Elevated CO_2 concentrations alter nitrogen metabolism and accelerate senescence in sunflower (*Helianthus annuus* L.) plants

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ABSTRACT

Elevated CO_2 concentrations were found to cause early senescence during leaf development in sunflower (*Helianthus annuus* L.) plants, probably by reducing nitrogen availability since key enzymes of nitrogen metabolism, including nitrate reductase (NR); glutamine synthetase (GS) and glutamate dehydrogenase (GDH), were affected. Elevated CO_2 concentrations significantly decreased the activity of nitrogen assimilation enzymes (NR and GS) and increased GDH deaminating activities. Moreover, they substantially rose the transcript levels of GS1 while lowering those of GS2. Increased atmospheric CO_2 concentrations doubled the CO_2 fixation and increased transpiration rates, although these parameters decreased during leaf ontogeny. It can be concluded that elevated atmospheric CO_2 concentrations alter enzymes involved in nitrogen metabolism at the transcriptional and post-transcriptional levels, thereby boosting mobilization of nitrogen in leaves and triggering early senescence in sunflower plants.

Keywords: leaf development; GS isoforms; transcript levels

Leaf senescence is a key developmental step in the life of annual plants. During this senescence process, cells undergo drastic metabolic changes and sequential degeneration of cellular structures, mainly chloroplasts. The main function of leaf senescence is the recycling of nutrients, especially nitrogen remobilization, which affects the nitrogen availability (Lim et al. 2007). Agüera et al. (2010) showed that leaf senescence in sunflower plants is accelerated by nitrogen deficiency. Research in this area focused on obtaining new cultivars capable of facing the changing climatic conditions on the grounds that elevated CO_2 concentrations affect nitrogen assimilation (Bloom et al. 2010). The rise might be mitigated by crop plants, where photosynthesis converts atmospheric CO_2 into carbohydrates and other organic compounds. The extent of this mitigation remains uncertain, owing to the complex relationship between carbon and nitrogen metabolism in plants (Reich et al. 2006). Elevated levels of atmospheric CO_2 inhibit photorespiration in C3 plants increasing their photosynthetic efficiency, since carboxilation capacity of ribulose-1-5-biphosphate carboxylase/oxygenase (Rubisco) enzyme is not saturated by the current CO_2 concentration (Drake et al. 1997). Moreover, root absorption of NO_3^- and NH_4^+ from the soil and assimilation of NO_3^- and NH_4^+ into organic nitrogen compounds within plant tissues strongly influence primary productivity in plants. The assimilation of NO_3^- involves the sequential conversion of NO_3^- into NO_2^-, then into NH_4^+, through sequential reactions catalyzed by nitrate reductase (NR) and nitrite reductase (NiR), respectively. NH_4^+, the end-product of NO_3^- reduction, is assimilated by glutamine synthetase (GS) (Bernard and Habash 2009). Two different isoforms of GS were indentified

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in leaves, namely: cytosolic GS1 and chloroplastic GS2 (McNally and Hirel 1983). The GS1 and GS2 isoenzymes are differently regulated within specific cell types and organs, and in response to different developmental, metabolic and environmental cues (Zozaya-Hinchliffe et al. 2005). In addition to GS, other enzymes play key roles in maintaining carbon and nitrogen balance. Thus, GDH catalyzes the reversible amination/deamination between 2-oxoglutarate and glutamate. Its physiological role in nitrogen metabolism, however, is controversial (Forde and Lea 2007). Although some evidences suggest that glutamate dehydrogenase (GDH) plays a role in NH_4^+ assimilation, many other indicate that GDH functions primarily as a deaminating enzyme (Lea and Miflin 2003).

The purpose of this work was to study the effect of elevated atmospheric CO_2 concentration, on sunflower (*Helianthus annuus* L.) leaf senescence, with special emphasis on nitrogen metabolism enzymes (NR, GS and GDH) and the expression of GS1 and GS2 transcripts during leaf ageing.

MATERIAL AND METHODS

Sunflower (*H. annuus* L.) isogenic cultivar HA-89 (Semillas Cargill SA, Seville, Spain) plants cultivated in a growth chamber with a 16 h photoperiod (400 μmol/m^2/s of photosynthetically active radiation), a day/night regime of 25/19°C and 70/80% relative humidity. Plants were irrigated daily with a nutrient solution containing 10 mmol KNO_3 (Hewitt 1966). Plants were grown under the above-described conditions for 8 days and then transferred to different controlled-environment cabinets (Sanyo Gallenkam Fitotron, Leicester, UK) fitted with an ADC 2000 CO_2 gas monitor. The plants were kept under ambient CO_2 levels (400 μL/L) or elevated CO_2 concentration (800 μL/L) for another 34 days. High-purity CO_2 was supplied from a compressed gas cylinder (Air Liquid, Seville, Spain). Samples of primary leaves aged 16, 22, 32 or 42 days, were collected 2 h after the start of the light photoperiod. Whole leaves were collected and pooled in two groups: one was used to measure dry weight (DW), and the other was immediately frozen in liquid nitrogen and stored at –80°C. The frozen plant material was ground in a mortar pre-chilled with liquid N_2 and the resulting powder distributed into small vials that were stored at –80°C until enzyme activity and metabolite determinations. Net CO_2 fixation rate, transpiration and stomatal conductance were measured 2 h after the start of photoperiod in attached leaves, using a model CRS068 portable infrared gas analyzer (IRGA) with CIRAS software (USA). Gas exchange rates were determined under 400 μL/L or 800 μL/L CO_2 levels. The instrument was adjusted to maintain 150 cm^3/min constant flow, 25°C temperature, 80% relative humidity and 400 μmol/m^2/s lighting inside the leaf chamber. Measurements were made on primary leaves (16, 22, 32 and 42 days) after the IRGA stabilization period, using several plants per treatment. Leaf samples were acclimated in the leaf chamber for 5–10 min and the measurements were carried out during the following 3–5 min. For total organic C and N content determinations, leaves were ground with an Eppendorf grinder (Retsch MM301, New York, USA). Prior to analysis, the samples were dried at 70°C for 24 h. Approximately 3 mg of tissue was weighed into tin foil containers (2 × 5 mm) and analyzed for C and N on a CHN elemental analyzer (Interscience CE instruments, 11110 CHNS-O, EURO EA, Saint Nom, France). Frozen material was homogenized with chilled extraction medium (Agüera et al. 2006). The homogenate was centrifuged at 8 000 × *g* at 4°C for 2 min, and enzyme activities were measured immediately using the cleared extract. NR (EC 1.6.6.1) activity was assayed in the absence of Mg^{2+} to determine total NADH-NR activity, as described by Agüera et al. (2006). GS (EC 6.3.1.2) activity was measured with the transferase assay according to De la Haba et al. (1992). GDH (E.C. 1.4.1.2) deaminating activities were determined spectrophotometrically according to Loyola-Vargas and Sánchez de Jiménez (1984).

Total RNA from primary leaves was purified using the Tri-Reagent (Sigma Aldrich, St. Louis, USA), following the manufacturer's instructions. Total RNA (2.5 μg) was treated with DNAase (RQ1 RNAase-Free

Gene	Accesion number	Organism	Primers sequences (3'- 5')	
Actin	FJ487620	*Helianthus annuus*	agggcggtctttccaagtat	Forward primer
			tggtacgaccactggcataa	Reverse primer
GS1	AF005032	*Helianthus annuus*	ccaaagcctattcctggtga	Forward primer
			caaacacccgatcacaacag	Reverse primer
GS2	AF005223	*Helianthus annuus*	cttgaccctaagcccattga	Forward primer
			ggtttccgcaagtaatcctg	Reverse primer

DNase, Promega) and used to generate first-strand cDNA by Reverse Transcriptase III (Invitrogen) using Oligo dT primer, in a total volume of 20 µL. The cDNA was appropriately diluted and the PCR reactions were done using the specific primers listed in the table below. The identity of the amplified fragments was verified by sequenciation.

Expression analysis was carried out by semi-quantitative PCR using GoTaq Flexi DNA polymerase (Promega). Expression levels were normalized using the expression of the housekeeping gene actin as internal control. Gene expression levels were determined by image analysis using Quantity One, version 4.6.3 (BioRad, California, USA) after gel electrophoresis of the PCR products, and referred to the level of expression of actin gene in the same sample.

Values are given as the means ± SD of duplicate determinations from three separate experiments. All results were statistically analyzed using the Student's *t*-test and they were conducted at a significance level of 5% ($P < 0.05$).

RESULTS AND DISCUSSION

Available evidence indicates that high atmospheric CO_2 concentrations during leaf ontogeny alter the activity and expression of some enzymes that play a key role in the nitrogen metabolism (NR, GS and GDH) (Figure 1) in sunflower plants. In fact, plants grown under elevated CO_2 concentration exhibited a significant ($P < 0.05$) lower NR (Figure 1a) and GS activities (Figure 1b) than those grown under ambient atmospheric CO_2 conditions, throughout development. Stitt and Krapp (1999) initially assumed that some plant species will require an increased rate of nitrate assimilation to support an increased plant growth under elevated CO_2 concentrations. However, CO_2 enrichment was shown to inhibit NO_3^- assimilation in wheat and *Arabidopsis* plants (Bloom et al. 2010). NO_3^- assimilation is powered by the reduced form of nicotinamide adenine dinucleotide (NADH). Photorespiration boosts the release of malic acid from chloroplasts and increases the

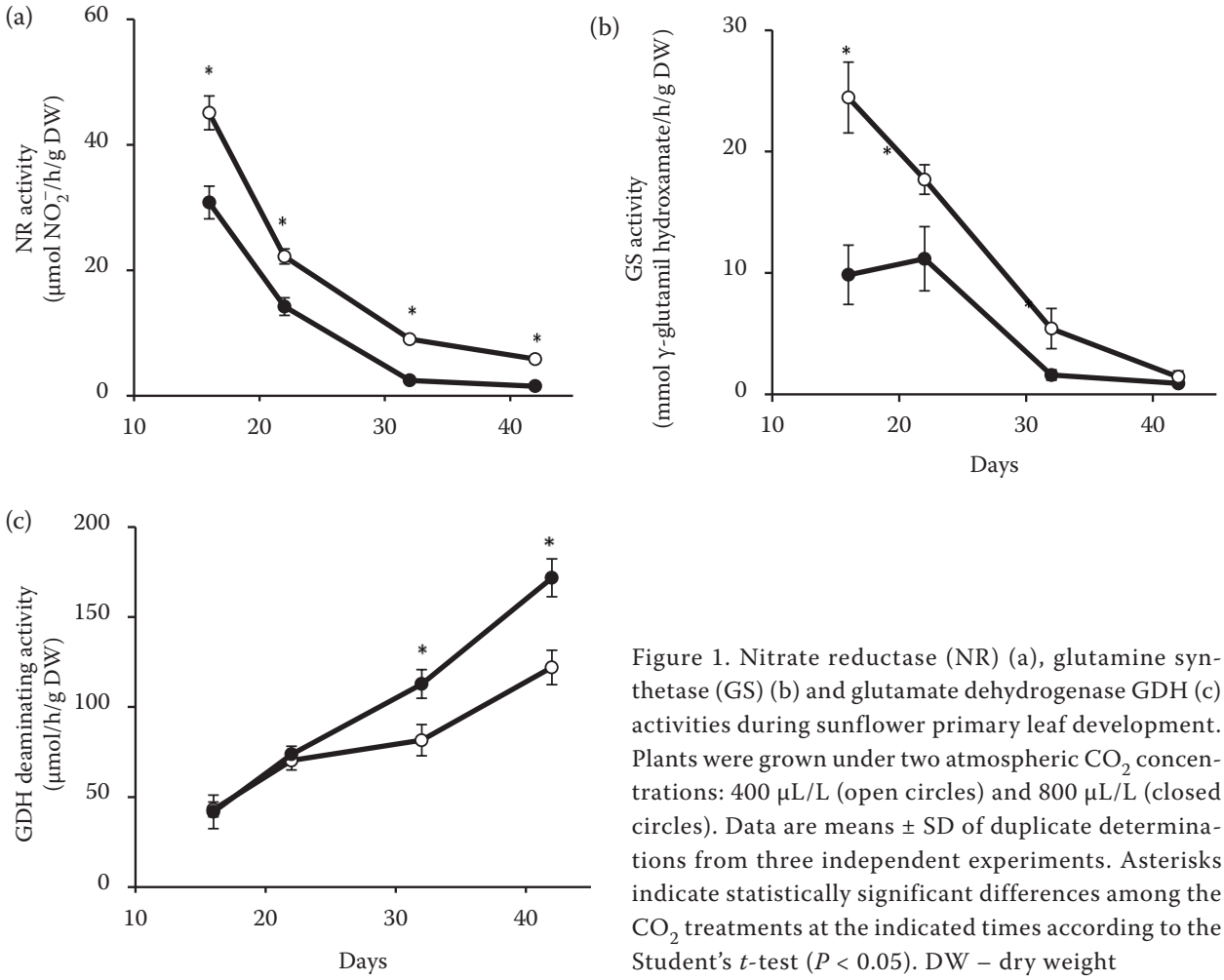

Figure 1. Nitrate reductase (NR) (a), glutamine synthetase (GS) (b) and glutamate dehydrogenase GDH (c) activities during sunflower primary leaf development. Plants were grown under two atmospheric CO_2 concentrations: 400 µL/L (open circles) and 800 µL/L (closed circles). Data are means ± SD of duplicate determinations from three independent experiments. Asterisks indicate statistically significant differences among the CO_2 treatments at the indicated times according to the Student's *t*-test ($P < 0.05$). DW – dry weight

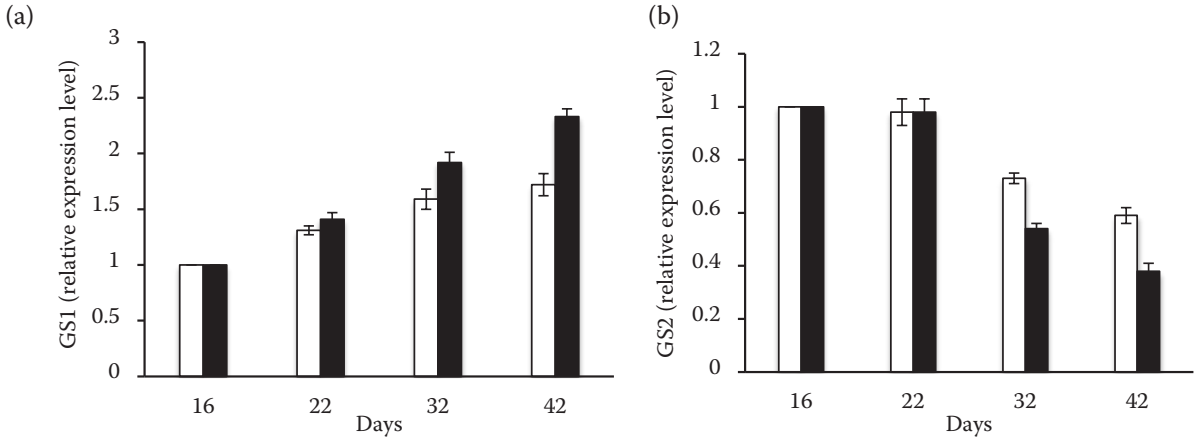

Figure 2. Changes in GS1 (a) and GS2 (b) relative expression level during sunflower primary leaf development. Plants were grown under two atmospheric CO_2 concentrations: 400 μL/L (white bars) and 800 μL/L (black bars). Data are means ± SD of duplicate determinations from three independent experiments. Asterisks indicate statistically significant differences among the CO_2 treatments at the indicated times according to the Student's *t*-test ($P < 0.05$)

availability of cytoplasmic NADH (Igamberdiev et al. 2001), which enables the first step of NO_3^- assimilation (Quesada et al. 2000). Elevated CO_2 atmospheric concentrations reduce photorespiration and thereby diminish the amount of NADH available to power NO_3^- reduction, which may account for the decreased levels of NR activity observed in sunflower plants grown under elevated CO_2 (Figure 1a). On the other hand, six transporters of the *Nar1* family are involved in NO_2^- translocation from the cytosol into the chloroplast in *Chlamydomonas*, and some of these transport both NO_2^- and HCO_3^- (Mariscal et al. 2006). Bloom et al. (2002) showed that HCO_3^- inhibits NO_2^- influx into isolated wheat and pea chloroplasts, indicating that an analogous system is operating in higher plants. A decreased NO_2^- influx into the chloroplast might therefore be the result of increased CO_2 levels, which may also account for the reduced ($P < 0.05$) GS activity observed in sunflower plants grown under elevated CO_2 concentrations (Figure 1b). Studies have shown that both the chloroplastic and the cytosolic isoforms of GS are affected by abiotic stress (Bernad and Habash 2009). Our results indicate that elevated CO_2 atmospheric concentrations significantly increase ($P < 0.05$) GS1 relative expression (Figure 2a), but decrease ($P < 0.05$) GS2 transcript levels (Figure 2b), in sunflower leaves. During this senescence process, cells undergo drastic metabolic changes and sequential degeneration of cellular structures, starting with the chloroplasts. These organelles play a dual role, as a main source for nitrogen and as a regulator of their own degradation during senescence (Zapata et al. 2005). Increased atmospheric CO_2 levels may boost processes leading to accelerated senescence in sunflower leaves, including dismantling of chloroplasts, where GS2 operates (McNally and Hirel 1983). Several studies have shown that GS1 isoforms are involved in nitrogen remobilization during leaf senescence in grasses (Swarbreck et al. 2011). In C3 plants leaves, the largest part of the NH_4^+ assimilated under ambient CO_2 concentration is originated in the process of photorespiration, rather than from de novo assimilation of NO_3^- or NH_4^+ (Stitt and Krapp 1999) and elevated CO_2 concentration decreases photorespiration (Foyer et al. 2009). NH_4^+ from photorespiration is assimilated by the GS2 isoform (Lam et al. 1996), which agrees with the low levels of GS2 transcripts found in leaves of the plants grown under elevated CO_2 concentrations relative to the control (Figure 2b). On the other hand, GDH deaminating activity (Figure 1c) peaked in senescent leaves (42 days) with both treatments; activity values after 22 days were significantly higher ($P < 0.05$) at the elevated CO_2 concentration (Figure 1c). Lea and Miflin (2003) showed that GDH worked primarily in the deamination reaction leading to the production of NH_4^+ in mitochondria. Therefore, increase in GDH deaminating activity with the increment in CO_2 levels and leaf age was expected. These conditions, which boost nitrogen remobilization, are typical of senescence (Lehmann and Ratajcak 2008). Díaz et al. (2008) found induction of *gdh2* expression and

Table 1. CO_2 fixation rate, transpiration rate, stomatal conductance and C:N ratio during sunflower primary leaf development. Plants were grown under different atmospheric CO_2 concentrations: CO_2 ambient (400 µL/L) and CO_2 elevated (800 µL/L)

Days	CO_2 fixation (µmol $CO_2/m^2/s$)		Transpiration (mmol $H_2O/m^2/s$)		Stomatal conductance (mmol $H_2O/m^2/s$)		C:N ratio	
	ambient	elevated	ambient	elevated	ambient	elevated	ambient	elevated
16	3.0 ± 0.4	6.8 ± 1.0*	3.2 ± 0.3	3.2 ± 0.4	345.3 ± 5.1	305.7 ± 43.0	8.0 ± 0.7	9.0 ± 0.7*
22	3.4 ± 0.2	7.5 ± 1.1*	1.8 ± 0.1	2.3 ± 0.1*	181.0 ± 7.4	208.0 ± 36.9	8.2 ± 0.1	11.6 ± 2.0*
32	2.5 ± 0.5	4.5 ± 0.9*	1.1 ± 0.2	1.9 ± 0.2*	81.8 ± 9.0	160.1 ± 33.7*	11.3 ± 1.1	14.0 ± 1.8*
42	1.2 ± 0.2	3.0 ± 0.4*	1.1 ± 0.1	1.4 ± 0.1*	78.5 ± 2.0	95.1 ± 9.0	12.4 ± 0.9	18.3 ± 1.5*

Data are means ± SD of duplicate determinations from three independent experiments. Asterisks indicate statistically significant differences among the CO_2 treatments at the indicated times according to Student's *t*-test ($P < 0.05$)

GDH activity with ageing in *Arabidopsis*, which suggests that GDH participates in amino acid degradation and nitrogen recycling in this plant.

As can be seen from Table 1, the elevated CO_2 concentrations significantly ($P < 0.05$) increased photosynthetic and transpiration rates. In contrast, stomatal conductance only showed significant differences in 32 days-old leaves, although these parameters decreased during ageing of sunflower primary leaves. The stomatal response to atmospheric changes was extensively studied on a wide variety of species. Although stomata in most species close when the CO_2 concentration rises beyond certain levels, the response of plants to high CO_2 levels varies widely, and some species are even unaffected (Drake et al. 1997, Larios et al. 2004). The absence of a stomatal response to atmospheric CO_2 may be either genetically determined or the result of adaptation to an atmosphere with a high relative humidity (Morison 1998). Larios et al. (2004) found that exposure of sunflower leaves to increasing CO_2 concentrations caused concomitant increases in photosynthetic CO_2 assimilation and soluble sugars and reduction in nitrate content. Elevated levels of atmospheric CO_2 were previously reported to decrease photorespiration rates in C3 plants and to potentially increase their photosynthetic efficiency as a result (Long et al. 2006). In our plants, the elevated CO_2 concentration led to an increased ($P < 0.05$) C:N ratio during ageing of sunflower primary leaves (Table 1). Consequently, an increase in atmospheric CO_2 concentrations alters carbon and nitrogen contents, and leads to a gradual nitrogen limitation by which leaves accumulate carbohydrates faster than the plants can acquire nitrogen, thereby causing the nitrogen contents of leaves to decrease (Reich et al. 2006). Urban et al. (2012) found that elevated CO_2 treatment resulted in decrease of the Rubisco content in *Picea abies*, however, higher proportion of Rubisco are present in its active carbamylated Rubisco forms in comparison to ambient CO_2 plants. In these plants, the Rubisco content linearly correlates with leaf nitrogen content, irrespective of CO_2 concentration treatments. Limited nitrogen availability leads to early senescence and increases the oxidation state of cells in sunflower leaves (Agüera et al. 2010, De la Mata et al. 2012). In addition, according to Schildhauer et al. (2008), the supply of nitrogen can reverse senescence by altering the expression of genes coding for plastidic GS.

In conclusion, elevated atmospheric CO_2 concentrations during leaf development in sunflower (*H. annuus* L.) lead to early senescence through a decrease in nitrogen availability resulting from the effects of key enzymes of nitrogen metabolism on transcriptional (GS1 and GS2) and post-transcriptional levels (NR, GS and GDH).

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