (Larson and Mayland, 2007; Milchunas et al., 2005; Powell et al., 2003). In the present study, plants showed decreased CP content under elevated CO_2 and temperature (Fig. 4A); similar results were previously obtained by Milchunas et al. (2005). The decrease in CP levels under elevated CO_2 and temperature may reduce alfalfa forage quality. The CP content drop was not a dilution effect as a result of non structural carbohydrates (NSC) accumulation under elevated CO_2 (Fig. 4C). Usually, increased NSC (including total soluble sugars) has been related to higher forage digestibility and quality (Fisher et al., 2002). In the present study, NSC content remained unchanged at elevated CO_2 and temperature and therefore forage quality was not improved in this aspect. The decrease in CP content was possibly due to the reallocation of N resources from shoots (especially leaves) to other parts of the plant (Rogers and Ellsworth, 2002; Ellsworth *et al.,* 2004; Tissue *et al.,* 1997).

Some authors state that CP content is usually negatively correlated with yield (Casler 2001; Casler and Vogel, 1999). In the present study a similar result was obtained, especially in plants grown at elevated CO_2 and temperature. The most productive plants were also the less digestible. In order to explain the compromise between forage yield and quality for the treatments, we calculate the CP/SDM ratio. This parameter decreased with the combination of elevated CO_2 and temperature regardless of inoculated strain, showing that forage produced under elevated CO_2 and temperature was of less quality than that produced at ambient CO_2 and temperature (Fig. 4C). The lower CP/SDM in alfalfa inoculated with 102F34 and 102F78 and grown under elevated CO_2 and temperature was due to a very strong decrease in CP and yield enhancement. In 102F78 inoculated plants the same trend was observed, but these plants presented more CP content and almost the same yield, becoming the forage of higher quality. However, the higher IVDMD of plants inoculated with 102F34 under any CO_2 or temperature condition, makes them more suitable for growth at future climate change scenarios. Reduction in IVDMD and quality (CP content) under elevated CO_2 and high temperature would result in the need of a greater forage consumption compared to biomass grown under present CO_2 ambient concentration to achieve similar comparable weight gain.

Conclusions

Combined effect of CO_2 and temperature on alfalfa SDM depends on *S. meliloti* strain; the 102F34 inoculated plants proved to be the most productive, followed by 102F78, and then by 1032GMI. Elevated CO_2 and temperature (separately or in combination) increased NDF and ADF in plants inoculated with 102F78 and 1032GMI, leading to the drop in IVDMD. 102F34 inoculated plants did not enhance NDF or ADF and hence IVDMD was unaffected. CP content was negatively related to SDM. Plants inoculated with 102F78 showed similar SDM production than 102F34 ones but with higher CP content at elevated CO_2 and temperature. In the studied climate change conditions, 102F78 produced more quality forage respect to the yield. However, the higher digestibility of plants inoculated with 102F34 under any CO_2 or temperature conditions, makes them more adequate for growth at climate change scenarios.

Acknowledgements

This work was supported by Ministerio de Ciencia e Innovación (MICINN BFU2008-01405), Fundación Universitaria de Navarra (PIUNA-2008) and Fundación Caja Navarra. A. Sanz-Sáez was the recipient of a research grant from Asociación de Amigos de la Universidad de Navarra. Thanks to PhD. Fernando Muñoz of Centro de Investigación y Tecnología Agroalimentaria de Aragón, Zaragoza (Spain) for the technical and theoretical support in fibres quantification. The assistance of A. Urdiain, M. Oyarzun and L. González is also appreciated.

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DISCUSIÓN GENERAL

Discusión General

El presente trabajo pretende abordar el efecto del CO_2 elevado, alta temperatura y adición de N, en la aclimatación fotosintética, fijación de N_2, producción y calidad de alfalfa nodulada. En experimentos en los que se exponen plantas a determinados factores ambientales, como en caso del CO_2 elevado o la alta temperatura, es necesario controlar el resto de variables ambientales en cámaras climáticas para poder estudiar su efecto aislado. Sin embargo, la respuesta de la planta a estos factores puede variar si se compara en condiciones de campo. Los invernaderos de gradiente térmico, empleados en este trabajo, son una instalación que permite estudiar el efecto de la temperatura y CO_2 elevados en condiciones cercanas a la realidad, pues son capaces de mantener en todo momento unas condiciones dinámicas de luz, temperatura y humedad iguales a las ambientales.

En estudios en cámaras climáticas, se observó que las plantas de alfalfa noduladas que obtienen el N exclusivamente de la fijación de N_2, aclimatan su fotosíntesis tras exposiciones a largo plazo de CO_2 elevado. Sin embargo, la adición de N mineral a plantas crecidas en las mismas condiciones evitó la aclimatación fotosintética y repercutió en un mayor crecimiento de estas plantas. Por lo tanto, se puede concluir que las plantas de alfalfa exclusivamente fijadoras de N_2 no fueron capaces de cubrir la demanda de N exigida en condiciones de CO_2 elevado, con mayor crecimiento, produciéndose así la aclimatación fotosintética. Esto refuerza la hipótesis de que una de las posibles causas de la aclimatación fotosintética es la baja disponibilidad de N, posiblemente debido a un descenso de la fijación de N_2 en condiciones de CO_2 elevado. El estudio en mayor profundidad del metabolismo de la hoja y el nódulo en elevado CO_2, reveló que existe relación entre la aclimatación fotosintética en la hoja y la reducción del metabolismo nodular. El CO_2 elevado afectó significativamente al metabolismo nodular, concretamente del bacteroide, reduciendo el contenido de isocitrato sintasa, enzima clave del ciclo de los ácidos tricarboxílicos de la bacteria. Otra enzima que redujo su contenido en estas condiciones fue la anhidrasa carbónica de las células noduladas que se relacionó con una mayor permeabilidad del córtex del nódulo al O_2, que podría haber reducido la fijación de N_2. También disminuyó su contenido debido al CO_2 elevado el transportador ABC, encargado de

translocar compuestos nitrogenados derivados de la fijación de los bacteroides a las células del nódulo. La reducción de estas proteínas nodulares podría haber causado el descenso en la fijación y transporte de N provocando el déficit de este elemento favoreciendo la aclimatación fotosintética.

Siendo la disponibilidad de N uno de los factores que limita la respuesta al CO_2 y temperatura elevados, la selección de cepas de *Sinorhizobium meliloti* altamente eficientes en la fijación de N_2, podría mejorar el contenido de N en la planta y evitar la aclimatación fotosintética aumentando también la producción. Ésta hipótesis fue probada en plantas de alfalfa crecidas en verano y en otoño en invernaderos de gradiente térmico para estudiar asimismo la influencia de la estación. La inoculación con diferentes cepas de *Sinorhizobium meliloti* (102F78, 102F34 y 1032GMI) varió la respuesta de la alfalfa al CO_2 elevado, alta temperatura y estación; sin embargo, ninguna de las cepas consiguió evitar la aclimatación fotosintética, especialmente en los tratamientos de alta temperatura. Las plantas crecidas en otoño presentaron mayor producción que en verano, posiblemente debido a que las altas temperaturas de ésta estación afectaron negativamente a la fijación de N_2 induciendo un aporte deficiente de este elemento a la planta. En las dos estaciones las plantas que más producción presentaron fueron las crecidas en condiciones de CO_2 y temperatura elevados. En verano, las plantas que más produjeron fueron las inoculadas con la cepa 102F78, demostrando ser la mejor adaptada a las altas temperaturas. El mayor crecimiento de estas plantas en CO_2 elevado, no se debió a un aumento de la fijación de N_2 o del número de nódulos, sino que posiblemente se debió a un menor consumo de carbono en la fijación biológica de N_2. En otoño, las plantas inoculadas con la cepa 102F34 fueron las más productivas debido a un aumento en la materia seca nodular en condiciones de CO_2 y temperatura elevados. De estos resultados se desprende que la respuesta de la alfalfa nodulada al CO_2 elevado depende de la cepa de *Sinorhizobium meliloti* inoculada y de la estación de crecimiento.

El proceso de la aclimatación fotosintética al CO_2 elevado está ampliamente estudiado en la bibliografía, pero no existe un consenso sobre los parámetros fisiológicos y bioquímicos más sensibles y repetitivos utilizados para detectarla. En el presente estudio se concluye que los parámetros fotosintéticos más adecuados para

detectar la aclimatación bajo las condiciones estudiadas fueron la fotosíntesis medida a concentraciones de CO_2 de crecimiento (A_{growth}) y la medida a 400 μmol mol^{-1} de CO_2 (A_{400}). La velocidad máxima de carboxilación de la rubisco (Vc_{max}) demostró ser menos sensible que la actividad *in-vitro* de la rubisco, posiblemente debido a que este parámetro suele subestimar el dato real en condiciones de temperatura elevada. El descenso del contenido foliar de N es otro buen indicador de aclimatación fotosintética al CO_2 elevado. Sin embargo, en el presente estudio, sólo descendió en las plantas crecidas en verano, estación en la que las plantas se mostraron mas aclimatadas. Otro parámetro que fue sensible en la detección de la aclimatación fue la disminución del contenido de subunidad grande (RLS) y pequeña (RSS) de la rubisco. Este descenso posiblemente fue el causante de la caída de la actividad rubisco y, por lo tanto, de la aparición de la aclimatación fotosintética. Por otro lado, la caída en el contenido de rubisco, no se vio acompañada por un descenso en la expresión génica de ambas subunidades, no existiendo correlación entre expresión y contenido. Esto podría deberse a la existencia de un retraso entre la expresión y la traducción de la proteína, lo que debería tenerse en cuenta a la hora de seleccionar la hora de las cosechas o muestreos en este tipo de estudios en alfalfa.

En el presente trabajo, se estudió así mismo el efecto del CO_2 en la fotosíntesis, contenido y expresión génica de rubisco en plantas de alfalfa noduladas a lo largo del día para comprobar la existencia del retraso anteriormente comentado y determinar la hora de cosecha más adecuada para detectar la aclimatación fotosintética. Las plantas crecidas en CO_2 elevado mostraron aclimatación fotosintética durante todo el día. Observándose un aumento de la fotosíntesis al mediodía en las plantas crecidas en CO_2 ambiente, debido a la mayor intensidad de luz y temperatura, que no fue seguida por la plantas de CO_2 elevado, siendo esta la hora a la que más aclimatación fotosintética se detectó. Esta caída de la fotosíntesis estuvo relacionada con el descenso en el contenido de las dos subunidades de rubisco a esa hora, especialmente con la subunidad pequeña (RSS), considerada limitante de la fotosíntesis. El descenso en el contenido de rubisco no se asoció con una caída en la expresión génica, no observándose correlación entre el contenido y la expresión. Las plantas crecidas en CO_2 ambiente presentaron un aumento de la expresión génica de ambas subunidades al principio de la noche, pudiendo ser este pico el que explicaría el aumento en el contenido de rubisco al mediodía. En las plantas

crecidas en CO_2 elevado no se da este aumento al inicio de la noche. Esto podría explicar el descenso en el contenido de las dos subunidades de rubisco al mediodía. Por lo tanto, el presente trabajo demuestra la existencia de un desfase horario de larga duración entre la expresión y contenido de la rubisco en alfalfa. Asimismo, se estimó que el mediodía era la mejor hora para detectar la aclimatación fotosintética tanto desde el punto de vista fotosintético como de contenido de rubisco.

Como se viene observando, en el presente estudio, el CO_2 elevado y la alta temperatura afectaron a la bioquímica de la alfalfa. Un posible efecto descrito en la bibliografía es el descenso en la calidad y digestibilidad de la alfalfa como forraje para la alimentación animal cuando se crece en condiciones de CO_2 y temperatura elevados. La aplicación de estos dos tratamientos en combinación o por separado, aumentaron el contenido de fibras en las plantas inoculadas con las cepas 102F78 y 1032GMI, descendiendo su digestibilidad. Sin embargo las plantas inoculadas con la cepa 102F34 no aumentaron su contenido en fibras, por lo que se alteró la digestibilidad de estas plantas. De acuerdo con la bibliografía, se consideró a la proteína cruda (CP) como el mejor indicador de calidad de forraje. El CO_2 y la temperatura elevados descendieron el contenido en CP, y por tanto, la calidad del forraje de las plantas inoculadas con cualquier cepa, siendo las plantas inoculadas con la 102F34 las que presentaron una caída más acusada. Teniendo en cuenta los resultados obtenidos, se relacionó negativamente la calidad con la producción de parte aérea. Las plantas inoculadas con la cepa 102F78 fueron las que mejor balance entre calidad y producción presentaron. Sin embargo, la mayor digestibilidad de las inoculadas con 102F34 las hizo más adecuadas para la producción de forraje en una situación de CO_2 y temperatura elevados.

CONCLUSIONES GENERALES

Conclusiones Generales

1. Las plantas de alfalfa noduladas, exclusivamente fijadoras de N_2 presentaron aclimatación fotosintética. El suministro de N por parte de los nódulos fue insuficiente para satisfacer la demanda originada por el CO_2 elevado. Las plantas de alfalfa regadas con dosis elevadas de NH_4NO_3 no presentaron aclimatación fotosintética, confirmándose así que el nitrógeno es un nutriente clave para la respuesta de las plantas al CO_2 elevado.

2. El CO_2 elevado afectó negativamente al metabolismo nodular, mostrando descensos en el contenido de enzimas claves como la citrato sintasa. La disminución en nitrogenasa reductasa y transportador ABC, encargado retransportar los compuestos nitrogenados de la bacteria a la célula del nódulo, podrían indicar un aporte insuficiente de N hacia la planta.

3. La inoculación con distintas cepas de *Sinorhizobium meliloti* resultó en diferentes respuestas en la producción, dependiendo de las condiciones de CO_2 elevado, alta temperatura o diferentes épocas del año. Las plantas inoculadas con la cepa 102F78 fueron las mas productivas en verano, demostrando ser las mejor adaptadas a las altas temperaturas. Sin embargo, en otoño, las plantas más productivas fueron las inoculadas con la cepa 102F34.

4. Otoño fue la época más productiva, debido a las temperaturas más suaves. En verano, las altas temperaturas pudieron afectar negativamente la fijación de N_2 reduciendo el aporte de N a la planta y limitando el crecimiento.

5. A la hora de detectar la aclimatación fotosintética, los parámetros más sensibles fueron la fotosíntesis medida a concentraciones de CO_2 de crecimiento y la fotosíntesis medida a 400 μmol mol^{-1}. La actividad *in-vitro* de la rubisco resultó ser un parámetro mas sensible que la velocidad máxima de carboxilación de la rubisco.

6. El intercambio de carbono neto (fotosíntesis y respiración) se correlacionó con el contenido de subunidad grande y pequeña de la rubisco, pero no con la expresión

génica de estas dos subunidades a lo largo del día. Se observó un retraso de larga duración entre la expresión de las subunidades de la rubisco y su síntesis proteica.

7. El contenido en fibras se vio incrementado por el CO_2 elevado y la alta temperatura en las plantas inoculadas con las cepas 102F78 y 1032GMI provocando el descenso de la digestibilidad del forraje. El aumento de la producción en condiciones de CO_2 y temperatura elevados, se relacionó negativamente con la calidad del forraje. Las plantas inoculadas con la cepa 102F34 se consideraron las más adecuadas para su crecimiento en condiciones de alto CO_2 y temperatura debido a su mayor producción y digestibilidad.