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Increased photosynthetic acclimation in alfalfa associated with arbuscular mycorrhizal fungi (AMF) and cultivated in greenhouse under elevated CO_2

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ABSTRACT

Medicago sativa L. (alfalfa) can exhibit photosynthetic down-regulation when grown in greenhouse conditions under elevated atmospheric CO_2. This forage legume can establish a double symbiosis with nitrogen fixing bacteria and arbuscular mycorrhizal fungi (AMF), which may increase the carbon sink effect of roots. Our aim was to assess whether the association of alfalfa with AMF can avoid, diminish or delay the photosynthetic acclimation observed in previous studies performed with nodulated plants. The results, however, showed that mycorrhizal (M) alfalfa at the end of their vegetative period had lower carbon (C) discrimination than non-mycorrhizal (NM) controls, indicating photosynthetic acclimation under ECO_2 in plants associated with AMF. Decreased C discrimination was due to the acclimation of conductance, since the amount of Rubisco and the expression of genes codifying both large and small subunits of Rubisco were similar or slightly higher in M than in NM plants. Moreover, M alfalfa accumulated a greater amount of soluble sugars in leaves than NM plants, thus favoring a down-regulation effect on photosynthetic rates. The enhanced contents of sugars in leaves coincided with a reduced percentage of arbuscules in roots, suggesting decreased sink of carbohydrates from shoots to roots in M plants. The shorter life cycle of alfalfa associated with AMF in comparison with the NM controls may also be related to the accelerated photosynthetic acclimation in M plants. Further research is needed to clarify to what extent this behavior could be extrapolated to alfalfa cultivated in the field and subjected to periodic cutting of shoots under climatic change scenarios.

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Introduction

The level of atmospheric CO_2 has been constantly increasing since the industrial revolution. This atmospheric CO_2 increase is expected to double within the next hundred years (Watson et al., 1990). The enhanced CO_2 concentration increases the potential net photosynthesis in C3 plants (Drake et al., 1997) and therefore can improve growth and above-ground biomass production (Oliveira et al., 2010) over short-term exposures. CO_2 exchange rates would depend mainly on how the enzyme Rubisco, which catalyzes the fixation of CO_2 into photosynthetic metabolism, acclimates or adjusts in high CO_2 environments. In alfalfa, the positive response of plants to CO_2 is not maintained over the long term, and photosynthesis declines (Aranjuelo et al., 2009; Erice et al., 2006a). This process, referred to as down-regulation or some degree of photosynthetic acclimation, may be due to stomatal limitations resulting from lower stomatal conductance at elevated CO_2 (ECO_2) concentrations (Sánchez-Díaz et al., 2004) and/or to decreased activity and/or amount of Rubisco under ECO_2 (Aranjuelo et al., 2005; Erice et al., 2006b; Sanz-Sáez et al., 2013). When the synthesis of carbohydrates in plants exposed to ECO_2 exceeds the capacity to produce new sinks, their photosynthetic rate declines as a consequence of a product feedback inhibition (Thomas and Strain, 1991).

Roots and their microbial symbionts are sinks for photoassimilates. Thus, symbionts associated with plant roots may reduce the

Abbreviations: *A*, net photosynthesis; ACO_2, ambient CO_2; AMF, arbuscular mycorrhizal fungi; DM, dry matter; ECO_2, elevated CO_2; *g*, stomatal conductance; LDM, leaves dry matter; M, mycorrhizal; NDM, nodule dry matter; NM, non-mycorrhizal; *RbcL*, Rubisco large subunit gene expression; *RbcS*, Rubisco small subunit gene expression; RDM, root dry matter; RLS, Rubisco large subunit content; RSS, Rubisco small subunit content; SDM, shoot dry matter; TSP, total soluble proteins; TSS, total soluble sugars; $\delta^{13}C$, ^{13}C isotopic composition.

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magnitude of CO_2-induced acclimation (Fitter et al., 2000). Mycorrhizal fungi colonize the roots of over 90% of plant species mostly to the mutual benefit of both plant host and fungus (Smith and Read, 2008). The most common are the arbuscular mycorrhizal fungi (AMF), which are formed by the majority of plants, including alfalfa. AMF may significantly influence host plant physiology in a high carbon dioxide world because decreased concentrations of phosphorus (P) in tissues of plants grown under ECO_2 can be alleviated by the increased uptake of P by mycorrhizal roots (Cavagnaro et al., 2011). Moreover, AMF can improve nitrogen (N) nutrition of host plants, which can be determinant to photosynthesis under ECO_2. In fact, acclimation of photosynthesis to ECO_2 has been reported to be more pronounced when plants are N-limited (Sanz-Sáez et al., 2010). In legumes, AMF may exert an additional effect on the N nutrition because, once mycorrhizal symbiosis is functional, the rate of N_2 fixation in nodules can be improved (Mortimer et al., 2008).

The objective of our study was to assess whether the association of alfalfa with AMF can avoid, diminish or delay the photosynthetic down-regulation previously observed in nodulated alfalfa cultivated in greenhouse under ECO_2.

Materials and methods

Experimental design

Seeds from alfalfa (*Medicago sativa* L. cv. Aragón) were surface disinfected in a 0.1% (w/v) $HgCl_2$ solution for 10 min, washed with sterile water to remove any trace of chemicals and placed in Petri dishes to germinate. One week later, seedlings were transferred to 2 L pots (four plants per pot) filled with a mixture of vermiculite–sand–light peat (2.5:2.5:1, v:v:v). Peat (Floragard, Vilassar de Mar, Barcelona, Spain) was previously sterilized at 100 °C for 1 h on three consecutive days. At transplanting, half of the plants (15 pots) were inoculated (13 g per pot) with the mycorrhizal inoculum AEGIS Endo Gránulo commercialized by Atens (Tarragona, Spain) (M plants). The inoculum was a mixture of *Rhizophagus intraradices* (Schenck and Smith) Walker & Schüβler comb. nov. and *Funneliformis mosseae* (Nicol. and Gerd.) Walker & Schüβler comb. nov. that contained around 100 spores and other infective propagules (mycelium, spores and roots) per gram of product. A filtrate was added to pots (15 pots) that did not receive the mycorrhizal inoculum (NM plants) in an attempt to restore other soil free-living microorganisms accompanying AMF. For each pot, the filtrate was obtained by passing 13 g of mycorrhizal inoculum in 20 mL of distilled water through layer 15–20 μm filter papers (Whatman, GE Healthcare, UK). During the first month after transplanting, all plants were inoculated three times with *Sinorhizobium meliloti* 102F78 (The Nitragin Co. Milwaukee, WI, USA) maintained on yeast extract mannitol agar (YEMA; 0.5 g L^{-1} K_2HPO_4, 0.2 g L^{-1} $MgSO_4 \cdot 7H_2O$, 0.1 g L^{-1} NaCl, 10 g L^{-1} mannitol, 0.4 g L^{-1} yeast extract and 15 g L^{-1} agar in distillate water, pH 6.8–7.0).

Plants were grown at 25/15 °C (d/n) temperatures, 50/85% (d/n) relative humidity and received natural daylight supplemented with irradiation from sunlight-type Osram HQI-TS 400 W/D (Osram GmhH, Augsburg, Germany) that provided a minimum photosynthetic photon flux of around 500–600 μmol m^{-2} s^{-1} during a 14 h photoperiod. Four weeks after transplanting, three non-inoculated (NM) pots and three pots inoculated with AMF (M) were harvested (first harvest, five-week old plants) for determining mycorrhizal establishment. At that time (four weeks after transplanting, Erice et al., 2006a,b), the remaining pots were divided into two groups: (1) non-mycorrhizal (NM) (six pots) and mycorrhizal (M) (six pots) plants cultivated at ambient (ACO_2; set at 395 μmol mol^{-1}, measured experiment mean 395 ± 20 μmol mol^{-1}) CO_2 concentration and (2) NM (six pots) and M (six pots) plants subjected to elevated (ECO_2; set at 700 μmol mol^{-1}, measured experiment mean 710 ± 22 μmol mol^{-1}) CO_2 concentration. Atmospheric CO_2 concentrations were constantly monitored by using a 'Guardian Plus Infra-Red Gas Monitor' (Edinburgh Instruments Limited, Livingston, UK). CO_2 treatments were switched weekly between the greenhouses and the pots were randomized within treatments to eliminate the edge effects. Two harvests were performed after starting different atmospheric CO_2 treatments: two weeks later (second harvest, seven-week-old plants) and four weeks later (third harvest, at the end of the vegetative period, nine-week old plants). All plants were alternately irrigated with 200 mL of distilled water and 200 mL of nutrient solution (Goicoechea et al., 1997). Water was added to avoid salt accumulation and ensure optimal irrigation. The basal nutrient solution was N-free so that the N source was mainly relied on N_2 fixation.

Growth parameters and mycorrhizal analyses

Dry matter (DM) of leaves (L), shoots (S), roots (R) and nodules (N) was determined after drying plant material into the oven at 80 °C until weight was constant. Root samples were cleared and stained (Phillips and Hayman, 1970) and mycorrhizal colonization was determined by examining 1 cm root segments (n = 45 per pot) under the microscope. Results were expressed as percentage of infection (Hayman et al., 1976). The presence or absence of arbuscules was also assessed as the ratio between root segments showing arbuscules and total roots showing any mycorrhizal structure; results were expressed as percentages.

Biochemical determinations in leaves and roots

Starch, total soluble sugars (TSS) and total soluble proteins (TSP) analyses were carried out with 200 mg of fresh leaves and roots that were ground in an ice-cold mortar and pestle containing potassium phosphate buffer (50 mM, pH 7.0). The homogenates were filtered through four layers of cheese cloth and centrifuged at 28,710 × g at 4 °C for 15 min. The supernatant was collected and stored at 4 °C for TSS and TSP determinations. The pellet was used to determine starch that was estimated after iodine reaction (Jarvis and Walker, 1993). TSS in leaves and roots were analyzed with the anthrone reagent in a U-2001 spectrophotometer (Hitachi Instruments, Inc., USA) (Yemm and Willis, 1954). Leaf and root TSP were measured by the protein dye-binding method of Bradford (1976) using bovine serum albumin (BSA) as a standard.

Carbon (C) isotopic composition ($\delta^{13}C$), %C and %N and the C/N ratio of the leaf dry matter

Nine-week-old plants were harvested (3rd harvest) and the collected plant material was dried in an air-forced oven at 60 °C until weight was constant. Then samples were analyzed for isotopic composition ($\delta^{13}C$) together with %C, %N and C/N ratio. One milligram of ground sample was used for each determination, and six replicates were analyzed for each treatment. The $^{13}C/^{12}C$ ratio of plant material were conducted at the Serveis Cientifico-Tècnics, University of Barcelona (Barcelona, Spain) using an elemental analyzer (EA1108, Series 1, Carbo Erba Instrumentazione, Milan, Italy) coupled to an isotope ratio mass spectrometer (Delta C, Finnigan, Mat., Bremen Germany) operating in continuous flow mode (Nogués et al., 2008).

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 vapor and oxygen from gas samples were removed and the carbon dioxide, argon, and nitrogen

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