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Nitrogen Modulates the Diurnal Regulation of Nitrate Reductase in Wheat Plants – Projections Towards Climate Change

R. Morcuende, P. Pérez, R. Martínez-Carrasco and E. Gutiérrez

Institute of Natural Resources and Agrobiology of Salamanca, IRNASA–CSIC, Apartado 257, 37071 Salamanca, Spain; e–mail: rosa.morcuende@irnasa.csic.es

Abstract: This study investigates whether the diurnal regulation of nitrate reductase activity in the flag leaf of wheat is affected by combined increases of CO_2 and temperature in the air and to ascertain whether the nitrogen supply modifies these effects. Spring wheat was grown at ambient (360 μmol mol^{-1}) or elevated (700 μmol mol^{-1}) CO_2, under ambient and 4°C warmer temperatures, and with two levels of nitrogen supply in field temperature gradient chambers. At ear emergence, NR activity reaches a maximum in the early part of the light period and declines later in the light period and during the first part of the night. Although elevated CO_2 did not increase NR activity, it led to a modification of the diurnal regulation. During the last part of the photoperiod the decline of the activity was faster in plants grown in ambient CO_2, in which the accumulation of amino acids was higher. The maximum reached in the first hours of the light period in plants grown in elevated CO_2 and nitrogen abundance was related to a higher accumulation of soluble carbohydrates. The dark inactivation of NR was prevented in plants grown in elevated CO_2 with low nitrogen. Additionally, the higher decline of NR activation in plants grown with ample nitrogen supply and higher temperatures was related to the accumulation of amino acids. It is concluded that nitrogen plays a role in the activity and post-translational regulation of NR under the future climatic scenario.

Key words: carbohydrates, diurnal rhythm, elevated CO_2, nitrate reductase, nitrogen, temperature, wheat

INTRODUCTION

Due to anthropogenic activities the concentration of carbon dioxide in the atmosphere is increasing and is projected to double from its current level by the end of this century. As a consequence, the earth´s mean annual surface temperature is also rising and is predicted to increase as much as 1.5–4.5ºC within this century (Schneider, 2001). Both CO_2 and temperature are key factors affecting photosynthesis, plant growth and development, and their predicted changes will have a significant impact on plant productivity.

The atmospheric CO_2 enrichment initially improves carbon fixation by plants. However, in the long term plants growing in elevated CO_2 often show an acclimatory down-regulation of the photosynthetic capacity of leaves (Drake et al., 1997; Pérez et al., 2005; Martínez-Carrasco et al., 2005) characterized by a reduction in the amount and activity of ribulose–1,5–bisphosphate carboxylase –Rubisco– (Drake et al., 1997), which is often associated with decreased expression of genes encoding the small sub-

unit of Rubisco (Drake et al., 1997), mediated by the increased level of carbohydrates in leaves under elevated CO_2 or low nitrogen contents (Riviere-Rolland et al., 1996; Geiger et al., 1999; Pérez et al., 2005).

Nitrate reductase –NR– catalyses the first reaction in the pathway of nitrate assimilation and is regulated by a hierarchy of sophisticated mechanisms leading to changes in transcription, post-translational modification and protein turnover (Scheible et al., 1997a). The expression of the enzyme is induced by nitrate and sugars (Krapp et al., 1993; Vincentz et al., 1993), and repressed by glutamine or closely related metabolites (Vincentz et al., 1993). Elevated CO_2 leads to an accumulation of carbohydrates (Drake et al., 1997; Pérez et al., 2005) and, as long as the rate of nitrate assimilation and amino acid synthesis are regulated in response to changes in the availability of carbohydrates, an increase of the enzyme activity and gene expression under elevated CO_2 concentrations may be expected. However, there is no consistent evidence for an increase of NR activity in elevated CO_2. Although CO_2 enrichment led to a small increase of NR activity (Fonseca et al., 1997; Geiger et al., 1998), sometimes it has also decreased (Ferrario-Mery et al., 1997; Geiger et al., 1999). The anomalous decline of NR activity could be explained because plants growing under such conditions might become nitrate limited, and nitrate is required for induction of gene expression for the enzyme (Crawford, 1995; Scheible et al., 1997a). Alternatively, it could be a consequence of preferential assimilation of ammonium under elevated CO_2 conditions, resulting in formation of glutamine and repression and/or post–translational inactivation and degradation of NR (Scheible et al., 1997a; Morcuende et al., 1998). Moreover, NR is subject to diurnal changes in the level of transcripts and activity (Scheible et al., 1997a), and any change in the level of carbohydrates and nitrogen metabolites could play a role in its regulation (Scheible et al., 1997a). It could be hypothesized that the increases of atmospheric CO_2 concentration and temperature predicted with climate change could lead to changes in the pool of such metabolites and it is quite likely that the diurnal regulation of NR will be modified.

The purpose of the present study was to assess whether NR activity and its diurnal changes in flag leaves of wheat are affected by combined increases in atmospheric CO_2 and temperature and to ascertain whether nitrogen supply modifies these effects, as long as the response to enhanced CO_2 depends on nitrogen availability. With this objective the NR activity and the amount of amino acids were determined. The leaf carbohydrate content previously reported by Pérez et al. (2005) is also considered. The flag leaf at ear emergence was selected for this study as a stage when acclimation to elevated CO_2 is more likely than in younger plants and the ear provides an active sink for assimilates. In order to approximate the natural environment of Mediterranean wheat crops, this experiment was conducted in the field under temperature gradient chambers.

MATERIALS AND METHODS

Plant cultivation

This field experiment was conducted in a clay-sand soil located at the farm of the CSIC Institute of Natural Resources and Agrobiology, in Salamanca, Spain (41°N, 800 m above sea level). The climate corresponds to a Mediterranean type.

Spring wheat (*Triticum aestivum* L cv. Alcázar) was sown at a rate of 180 kg ha^{-1} and 0.13 row spacing on 13 February. Before sowing, N (as NH_4NO_3), P and K fertilizers (80, 40 and 40 kg ha^{-1}, respectively) were applied. The crop was watered weekly through a drip irrigation system providing amounts of water equivalent to the average rainfall in this area during the period of the experiment (198 mm between February and June).

After seedling emergence, two temperature gradient chambers (Pérez et al., 2005), based on those described by Rawson et al. (1995), were mounted over the crop on 23 March. One chamber was kept at the ambient air CO_2 concentration (360 μmol mol^{-1}) and another at 700 μmol mol^{-1} (elevated CO_2) by injecting pure CO_2 at the two inlet fans during the light hours. The temperature difference between the extreme modules in a chamber was set at 4°C. Additional (40 kg ha^{-1}) nitrogen was added to one of the longitudinal halves of each chamber 34 days after sowing, such that two levels of this nutrient (80 and 120 kg ha^{-1}) were compared. The samplings were repeated in four consecutive sections within the two module halves.

On day 3 after the beginning of ear emergence in the whole experiment (22 May) ear emergence was advanced about 3 days by warm temperatures – flag leaves (two per replicate) were harvested and immediately plunged into liquid nitrogen just before dawn, 4–6 h later, 1–2 h before dusk and 2–3 h into the dark period; light intensities were < 10, 1700, 100 and < 10 μmol m^{-2} s^{-1}, respectively.

Nitrate reductase activity and amino acids analyses

Nitrate reductase activity in the absence or presence of 10 mM Mg^{+2} was determined as in Scheible et al. (1997a) with either 5 mM EDTA or 10 mM magnesium acetate in the assay buffer. The activation state of the enzyme is given by the ratio of its activity in the presence and the absence of 10 mM Mg^{+2} multiplied by 100%.

Frozen leaf subsamples (100 mg fresh weight) stored in liquid nitrogen were extracted three times in 1 ml 80% ethanol with 10 mM Hepes–KOH pH 7.5 at 80°C for 30 min and the extracts were pooled. Then, the residue was extracted three times in 1 ml water at 80°C for 30 min and the extracts were pooled. Amino acids were analysed in the ethanol extracts according to Hare (1977). The same extract was also used to measure the soluble carbohydrates as previously reported (Pérez et al., 2005), while the residue from the ethanol water extractions was used for the assay of starch (Pérez et al., 2005).

Experimental design and statistical analyses

Analyses of variance were performed as in a nested design according to Snedecor & Cochran (1967), with temperature and nitrogen as a stratum included in CO_2 and replicates as a stratum included in that for temperature and nitrogen. Time effects were evaluated by including the hour of the day as a further stratum in the analysis as described by Pérez et al. (2005).

RESULTS

The study of the diurnal changes of NR activity, a key enzyme in the control of nitrate assimilation rate, together with the amount of carbohydrates and amino acids,

will help in understanding the mechanisms involved in the diurnal regulation of the enzyme in the flag leaf of wheat three days after ear emergence in plants grown in the field under temperature gradient chambers.

To measure total NR activity, magnesium was omitted from the assay – see material and methods (Figs. 1A, B). In general, total NR activity showed a maximum during the first hours of the light period and declined during the rest of the photoperiod and in the first hours of darkness. In plants grown with ample nitrogen supply the NR activity rose by the end of the night at both air CO_2 concentrations and in plants grown with low nitrogen supply at higher temperatures the activity was slightly increased at the end of the night in ambient CO_2 and slightly decreased in elevated CO_2 while the reverse was true for those grown at ambient temperatures. CO_2 enrichment did not increase the NR activity 4–6 h into the light period as compared to plants grown in ambient CO_2 and decreased NR at the end of the light period and the end of the night.

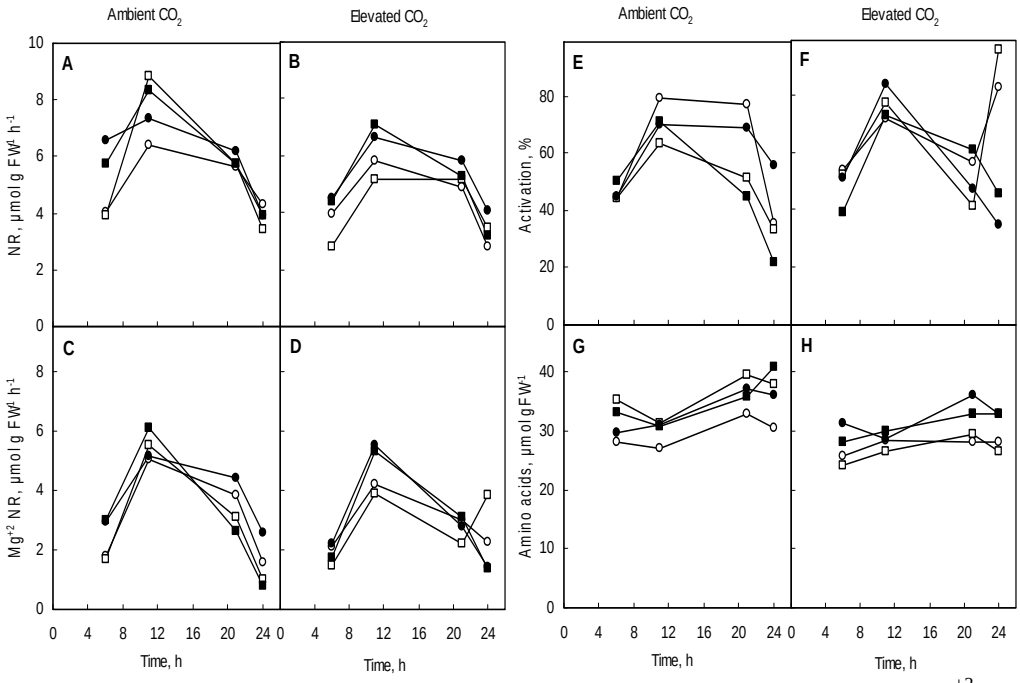

Figure 1. Diurnal changes in NR activity in the presence and absence of Mg^{+2}, its activation state and the amino acid contents of flag leaves of wheat in response to CO_2, temperature and nitrogen 3 days after ear emergence. Plants were grown in the field under temperature gradient chambers at either ambient (360 µmol mol^{-1}) or elevated (700 µmol mol^{-1}) CO_2, ambient (circles) or ambient + 4°C (squares) temperatures and 80 (open symbols) or 120 (closed symbols) kg nitrogen h^{-1}. Light intensities before dawn, 4–6 h later, 1–2 h before dusk and 2–3 h into the dark period were < 10, 1700, 100 and < 10 µmol m^{-2} s^{-1}, respectively. Each point is the mean of four replicates.

In plants grown at ambient CO_2 the decline of the activity at the end of the photoperiod was faster than in those grown in elevated CO_2. With a high nitrogen supply, the activity of the enzyme was higher at the end of the night, and in elevated CO_2, was also higher during the first hours of light. High temperatures drastically decreased the NR activity at the end of the photoperiod in ambient CO_2 as well as in

elevated CO_2 in plants with an ample nitrogen supply. In plants grown at ambient CO_2 and low nitrogen supply the elevation of temperature increased the activity during the first hours of the light period.

The activity of the enzyme was also analysed in the presence of 10 mM Mg^{+2} and showed a similar pattern of diurnal changes as the activity in the absence of magnesium in plants grown in ambient CO_2 and in elevated CO_2 combined with ample nitrogen supply. In plants grown in elevated CO_2, with low nitrogen supply the activity decreased by the end of the night, while at high temperatures NR increased in the first hours of darkness. In plants grown in elevated CO_2, nitrogen abundance increased the activity 4–6 h into the light period (Fig. 1D). In plants grown in ambient CO_2, NR in the presence of magnesium (Fig. 1C) was higher at the end of the night with high than low nitrogen supply.

Two hours into the dark period, low nitrogen increased the activation state of NR in plants grown at elevated, but not at ambient CO_2. The loss of NR activation at the end of the light hours was lower in plants grown in ambient than elevated CO_2. High temperatures decreased the activation state of the enzyme in plants grown in ambient CO_2 and nitrogen abundance.

In general, the amino acid contents in plants grown in ambient CO_2 (Fig. 1G) slightly decreased after the onset of the light period, accumulated to high levels along the rest of the photoperiod, and then gradually decreased during the night. In elevated CO_2 (Fig. 1H), at high temperatures the amino acids rose during the photoperiod and then slightly decreased in the night; at ambient temperatures with low nitrogen the level of amino acids was hardly modified during the whole diurnal cycle while with nitrogen abundance slightly decreased after 4–6 h into the light period, rose during the rest of the light hours and then decreased in the night. With nitrogen abundance the level of amino acids increased in plants grown in elevated CO_2 (Fig. 1H). In ambient CO_2, higher temperatures increased the amino acid content in low nitrogen during the whole diurnal cycle, while with nitrogen abundance it was only increased in the night.

As previously shown by Pérez et al. (2005), the levels of glucose, fructose and fructans increased during the first part of the light period and decreased thereafter. Sucrose increased from the end of the dark period to the first part of the day, remained at this level until the end of the light period and then declined during the first hours of the night. Starch increased throughout the light period and was mobilised at night. Elevated CO_2 significantly increased glucose and fructans during the dark period. CO_2 enrichment also increased fructose, sucrose and starch during the day and night. The higher carbohydrate levels at the end of the night under elevated CO_2 suggest that the extra carbohydrate accumulated during the day was not fully mobilised during the dark period. High temperatures decreased the amount of fructose, except in plants at elevated CO_2 and low nitrogen and, in the light hours, they decreased sucrose levels. High temperatures also decreased starch contents in plants with ample nitrogen in the light hours and, in plants with ample nitrogen and elevated CO_2, also 2 h after the start of the night.

DISCUSSION

A hierarchy of transcriptional, post-transcriptional and post-translational regulations adjust the activity of NR to the plant nitrogen status (Crawford, 1995). In

higher plants NR is rapidly and reversibly modulated by light/dark transitions, the inactivation occurring by phosphorylation of a serine residue of the protein followed by binding of an inhibitory 14–3–3 protein (Bachmann et al., 1996; Moorhead et al., 1996).

In the present study NR activity shows a diurnal rhythm in the presence and absence of magnesium, reaches a maximum in the early part of the light period and declines later in the light period and during the first part of the night, in agreement with studies with another plant species (Scheible et al., 1997a; Geiger et al., 1998; Sicher, 2001). Although elevated CO_2 did not increase NR activity, it led to a modification of the diurnal regulation during the last part of the photoperiod. Geiger et al. (1998) showed that elevated CO_2 does not significantly increase NR activity at the daily maximum after 3–4 h illumination, but instead modifies the diurnal regulation of NR in the leaf. Galangau et al. (1988) suggested that the decline of NR activity at the end of the photoperiod in tobacco and tomato leaves grown under ambient CO_2 conditions cannot be solely due to decreased transcription, because a similar decrease occurs in transformants that are over-expressing the *Nia* gene under the control of the constitutive 35S promoter (Vincentz & Caboche, 1991). The decline was related to a feedback mechanism by accumulation of leaf metabolites. Scheible et al. (1997a) associated the decline of NR activity with a decrease of nitrate and accumulation of amino acids, in particular glutamine, during the last part of the photoperiod. Our results are consistent with such suggestion, as long as the decline of the activity was faster in plants grown at ambient CO_2 concentrations, in which the accumulation of amino acids was higher (Fig. 1G). It has been shown that glutamine is the amino acid which accumulates in higher amounts in the second part of the photoperiod (Scheible et al., 1997a; Geiger et al., 1998; Sicher, 2001), and either glutamine or related downstream metabolites repress NR (Vincentz et al., 1993).

The higher NR activity at the end of the night in plants grown with ample nitrogen supply was related to a gradual decline of amino acids during the night (Figs. 1G, H) and further recovery of the level of nitrate and NR transcripts (Scheible et al., 1997a). This is consistent with the known induction by nitrate of the expression of the enzyme (Crawford, 1995). The maximum activity reached in the first hours of the light period in plants grown in elevated CO_2 and nitrogen abundance was related with a higher accumulation of soluble carbohydrates, in special, glucose and sucrose (see Pérez et al., 2005). Sugars increase the expression (Krapp et al., 1993; Vincentz et al., 1993), activity (Morcuende et al., 1998) and post–translational activation of NR (Kaiser & Huber, 1994; Morcuende et al., 1998).

The dark inactivation of NR was prevented in plants grown in elevated CO_2 with low nitrogen (Fig. 1F), suggesting that plant nitrogen status modulates post-translational regulation of this enzyme under CO_2 enrichment. It has been shown that glutamine increases the post-translational inactivation and degradation of NR (Morcuende et al., 1998) and a lower level of glutamine will prevent the inactivation of NR during the first hours of light. Some evidence allows us to associate NR inactivation in elevated CO_2 with the level of this amino acid. First, in plants grown in elevated CO_2 with low nitrogen the amino acid levels in the last part of the light period and first hours of dark were either hardly modified, or slightly increased, to decline afterwards (Fig. 1H). Consequently, a lower accumulation of glutamine is likely under such conditions, and may result from NH_4NO_3 fertilization and the elevated CO_2

enhancement of ammonium assimilation (Geiger et al., 1999). Second, plants grown in the mentioned conditions accumulated high amounts of storage carbohydrates, fructans and starch, while the level of the rest of sugars did not increase, as shown by Pérez et al. (2005). Scheible et al. (1997b) showed that the transcript level of the ADPglucose pyrophosphorylase gene (*AgpS*) was repressed by nitrate, which is also a negative signal for fructans biosynthesis (Morcuende et al., 2004). These results suggest a low level of nitrate leading to lower synthesis of amino acids and glutamine accumulation. In addition, the higher decrease of NR activation in plants grown with ample nitrogen supply and 4ºC above ambient temperatures is consistent with a higher accumulation of amino acids in such conditions, and the post-translational NR inactivation in the dark was associated with an increase of glutamine (Morcuende et al., 1998).

CONCLUSION

Our study shows that atmospheric CO_2 enrichment modifies the nitrogen metabolism in the flag leaf of wheat at ear emergence and nitrogen supply modulates the post-translational regulation of NR in elevated CO_2, as long as prolonged growth at elevated concentrations of CO_2 leads to lower nitrogen status of the plants. However, further investigations will be necessary to improve our understanding of the biochemical and molecular mechanisms involved in the response of wheat crops to the future climatic scenario.

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