OnLine Journal of Biological Sciences 2 (7) 463-469, 2002
ISSN 1608-4127

Interactive Effects of Nitrogen Starvation and Different Temperatures on Senescence of Sunflower (*Helianthus annus* L.) Leaves Associated with the Changes in RNA, Protein and Activity of Some Enzymes of Nitrogen Assimilation

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Abstract: Interactive effects of N-starvation and different temperatures on senescence of sunflower (*Helianthus annus* L. cv Giza) leaves associated with the changes in RNA, protein and activity of some enzymes involved in N assimilation, photosynthesis and respiration were studied. Sunflower plants were grown in controlled environment in two temperatures regimes in the presence and absence of N. It was found that senescence was initiated at time of full leaf elongation based on declines in total RNA and soluble protein. Removal of N from the growth medium just at the time of full leaf elongation enhanced the rate of senescence and a significant decrease was observed in the amount of RNA, protein and in terms of any indices measured of growth parameters such as leaf area, specific leaf weight and relative growth rate particularly in cool conditions. The net loss of the soluble proteins in the leaves was paralleled by an increase in the total amino acids in cool more than warm conditions. Furthermore, carbohydrates content such as starch, sucrose and glucose were accumulated in N-starved leaves either in cool or warm conditions more than in plants grown in $+NO_3^-$. In addition, on leaf area basis, activity of photosystems I and I+II were some what greater in plants grown under cool than warm conditions and in the presence than in absence of NO_3^-. During natural senescence with adequate plant NO_3^- supply the activity of nitrate reductase (NR) increased and that of glutamine synthetase (GS) and glutamate synthase (GOGAT) was stable or increased, but decreased under N-starvation. On the other hand, the activity of NADH-glutamate dehydrogenase was relatively stable during senescence and was not affected by N starvation indicating that N assimilation could be carried out and might be increased during senescence if N was present and suggesting that proteolytic activity must be regulated during senescence. Furthermore, the activities of phosphoglycolate phosphatase (PGP) and glycolate oxidase (GO) enzymes associated with photorespiration, were decreased to low levels during senescence in the presence of NO_3^- either at 13 or 30 °C and decreased sharply to very small amounts in the absence of NO_3^-, whereas the activity of phosphoenolpyruvate phosphate (PEP) activity was relatively constant in the presence of NO_3^- and declined during senescence when NO_3^- was omitted.

Key words: Sunflower, nitrogen, changes in RNA

Introduction

Nitrogen is considered as one of the most widely distributed elements in nature and in the living organism and as one of the major limiting nutrients in plant growth and development. Inorganic nitrogen sources such as nitrate and ammonia function as substrates for reduction and subsequent incorporation into organic substances such as protein, nucleic acids, enzymes and growth regulators, which are utilized by different pathways for various aspects of the life cycle of plants (Lea *et al.*, 1990; Solomonson and Barder, 1990). Lawlor *et al.* (1987) presented evidence that abundant nitrate fertilization of wheat in the field increases the ratio of photorespiration to photosynthesis, suggesting modifications to photorespiratory metabolism, but it is not clear why or how nitrogen nutrition affects the balance of oxygenation and carboxylation, or alters the capacity of associated metabolic processes. However, the balance between nitrate reduction and assimilation might be varied by environmental conditions and provides a mechanism by which the metabolism and cell composition respond to differences in nitrate supply. On the other hand, imposing N stress at the time of maximum leaf size had the desired effect of enhancing the rate of leaf senescence and had consistent effect of accelerating the decline in the amounts of protein content associated with several metabolic pathways, localized in various cellular compartments (Gepstein, 1988), indicating that nitrogen nutrition could be recognized as an important senescence factor due to the high amount of N that is mobilized from senescing leaves to developing sink tissues such as flowers and fruits (Guitman *et al.*, 1991; Smart, 1994; Crafts-Brander *et al.*,1998). However, as far as plants in the vegetative phase are concerned, very rare studies examined leaf senescence in relation to the demand for nitrogen or nitrogen sink activity. Thus, addition of nitrogen in the correct form and amount, and at a suitable time related to the demands of growth resulted in some modifications in the plant characteristics that optimize the production with economic use of fertilizer.

Furthermore, it is well known that temperature decreases the photosynthetic efficiency as it rises, probably due to the increase in the activity of ribulose diphosphate (RuDP). Crafts-Brander *et al.* (1998) have demonstrated that in wheat plants incubated in the dark, senescence would be more quickly at 35°C than at 25°C, indicating that temperature not only influences enzyme activity but also acts on senescence. Symptoms of rapid senescence induced by moderately elevated temperatures include slow cessation of gas exchange, gradual loss of chloroplast activity and integrity and diminished synthesis and enhanced proteolysis of leaf polypeptides (Ferguson *et al.*,1993; Xu *et al.*, 1995). However, these studies which clearly showed that at higher temperatures, the specificity of Rubisco for oxygen is increased and therefore the carbon flow through the photorespiratory pathway is increased, as well as the capacity of Calvin cycle is decreased as shown in spinach leaves incubated above 20 °C (Feller and Fisher, 1994). Accordingly, temperature environment also must be considered as an additional factor that governs the distribution of both wild and cultivated plants and influences the control of senescence (Herrmann and Feller, 1998) and consequently affects the growth and production of crops. Therefore, it appears reasonable to infer that sink-source and/or demand-supply relationships of nitrogen could regulate leaf senescence. The effects of both temperature environment and nitrogen supply on leaf senescence can be explained in one way if their effects on the nitrogen status of the plants are properly assessed. According to the above mentioned, the objective of this study was to examine the effects of nitrogen starvation and temperature stress on the plant growth and the activities of some enzymes involved in the nitrate metabolism such as nitrate reductase (NR.EC:1.6.6.1), glutamine synthetase (GS.EC:1.4.7.1), glutamate synthase, (GOGAT.EC:6.3.1.2) and NAD-glutamate dehydrogenase (GDH.EC: 1.4.1.2) to show the efficiency and capacity of leaves to assimilate nitrate under these conditions. Activities of enzymes associated with photorespiration such as phosphoglycolate phosphatase (PGP.EC:3.1.3.18), Gylcolate oxidase

(GO.EC:1.1.1.26) and phosphoenolpyruvate carboxylase (PEP,EC: 4.1.1.31) were also studied in order to asses which of these enzymes may place a limit on the maximum photosynthetic rate. Furthermore, the activities of photosystem I and I+II have been estimated to understand the response of plant to the change in the environmental temperature and nitrogen stress.

Materials and Methods

Growth conditions: Seeds of sunflower (*Helianthus annus* L. cv. Giza) were imbibed in warm water for 4 h, transferred to paper towels for 2 days and the germinated seeds were planted in plastic pots filled with vermiculite and grown on 10 April 2001 in growth chambers (located in the Botanical Garden, Faculty of Science, Alexandria University) with a 14 h photoperiod of 750 $m^{-2}s^{-1}$ photosynthetically active radiation (PAR). Light was provided by a mixture of fluorescent and tungsten lamps. The average temperature was 25/20 °C (day/night) and 60-70 % relative humidity. When the second leaves were fully expanded on 28 April 2001 (18 days after imbibition), the plants were divided into 2 groups. The first group and second group was maintained in the growth chamer under temperature regimes 30/26 °C and the second group was maintained under 13/10 °C (day/night) and irrigated with nutrient solution as described by Ono *et al.* (1996). For the control plants: 2.7mM KNO_3, 2.7mM $Ca(NO_3)_2$, 1.0mM $MgSO_4$, 0.89mM NaH_2PO_4, 0.33mM FeNa-EDTA, 6.7 μM $MnSO_4$ 0.67 μM $ZnSO_4$, 33 μM H_3BO_4, 0.33 μM Na_2MoO_4, 0.67 mM NaCl and 0.13 μM $CoSO_4$. For the N-starvation treatment, plants were supplied with the same nutrient solution lacking any nitrogen source. All other nutrients were constant, except that KCl and $CaCl_2$ were added in the $-NO_3^-$ treatment to keep concentration of K^+ and Ca^{2+} constant and to maintain ionic balance between $-NO_3^-$ and $+NO_3^-$ treatments. The replacement did not affect pH of the nutrient solutions. Nutrient solutions were renewed every 2 days. Growth rate of the plant was calculated as relative increase g. g^{-1} DW d^{-1} according to Scheible *et al.* (1997). At the desired time, plants were harvested and separated into roots, stems and leaves and fresh and dry weights were determined. Leaf area was determined by outlining each leaf on paper and measured using electronic planimeter (Delta T Devices, Burwell, UK).

Leaf sampling for biochemical analysis: Leaves were sampled at 0,4,8,12 and 16 days (after full leaf expanded) for each treatment. Nitrogen and carbon contents were analyzed with an elemental organic analyzer (NA 1500, C.E. Italy). Leaf nitrogen and carbon concentrations were expressed as % g^{-1} leaf dry weight and carbon/nitrogen (C/N) ratio was calculated as carbon concentrations divided by leaf nitrogen concentration. Chlorophyll was extracted from 1 cm^2 sections from the middle portion of 10 leaves in 5 ml of acetone. Chlorophyll concentration was determined as described by Holden (1976). Activity of photosystem I and photosystem I+II was measured by the method described by Lawlor *et al.* (1987). Soluble protein and amino acid contents in leaves tissue were extracted as follows: 1g fresh weight was ground in liquid nitrogen with a mortar and pestle, the homogenate was extracted in 1.7 ml extraction in medium containing 100 mM sodium phosphate (pH 7.5), 1% (W/v) polyvinylpolypyrrolidone and 0.1(v/v) β-mercaptoethanol. The crude extract was centrifuged at 15,000 × g for 30 min. The supernatant was used to measure soluble protein according to method of Bradford (1976) using bovine serum albumin as standard. The amount of total amino acids was determined by the ninhydrin method described by (Sugano *et al.*, 1975), using L-leucine as a standard. Total soluble sugars (TSS) were measured according to Irigoyen *et al.* (1992) by reacting 0.5 ml of supernatant with 3 ml freshly prepared anthrone reagent (50 mg anthrone + 80.5 ml H_2SO_4 + 19.5 ml H_2O) and placing in boiling water for 10 min. After cooling, the absorbance at 625 nm was determined. Starch was measured in the pellet according to Murata *et al.* (1968).

Total RNA determination: RNA was extracted from 0.5 to 0.6g of leaves and assayed as described by Camacho-Cristobal and Gonzales-Fontes (1999).

Extraction of enzyme: Leaves (approximately 1g fresh matter) were immediately homogenized using a pestle and mortar in 30 ml of sodium phosphate buffer (pH 7.5) for enzymes extract. Homogenate was centrifuged at 10, 000 × g for 30 min and the supernatant was used directly for the enzymes assays. Extraction was done at 4 °C

Enzyme assays: Nitrate reductase (NR) was assayed *in vitro* following the procedure described by Aslam *et al.* (1976). Glutamine synthetase (GS) was determined at 37 °C, with hydroxylamine as substrate instead of ammonia (pH 7.6). Reaction mixtures were assayed spectrophotometrically at 540nm (Mann *et al.*, 1980). Glutamate synthase (GOGAT) and glutamate dehydrogenase (GDH) activities were measured spectro-photometrically at 25 °C. Oxidation of NADH at 340 nm, was monitored for each enzyme spectro- photometrically as described by Ahmad and Hellebust (1987). Phosphoenol pyruvate carboxylase (PEP) activity was measured at room temperature by following NADH oxidation at 340 nm due to oxaloacetate reduction via malate dehydrogenase (Hatch and Oliver, 1978), Glycolate oxidase (GO) was measured as dye reduction after addition of DCPIP (dichlorophenol indophenol) according to Guerrier (1985). Phosphoglycolate phosphate (PGP) was assayed according to Guerrier (1985).

Statistical analysis: Data were subjected to Student's t-tests to find statistically the significant differences between the different measured parameters of the control and treated plants. All estimates of sample variability are given in terms of the SE.

Results and Discussion

During germination of sunflower seeds, the endosperm supplies sufficient nitrogen and other mineral elements under the optimum growth conditions, thereafter when seedlings used up their food stored material, seedlings were gradually dependent on external nutrient supply and other environmental conditions. The results showed that the effect of interaction between $+NO_3^-$ application and temperature (30 °C) on plant growth was significant in terms of any indices measured such as relative growth rate, area of leaves, specific leaf weight and root length, indicating that $+NO_3^-$ and warm conditions (30 °C) have the largest effect on plant growth (Table 1, 2). In contrast, absence of nitrogen ($-NO_3^-$) and cool conditions (13 °C) caused a strong reduction in each of the chosen growth parameters. Thus, N-starvation seems to be detrimental to the plant, also temperature appears to affect leaf development and senescence, through its effect on plant growth. An unexpected result obtained was the increase in root length when the plants were grown in the absence of NO_3^-. This result could be explained that in the absence of nitrogen, more carbohydrate was accumulated in the leaves either in cool or warm conditions. and a large portion of these constituents might be translocated from leaves to the roots, which were then grown faster and resulted in a decrease in the shoot-to-root ratio. Furthermore, the results of this study showed that C content was higher in the presence of $+NO_3^-$ particularly at 30 °C and remained more or less constant in $-NO_3^-$ either at 13 or 30 °C (Fig.1A). Also, the internal nitrogen content in relation to dry weight of leaves was higher in the presence of $+NO_3^-$ at 30 °C than in plants grown at 13 °C (Fig.1B), probably due to the fast uptake, accumulation and enhanced assimilation of NO_3^-. No significant increment of total N was detected under N-starvation either at 13 or 30 °C. Accordingly, it was found that C/N ratio remained unchanged in the presence of NO_3 over a wide range of temperatures (13–30 °C) (Fig.1C) even when total N is different (Fig.1B), probably due to a regulatory mechanism(s) that prevents further drop in the C/N ratio. Moreover, nitrogen

Table 1: Effect of the interaction between N-starvation and different temperatures on the growth parameters of sunflower plant. Plants were harvested at 0 and 8th day after full expanded second leaf. Leaf area are given for the whole plant. Data are means ± SE (n = 10)

		Leaf area (cm^2 $plant^{-1}$)		Specific leaf weight ($mg\ cm^{-2}$)		Relative growth rate ($g.\ g\ DW^{-1}d^{-1}$)	
Time (days)	Temperature (°C)	$+NO_3^-$	$-NO_3^-$	$+NO_3^-$	$-NO_3^-$	$+NO_3^-$	$-NO_3^-$
0	25	110 ± 4.6	110 ± 4.6	4.2 ± 0.4	4.2 ± 0.4	ND	ND
8	13	548 ± 12.6	120 ± 8.2	5.1 ± 0.6	5.0 ± 0.9	0.12 ± 0.01	0.12 ± 0.01
	30	855 ± 19.0	190 ± 6.9	9.0 ± 0.7	8.5 ± 0.9	0.17 ± 0.01	0.12 ± 0.01

ND, not determined

Table 2: Effect of the interaction between N-starvation and different temperatures on organs dry weights (mg dw. $plant^{-1}$) of sunflower plant. Plants were harvested at 0 and 8th day after full expanded leaf

Time (days)	Day/night Temp. (°C)	NO_3^- treatment	Leaves	Stem	Total Shoot	Roots	Root length (cm $plant^{-1}$)	Total Plant	Sh/Rt ratio
0	25/22	$+NO_3^-$	13.1±1.4	14.2±1.1	27.4	28.7±1.9	14.2± 0.9	56.1	0.95
		$+NO_3^-$	26.2±1.2	24.9±2.1	51.1	25.4±2.2	16.0±1.2	76.5	2.0
8	13/10	$-NO_3^-$	16.4±1.8	18.5±2.5	34.9	31.4±1.1	20.1± 1.1	66.3	1.1
		$+NO_3^-$	39.5±3.1	24.7±1.1	64.2	34.8±2.2	19.2±2.1	99.0	1.9
	30/26	$-NO_3^-$	29.3±2.1	23.7±2.2	53.0	41.3±1.5	24.8±1.6	94.3	0.6

Data are means ± SE (n=10), Sh: shoot system, Rt: root system.

Table 3: Effect of the interaction between N-starvation and different temperatures on the carbohydrate content of starch (St), total soluble sugars (TSS) and total carbohydrates (TC) from mature, fully-developed leaves of sunflower plant. Plants were harvested at 0 and 8th day after full expanded leaf. Data are means ± SE (n=3)

			Carbohydrate (glucose equivalents) (mg g^{-1} f.wt)		
Time (days)	NO_3 treatment	Temperature (°C)	St	TSS	TC
0	$+NO_3^-$	25	144.8±4.5	32.6±0.9	177.4±4.4
8	$+NO_3^-$	13	136.8±0.5	27.8±1.8	163.6±2.2
		30	110.9±7.7	18.2±1.1	129.1±3.9
	$-NO_3^-$	13	175.6±3.3	74.8±5.9	250.8±3.6
		30	218.4±5.8	58.2±2.7	276.2±2.4

St= starch, TSS=Total soluble sugars, Tc=Total carbohydrates

Table 4: Effect of the interaction between N-starvation and different temperatures on total RNA, soluble protein and amino acids content in the second leaves of sunflower plant. Data are means ± SE (n = 4)

		Total RNA (mg g^{-1} F.W.)		Soluble protein (mg g^{-1} F.W.)		Amino acids (mg g^{-1} F.W.)	
Temp. (°C)	Time/days	$+NO_3^-$	$-NO_3^-$	$+NO_3^-$	$-NO_3^-$	$+NO_3^-$	NO_3^-
13	0	1.20±0.04	1.20±0.04	34.2±1.2	34.2±1.1	52±2.8	52 ±2.8
	2	0.94±0.03	0.80±0.06	29.9±0.6	26.3±0.6	132±2.2	86 ±1.213
	4	0.79±0.02	0.56±0.02	28.1±0.4	21.4±0.5	164±7.1	106±1.6
	6	0.65±0.01	0.38±0.01	22.2±0.8	16.2±0.8	210±8.2	172±2.8
	8	0.58±0.01	0.24±0.01	20.4±0.2	13.9±0.2	208±3.1	170±3.2
30	0	1.20±0.04	1.20±0.04	34.2±1.2	18.2±0.2	52±2.8	52±2.8
	2	0.86±0.02	0.71±0.02	26.2±0.7	22.9±0.5	102±4.6	81±1.4
	4	0.69±0.10	0.43±0.03	25.4±0.6	16.4±0.8	111±6.4	84±2.4
	6	0.58±0.01	1.29±0.02	20.8±0.4	12.8±0.2	107±4.2	78±3.6
	8	0.53±0.01	0.17±0.01	18.2±0.2	10.9±0.2	103±2.1	67±1.8

Table 5: Effect of the interaction between N-starvation and different temperatures on the chlorophyll (*a+b*) content, chlorophyll *a*/*b* ratio, activity of photosystems I and I+II and chlorophyll / protein ratio in leaves of sunflower plant. Data are means ± SE (n = 5)

	Temperature (°C)			
	13		30	
Parameters	$+NO_3^-$	$-NO_3^-$	$+NO_3^-$	$-NO_3^-$
Chl *a* + *b* (g cm^{-2})	0.78±0.02	0.54±0.02	0.54±0.05	0.34±0.02
Chl *a* / *b* ratio	4.60±0.06	4.30±0.11	4.50±0.09	4.20±0.07
PSI (μmol $O_2 m^{-2} s^{-1}$)	16.20±0.51	11.50±0.93	14.30±0.34	9.90±0.61
PSI+II (μmol $O_2 m^{-2} s^{-1}$)	27.40±0.33	18.80±1.23	22.10±0.80	15.10±1.12
Total chl/protein ratio	2.10±0.12	2.10±0.46	2.00±0.25	2.10±0.42

starvation decreased the growth of plants amounted to 9.2 and 12.9 g DW $Plant^{-1}$ at 13 and 30 °C respectively. In contrast, the growth of NO_3^- supplied plants was increased markedly particularly at 30 °C (Fig. 1D), probably due to the higher net carbon assimilation rate (Ashraf, 1999).

Total soluble sugar and starch content: (Table 3) showed that in the absence of nitrogen, the carbohydrate content was much higher in the leaves either in cool or warm conditions. Also, accumulation of soluble sugar in the leaves was higher at 13 than 30 °C. In contrast, in the presence of NO_3^-, soluble sugar and starch were significantly decreased in the leaves particularly in

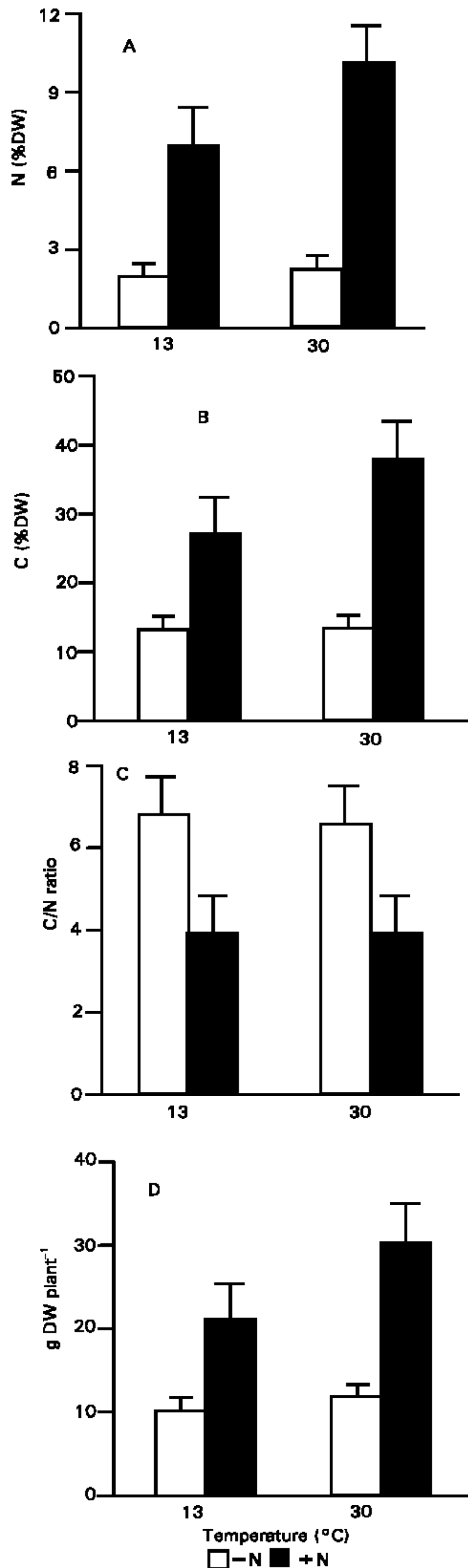

Fig.1: Effect of the interaction between N-starvation and different temperature on total N (A); total carbon (B), C/N ratio (c) and dry weight (D) of sunflower leaves. Values are mean ± SE(n = 5)

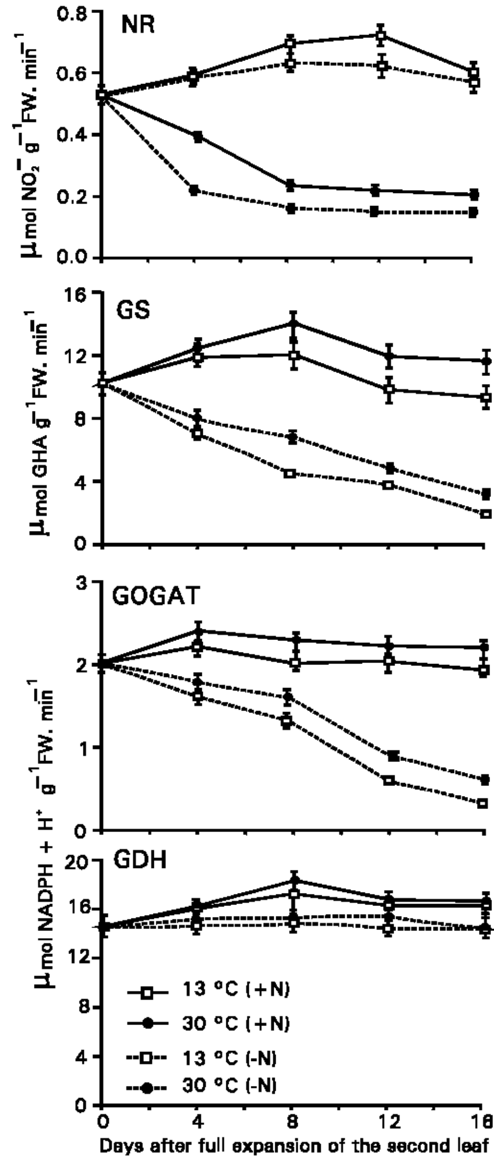

Fig. 2: Effect of interaction between N-starvation and different temperatures on the activity of nitrate reductase (NR), glutamine synthetase (GS), glutamate synthase (GOGAT) and glutamic dehydrogenase (GDH) enzymes in leaves of sunflower plant grown at 30 and 13 °C in the presence and absence of NO_3^-. Values are mean ± SE.

warm conditions, due to its consumption in NO_3^- assimilation. These, results were consistent with those reported by Crawford (1995) and Scheible *et al.* (1997) who found that total soluble sugar increased and starch decreased under cool conditions and in agreement with the result of Kaiser (1997) who found that in presence of NO_3, the total amount of structural carbohydrate remains constant whereas sugar and starch contents decreased markedly. Also, accumulation of soluble sugar except starch in the leaves was higher at 13 than 30 °C

RNA, soluble protein and amino acids content: Over the time course beginning at full expanded leaves of control (which is an

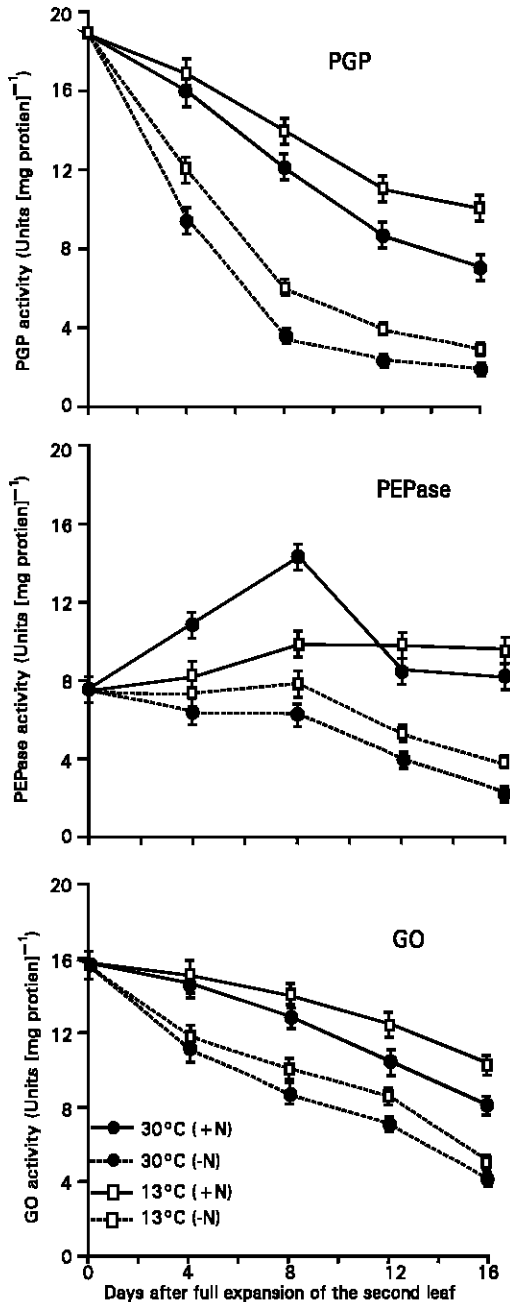

Fig. 3: Effect of the interaction between N starvation and different temperatures on the activity of phosphoenolpyruvate carboxylase (PEPase); phosphoglycolate phosphate (PGP) and glycolate oxidase (GO) enzymes in leaves of sunflower plants grown at 30 and 13 °C in the presence and absence of NO_3^-. PEPase and PGP activity units = μmol NADH min^{-1}. GO activity units = μmol glycolate oxidized min^{-1}. Values are mean ± SE.

indication of the onset of senescence process) there is a decrease in the total RNA and soluble protein (Table 4). For the plants grown in absence of -NO_3^-, the decrease in these constituents started earlier and the rate of decrease was faster than in the presence of nitrogen particularly in the early senescence, indicating that N-starvation enhanced the rate of leaf senescence. In the plants grown either with or without nitrogen, total RNA and soluble protein were decreased faster in warm than in cool conditions. According to Crafts–Brander *et al.* (1996), the effects of N-starvation and different temperatures might extend to the other metabolic pathways localized in various cellular compartments and associated with the decrease in amounts of proteins and increase in the rate of leaf senescence. As the leaves aged, soluble protein content decreased slightly in +NO_3 and markedly in –NO_3^- in the cool conditions (13 °C). The net loss of soluble proteins in the leaves was paralleled by an increase in the content of total amino acids. During senescence the total free amino acids content was substantially greater in cool conditions than in warm and with NO_3^- than without probably due to the decrease in the rate of protein synthesis (Table 4) as reported by Lawlor *et al.* (1987) who found that the rate of protein synthesis was decreased by about 35% in cool conditions. The results (Table 4), showed that in warm conditions and in the presence of NO_3^- the total amino acid content almost remained more or less constant. However, the relationship between total soluble amino acids and nitrate supplied depends on (1) the uptake and reduction of nitrate, (2) the rate of amino acids synthesis and (3) the rate of their consumption in protein synthesis.

Chlorophyll content and photosystem activity: Total chlorophyll content (*a+b*) per unit leaf area was much greater with nitrogen than without (Table 5) and was substantially greater in cool than warm conditions, suggesting that cool conditions may lead to greater remobilization of nitrogen occurred from thylakoid structure and other parts of the cells until late senescence. However, the Chl a/b ratio was not significantly affected by temperature or addition of nitrate. Furthermore, in the present study (Table 5), it was found that photosystem I activity per unit of leaf area was greater in the presence of NO_3 than in –NO_3^-, particularly in cool conditions. Also, PSI+II was larger in plants grown in cool growth conditions and presence of nitrogen than those grown in warm conditions and absence of NO_3^-. This is consistent with the results of Lawlor *et al.* (1987). The ratio of chlorophyll content /soluble protein was more or less similar under all conditions used In this connection, Evans and Terashima (1987) found that chlorophyll content was closely related to the soluble protein over all treatments and the ratio of chlorophyll/protein was found to be relatively constant until the end of senescence. Generally, N-starvation and temperature could be considered as environmental signals which directly affect leaf senescence and modify protein and pigment considerably (Smart, 1994).

Enzymes activity: At all times during the experiment, leaf nitrate reductase (N R), glutamine synthetase (GS), glutamate synthase (GOGAT) and glutamate dehydrogenase (GDH) enzymes associated with nitrate assimilation clearly responded positively to the variation in the temperature and presence of nitrate probably due to the effects of these factors on the synthesis of these enzymes. The response to presence or absence of NO_3^- was almost similar at low temperature and variable at high temperature (Lawlor, *et al.*, 1987). NR activity in leaves showed a significant response to the interaction between temperature, nitrogen level and leaf age. In this study, the maximum values were obtained in cool conditions either nitrogen was added or omitted, although NR enzyme is inducible by NO_3^-, suggesting that cool conditions stimulate the development of NR activity even in –NO_3^-. In contrast, the smallest values were obtained in warm condition and particularly in the absence of NO_3^- (Fig. 2). Furthermore, the increase in NR activity (which is rate limiting for nitrate reduction) in absence of nitrate, might be due to NO_3^-, which has been already absorbed before from full nutrient solution (control) and in a part due to nitrate transferred from the storage pool under cool conditions back to the metabolic pool in the case of N-starvation and lead to induction and increase of NR activity. Although the literature on the adaptive development of NR enzyme in response to NO_3 application is abundant, the

information of the influence of exogenously supplied NO_3 on the activity of glutamine synthetase (GS), glutamate synthase (GOGAT) and glutamic dehydrogenase (GDH) enzymes involved in the assimilation of ammonium is rare and mostly dealing with the influence of NH_4 rather than of NO_3 feeding. Glutamate dehydrogenase (GDH) also has been considered to play a major role in ammonia assimilation it is considered not to contribute to the reductive amination of 2-oxoglutarate because of its low affinity for NH_4, since GDH is more involved in glutamate degradation. Therefore, an increase in GDH activity is a common phenomenon in many senescing organs and it is usually accompanied by a decrease in GS/GOGAT activity (Lea *et al.*,1990). In these results, GDH activity has been shown to increase under cool or warm conditions in the presence of NO_3^- until 8th day and was almost constant in absence of NO_3^- (Fig. 2) providing evidence that mitochondrial metabolism is maintained throughout the senescence process (Feller and Fisher, 1994; Crafts-Brander *et al.*, 1998). This response is in agreement with Lewis *et al.* (1989), suggesting a role of this enzyme in glutamate catabolism (Crafts-Brander *et al.*, 1996). However, in this study the high level of activities of GS and GOGAT enzymes were obtained in the presence of NO_3 either in cool or warm conditions (Fig. 2) suggesting that the primary amination reaction during NO_3 supply was due to GS and GOGAT activity. As leaves aged, the relative stability of GS and GOGAT activity in control obtained indicated that nitrogen assimilation capability was maintained during senescence via the GS/GOGAT cycle as the major route of NH_4 assimilation in plants and as the principle pathway from which glutamine and glutamate are formed (Lea *et al.*, 1990). On the other hand, the activities of these enzymes were significantly decreased under N-starvation (Fig. 2) probably due to the increase in the amount of glutamine in storage pool size, since GS has a substantially higher affinity for NH_4 and/or due to the decrease in the protein of these enzymes (Feller and Fisher, 1994). Compared to nitrate reductase (NR) activity, GS and GOGAT maintained high activities throughout the life of the leaf in the presence of N (Crafts-Brander *et al.*, 1998). Phosphoglycolate phosphate (PGP) and glycolate oxidase (GO) enzymes associated with photorespiration, were decreased to low levels during natural senescence and in the presence of NO_3 either at 13 or 30°C and decreased in the absence of NO_3^- (Fig. 3). Also, phosphoenolpyruvate caboxylase (PEP) was relatively constant in the presence of NO_3^- in cool conditions, whereas in warm conditions (30°C) the activity of PEPase increased between 0 and 8 days after full leaf elongation and declined thereafter. Under N-starvation, the pattern of the activity was similar either at 13 or 30°C within 8 days after full leaf elongation, then activity declined at the end of experiment (Fig. 3).

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