Effects of water deficits on the activity of nitrate reductase in leaves and roots of sunflower and white lupin plants growing under two nutrient supply regimes

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Abstract

The effects of a two weeks soil drying period on the activity of nitrate reductase (NR; EC 1.6.6.6) were studied on *Helianthus annuus* L. and non-nodulated *Lupinus albus* L. plants, growing under two nutrient supply regimes. NR activity was assessed in leaf and root extracts, by measuring the activity of the unphosphorylated active form (NR_{act}), the maximal extractable activity (NR_{max}) and the activation state. To get insight into potential signalling compounds, nitrate, amino acids and soluble sugars concentrations were also quantified. On both species, foliar NR_{act} and NR_{max} were negatively affected by soil drying and reduced supply of nutrients, the observed changes in NR activity being linearly-correlated with the depletion of nitrate. Similar results were obtained in the roots of sunflower. Conversely, in white lupin roots NR_{max} was found to be independent of tissue nitrate concentration. Regardless of the species and organ, the activation state of the enzyme was unaffected by the nutrient supply regime. In well-watered sunflower roots only about 50% of the existing NR was unphosphorylated, but the activation state increased significantly in response to drought. In contrast, lupin roots always exhibited NR activation state values close to 80% or even higher. At the leaf level, NR activation state was hardly changed in response to soil drying. The contribution of changes in the concentrations of soluble

sugars and amino acids to explain the observed variations in NR activity are discussed.

Abbreviations

FW, Fresh weight; NR, Nitrate reductase; NR_{act}, activity of the unphosphorylated form of nitrate reductase; NR_{max}, maximal extractable activity of nitrate reductase

Introduction

The availability of water is among the major limiting factors for plant growth, and drought-induced nitrogen deficiency may have a contributory role to explain the observed growth limitation under water deprivation (Heckathorn et al. 1997). Besides limiting the acquisition by the roots of essential nutrients, including nitrate (BassiriRad and Caldwell 1992), drought may also restrict the ability of the plants to reduce and assimilate nitrogen, due to the inhibition of the activities of enzymes involved in nitrogen metabolism. The cytosolic NADH nitrate reductase (NR; EC 1.6.6.6), the first enzyme in the pathway of nitrate assimilation, is one of the enzymes which activity has been shown to decline in water-stressed leaves of several species, including wheat (Larsson et al. 1989), spinach (Kaiser and Brendle-Behnisch 1991), oat (Kenis et al. 1994), tomato (Brewitz et al. 1996), tobacco (Ferrario-Méry et al. 1998), maize (Foyer et al. 1998, Abd-El Baki et al. 2000), and sunflower (Azedo-Silva et al. 2004). Although nitrate reduction may also occur on the roots (Stöhr and Mäck 2001, Scheurwater et al. 2002), studies on the effects of water deficits on NR activity in roots are rare and have provided conflicting evidence: whereas dehydration has been reported to negatively affect the activity of NR in wheat (Larsson et al. 1989), and sunflower roots (Azedo-Silva et al. 2004), contrasting results were obtained by Abd-El Baki et al. (2000), who found that NR

activity was not affected when maize roots were dehydrated under osmotic stress. These contradictory results highlight the need to pursuit the investigation on the effects of drought on root NR activity, extending those studies to a wider number of species, namely those in which the roots have been suggested to be the predominant site of nitrate reduction, such as temperate legume species (Pate et al. 1979, Pajuelo et al. 2002).

NR is a highly regulated enzyme, its activity being dependent on several internal signals. Nitrate is the primary signal that induces the transcription of NR genes (Crawford 1995, Kaiser et al. 2002), but sugars and reduced nitrogen metabolites also play a role in the regulation of NR activity, the transcription of NR genes being enhanced by sugars (Sivasankar et al. 1997, Klein et al. 2000, Larios et al. 2001), and repressed by glutamine or closed related metabolites (Scheible et al. 1997, Sivasankar et al. 1997). NR is also subjected to post-translational regulation, including inactivation through protein phosphorylation and subsequent Mg^{2+}-dependent binding of inhibitory protein (Kaiser et al. 1999, Kaiser and Huber 2001, Lillo et al. 2004). The regulation of NR by nitrate seems to occur only at the transcriptional level (Li and Oaks 1993, Kaiser and Huber 2001, Kaiser et al. 2002). However, sugars and amino acids have been reported to regulate enzyme activity also at the post-translational level, NR being activated by sugars (Kaiser and Brendle-Behnisch 1991, Morcuende et al. 1998, Kaiser and Huber 2001, Kaiser et al. 2002, Iglesias-Bartolomé et al. 2004, Lillo et al. 2004), and inactivated by end products of nitrogen assimilation, such as glutamine (Scheible et al. 1997, Morcuende et al. 1998).

Taking into account the way NR activity is regulated, the negative effects of water deficits on the activity of this enzyme may result either from decreased NR protein, or decreased activation of the existing protein. Maximal NR activity

(NR_{max}), determined in the presence of excess EDTA and though to reflect NR protein content (Kaiser and Huber 2001), has been found to be unaffected by dehydration in tomato leaves (Brewitz et al. 1996). However, the majority of studies evidenced a strong negative effect of water deficits on NR_{max}, not only in leaves (Larsson et al. 1989, Ferrario-Méry et al. 1998, Foyer et al. 1998, Abd-El Baki et al. 2000, Azedo-Silva et al. 2004), but also in roots (Azedo-Silva et al. 2004). As to the effects of water deficits on the post-translational regulation of the enzyme, no clear picture has emerged so far. Drought-induced phosphorylation-dependent inactivation of NR has been found to occur in some cases (Kaiser and Brendle-Behnisch 1991, Brewitz et al. 1996, Foyer et al. 1998). However, contradictory evidence was provided by other studies, in which NR activation was found to be unaffected by dehydration (Ferrario-Méry et al. 1998, Abd-El Baki et al. 2000), or even to increase (Azedo-Silva et al. 2004). These contradictory results indicate that more work is needed in order to elucidate how the activity of NR is modulated by water deficits.

In the present work, the maximal activity, the activity of the unphosphorylated active form, and the activation state of nitrate reductase were assayed both in roots and leaves of sunflower and white lupin plants subjected to a two-week period of slowly-imposed soil drying and subsequent re-watering. In order to gain insight into potential signalling compounds, nitrate, amino acids, hexose and sucrose concentrations were also measured. With the purpose of evaluating whether the relationship between NR activity and signalling compounds, observed during the water deprivation period, was merely correlative or could be reproduced when those compounds varied by causes other then soil drying, water deficits were imposed to plants growing under two nutrient supply regimes.

Materials and methods

Plant material, growth conditions and sampling

Seeds of *Lupinus albus* L. and *Helianthus annuus* L. were soaked overnight and then sowed in 3 l pots containing a mixture of unfertilised peat and vermiculite (1:1, v:v). The plants (one per pot) were grown in a naturally lit greenhouse, which coolers were set to control maximum temperature in order not to exceed 25ºC. The pots were watered with water for first four days after sowing. Afterwards, water was replaced by modified Hoagland solutions, which concentration was progressively increased until day 21 after sowing, when two nutrient supply regimes were established: no further increase in the concentrations of nutrients occurred in the solutions used to watered the plants referred as subjected to a deficient nutrient supply regime; in contrast, the concentration of nutrients in the watering solutions was increased twice in the case of the adequate nutrient supply regime. In the experiment with sunflower, the plants under an adequate supply of nutrients were watered with a nutrient solution containing 9 m*M* NO_3, as described by Azedo-Silva et al. (2004). In the case of white lupin, the nutrient supply regimes were established using solutions twice diluted when compared to those supplied to sunflower, taking into account that Pate et al. (1979) reported that a 5 m*M* NO_3 regime applied to non-nodulated white lupin plants produced plants whose growth rate and N assimilation rate matched closely those of symbiotic plants.

The onset of water stress imposition took place four weeks after sowing. Water stress imposition was applied by replacing only partially the water lost by evapotranspiration (determined gravimetrically). To achieve an approximately constant rate of soil drying, the percentage of water lost that was replaced by watering was progressively increased (from 25% to 75%) in order to compensate for the increase in transpiring leaf area. In the case of the experiment with sunflower, the water-stressed plants were subjected to a three week-long soil drying period. In the

experiment with white lupin plants, the soil drying period was extended for five additional days, in order to compensate for the lower transpiration rates of lupin compared to sunflower and thus to achieve a similar level of plant water deficit on both experiments. At the end of the soil-drying periods the pots were re-watered up to field capacity. The pots containing the plants referred as well-watered were daily brought to field capacity, throughout the experimental period.

Measurements and sampling of water stressed plants took place in the last day of the soil-drying period (WS), and two days after re-watering (RW). The measurements and sampling of well-watered plants (WW) were undertaken on the day following sampling of water-stressed plants. Because there are marked diurnal changes in the transcriptional and post-translational regulation of NR (Kandlbinder et al. 2000, Stöhr and Mäck 2001), sampling was conducted between the 4^{th} and 5^{th} hour of the photoperiod. Five plants were harvested per treatment. Only fully expanded, non-senescent leaves were sampled, whereas root samples represent pooled samples of the entire root system. Leaf and root samples were quickly frozen in liquid nitrogen, and stored at -80ºC until analysis.

Plant water status

Plant water status was assessed by measuring leaf water potential (Ψ) at the end of the dark period (predawn), using a pressure chamber (PMS Instruments, Corvallis, OR).

Quantification of nitrate, amino acids, soluble protein and sugars

Nitrate and amino acids were extracted, from intact leaf discs and powdered root samples, with 50% (v/v) ethanol, at 80ºC, during 10 min. Nitrate was quantified with a nitrite/nitrate colorimetric assay (Roche Diagnostics, Mannheim, Germany) modified to be used in 96 well plates, as described in Azedo-Silva et al. (2004). Total amino acids (except proline) were quantified by the ninhidrin method, modified in

order to eliminate interference resulting from the presence of sugars in the extracts (Magné and Larher 1992).

Soluble proteins were extracted by homogenizing root and leaf samples with 50 m*M* HEPES containing 0.1% Triton X-100. Proteins were quantified with the Bio-Rad Protein Assay Dye (Bio-Rad, Hercules, CA), using bovine serum albumin as a standard.

Soluble sugars were extracted, from intact leaf discs and powdered root samples, with 80% (v/v) ethanol, at 80ºC, for 20 min. Fructose, glucose and sucrose were quantified using a spectrophotometric enzyme-coupled assay (Stitt et al. 1989).

Extraction and assay of nitrate reductase activity

Frozen samples of leaves and roots were ground to a fine powder in a mortar pre-cooled with liquid nitrogen. NR activity was extracted and assayed as in Long and Oaks (1990), using leupeptin or chymostatin in the extraction buffer to stabilize the enzyme in leaves and roots, respectively. NR activity was measured either in the presence of 5 m*M* EDTA or 10 m*M* magnesium acetate (final concentrations in the respective assays). The activation state of NR was calculated as the activity determined in the presence of Mg^{2+}, which usually reflects the activity of the unphosphorylated active form (NR_{act}), divided by the maximal NR activity measured in the presence of 5 m*M* EDTA (NR_{max}), and is expressed as a percentage (MacKintosh et al. 1995, Ferrario-Méry et al. 1998).

Data analysis

Statistical analysis and graphic display were performed with SigmaStat (Version 3.0, SPSS Inc., Chicago, IL) and SigmaPlot (Version 8.02, SPSS Inc., Chicago, IL) software packages, respectively. Values shown are mean ± standard error of five replicates. Relationships between variables were described and tested for significance using simple linear and multiple regression techniques.

Results

Plant water status

As shown in Fig. 1, plant water status was not affected by the nutrient supply regime, but restricting water supply induced the development of plant water deficits: on the last day of water stress imposition predawn Ψ decreased by approximately 0.4 MPa below the values determined in well-watered plants, indicating that both species were subjected to a moderate level of water deficit. Two days following re-watering, leaf water status fully recovered to pre-stress levels.

Concentrations of nitrate and amino acids

The concentrations of nitrate determined in both sunflower and white lupin plants are shown in Fig. 2. In the absence of water deficits, the nitrate contents determined in plants adequately supplied with nutrients were about 10-fold higher in sunflower roots than those determined in the roots of white lupin plants. That difference was exacerbated at the leaf level, since nitrate accumulated to concentrations higher in the leaves than in the roots of sunflower, whereas the opposite occurred in white lupin plants. The species-dependent differences in tissue nitrate contents still persisted when white lupins adequately supplied with nutrients were compared to sunflower plants subjected to the deficient nutrient regime, despite those plants being supplied with a similar concentration of nitrate. Irrespective of the species, the concentration of nitrate in roots was approximately halved in response to the restriction in the amounts of nutrients supplied. Foliar nitrate contents were also decreased under conditions of deficient supply of nutrients, although to a smaller extent. As shown in Fig. 2, drought did not affect nitrate concentration in the roots of white lupin plants. In contrast, nitrate concentrations in white lupin leaves and in sunflower roots were halved in response to soil drying. The extent of drought-

induced nitrate depletion was even higher in the case of water-stressed sunflower leaves, which nitrate contents decreased to about 20% of the values determined in well-watered plants. Two days following re-watering, no significant difference in tissue nitrate concentration was detected between well-watered and re-watered plants, except in the case of sunflower leaves, in which a significant nitrate build-up occurred after stress relief, reaching values two-fold higher than those determined in control leaves.

In contrast with nitrate, total amino acids concentrations were hardly affected by the nutrient supply regime, as shown in Fig. 3. The concentrations of those products of nitrate reduction in leaves and roots of white lupin plants were also unaffected in response to soil drying. In contrast, the concentrations of amino acids in both leaves and roots of sunflower were about 50% higher in droughted plants than in well-watered ones, but tended to return to pre-stress levels two days following re-watering (Fig. 3). The build-up of amino acids in water-stressed sunflower plants did not result from drought-induced proteolysis, since the concentrations of soluble protein were similar in well-watered and water-stressed plants, irrespective of the organ and species (Fig. 4).

Concentrations of soluble sugars

As shown in Fig. 5, the two species under study exhibited similar hexose concentrations at the leaf level, but the concentrations of these mono-saccharides were much higher in the roots of sunflower than in the roots of white lupin. Only in the case of sunflower roots did the reduction in the amounts of nutrients supplied induce a significant increase in hexose concentrations. In response to soil drying, hexose concentrations were doubled on the roots. The extent of drought-induced accumulation of hexose was even higher at the leaf level, but following re-watering the foliar hexose content quickly returned to pre-stress levels.

In contrast to what occurred with hexose, sucrose concentrations were hardly affected by the nutrient supply regime (Fig. 6). Also contrasting with hexose, drought-induced sucrose accumulation occurred mainly at the root level. As shown in Fig. 6, the foliar sucrose concentrations were only increased in response to soil drying in the case of white lupin plants. However, even in this species, sucrose did not constitute the main sugar involved in drought-induced foliar sugar accumulation, since the extent of drought-induced accumulation of this di-saccharide did not surpass 50%. It is noteworthy that, although soil drying induced the accumulation of sucrose on the roots of both species, sucrose concentrations in the roots of water-stressed sunflower roots never surpassed the concentrations found in the roots of well-watered and re-watered white lupin plants (Fig. 6).

Activity of nitrate reductase

Similarly to what occurred with leaf nitrate concentrations (Fig. 2), in the absence of water deficits and under an adequate supply of nutrients, the maximal activities of NR determined in the presence of EDTA (NR_{max}) were higher in sunflower than in white lupin plants (Fig 7). Those differences were particularly accentuated at the leaf level, since in sunflower NR_{max} was lower in roots than in leaves, whereas similar values of NR_{max} were determined in leaves and roots of white lupin plants.

At the root level, the effect of the nutrient supply regime on NR_{max} was species-dependent: in response to the decreased supply of nutrients, NR_{max} was negatively affected in sunflower, but not in white lupin (Fig. 7). Irrespective of the species, NR_{max} values determined in the roots of water-stressed plants represented only about 10% of those found in roots of well-watered plants, but despite the strong negative effects of water deficits, they were fully reversed two days following re-watering (Fig. 7). In the case of sunflower roots, there was a clear relationship

between drought- and nutrient supply-induced changes in NR_{max}, and the concomitant changes in tissue nitrate concentrations (r^2=0.568, P<0.001, NR_{max} = 0.097 + 0.072 × [nitrate]). Amino acids also seemed to contribute to predicting NR_{max} in sunflower roots, as indicated by a stepwise procedure involving the concentrations of amino acids and nitrate as predictors in the model, which retained both variables as explanatory variables (r^2=0.681, P<0.001, NR_{max} = 0.591 + 0.078 × [nitrate] – 0.353 × [amino acids]). In contrast, NR_{max} and nitrate contents determined in white lupin roots were not correlated (r^2=0.082, P=0.139), and adding amino acids as predictor also did not allow to explain the observed variations in the activity of the enzyme (r^2=0.180; P=0.084).

As shown in Fig. 7, foliar NR_{max} decreased by about 20% in response to the reduction in the amounts of nutrients supplied, irrespective of the species and the watering regime. In response to the imposition of water deficits, foliar NR_{max} was halved on both species under study, but after re-watering the activity of the enzyme fully recovered, even surpassing values determined in the well-watered control in the case of sunflower leaves (Fig. 7). The observed changes in foliar NR_{max}, induced both by water deficits and nutrient deficiencies, were linearly correlated with the concomitant variations in foliar nitrate content both in sunflower (r^2=0.695, P<0.001, NR_{max} = 3.659 + 0.168 × [nitrate]) and white lupin (r^2=0.270, P=0.004, NR_{max} = 0.276 + 1.044 × [nitrate]). In contrast to what occurred at the root level, a stepwise procedure involving the concentrations of amino acids and nitrate as predictors in the model retained only the last one as exclusive and sufficient explanatory variable. Nitrate not only correlated with changes in NR_{max} observed within each species, but was also able to account for more than three thirds of the variation in maximum foliar NR activity when all the individual data points determined on both species were considered (r^2=0.780, P<0.001, NR_{max} = 1.349 + 0.248 × [nitrate]).

Contrasting with NR_{max}, the activation state of NR was not affected by the nutrient supply regime, irrespective of the species and the watering regime (Fig. 8). In the absence of water deficits, the percentage of the enzyme which was phosphorylated, and therefore inactive, was higher in sunflower than in white lupin plants, particularly at the root level. The increased activation rate of NR in lupin roots was apparently sufficient to compensate for their lower NR_{max}, and hence in well-watered plants adequately supplied with nutrients, the two species under study exhibited similar values of NR_{act} in their roots (Fig. 9), although NR_{max} was two-fold higher in sunflower than in white lupin roots (Fig. 7).

As shown in Fig. 8, foliar NR activation was hardly changed in response to drought, but the effects of the watering regime on the post-translational regulation of NR on roots were species-dependent. In white lupin roots, despite a slight tendency for the amount of inactive enzyme increasing under water deficits conditions, NR activation state never decreased below 80%. In contrast, only about 50% of NR existing in roots of well-watered sunflower plants was unphosphorylated, but the activation of NR increased significantly in response to soil drying. The observed changes in NR activation state in sunflower roots were linearly correlated with sucrose concentrations (r^2=0.354, P<0.001, NR activation = 40.18 + 9.432 × [sucrose]), rather than with hexose concentrations (r^2=0.014, P=0.549). Irrespective of the causes underlying it, the increase in NR activation state detected in roots of water-stressed sunflower plants was by far not sufficient to compensate for drought-induced depression in NR_{max}, and to avoid the decline in the activity of the unphosphorylated active form of NR, which pattern of variation in response to soil drying (Fig. 9) closely paralleled that exhibited by NR_{max} (Fig. 7).

Discussion

On most herbaceous plants, nitrate assimilation take place predominantly in leaves (Scheurwater et al. 2002). In accordance, both accumulated nitrate (Fig. 2) and NR activity (Fig. 9) were higher in leaves than in roots of sunflower. In contrast, temperate legumes are often considered to be root assimilators, and in the particular case of white lupin, Pate et al. (1979) estimated that over 90% of nitrogen assimilation in nitrate-grown plants was associated with root nitrate reduction. Non-conforming with the minor role in nitrate reduction attributed by Pate et al. (1979) to the shoots of white lupin, we have found that the activity of NR determined in leaves was similar to that in roots (Fig. 9). However, the results obtained in the present work are in agreement with recent works in which the shoots of white lupin have been reported to account for more than 50% of whole-plant *in vivo* nitrate reduction both in nitrate-grown (Cen et al. 2001) and nodulated plants (Fan et al. 2002). Nevertheless, it should be noted that *in vitro* NR activities, as determined by us, are measured under optimal conditions, including substrate saturation, and may considerably overestimate the rates of nitrate reduction *in situ* (Morcuende et al. 1998, Kaiser and Huber 2001, Kaiser et al. 2000). As shown in Fig. 2, the level of accumulated nitrate was about 10-fold lower in leaves than in roots of white lupin. Such rather low foliar nitrate concentrations have also been reported for carob, a legume-tree species, in which the transport of nitrate from root to shoot is limited by its low capacity for loading nitrate into the xylem (Cruz et al. 1993). Irrespective of the causes underlying it, the low nitrate concentrations detected in white lupin leaves may constitute a major limiting factor for nitrate reduction under *in vivo* conditions, which is likely to be much lower than *in vitro* NR activity.

In the present work, the decline in NR_{act} induced by the restriction in nutrient supply (Fig. 9) was not associated with increased inactivation of the enzyme (Fig. 8). This is in accordance with previous studies which indicated that increased nitrate

supply does not positively affect the activation state of NR (Li and Oaks 1993, Man et al. 1999, Kaiser and Huber 2001, Kaiser et al. 2002). As to the effects of water deficits on the post-translational regulation of nitrate reductase, the experimental evidence so far available is contradictory: the measured activation state of NR, which is supposed to reflect the phosphorylation of the enzyme, has been reported to decrease (Kaiser and Brendle-Behnisch 1991, Brewitz et al. 1996, Foyer et al. 1998), to be unaffected (Ferrario-Méry et al. 1998, Abd-El Baki et al. 2000), or even to increase (Azedo-Silva et al. 2004) in response to dehydration. The results obtained in the present study also did provide conflicting evidence as to how NR activation varies in response to dehydration: in sunflower roots NR activation state increased in response to soil drying, but no drought-induced activation of NR was detected, neither on lupin roots, nor on the leaves of both species under study (Fig. 8).

It is well established that low sugar leads to post-translational inactivation of NR (Kaiser and Brendle-Behnisch 1991, Kaiser and Huber 2001, Kaiser et al. 2002, Iglesias-Bartolomé et al. 2004, Lillo et al. 2004). Even so, Botrel and Kaiser (1997) concluded that sugar availability has little effect on the activation of NR in barley roots. That contention was based on the lack of correlation between NR activation state and changes in sugar levels resulting mainly from variations in hexose concentrations, whereas sucrose concentration remained low. Similarly, we found that hexose accumulation in water-stressed leaves (Fig. 5) was not accompanied by increased activation of foliar NR (Fig. 8). These results are not necessarily contradictory with the hypothesis of sugars playing a decisive role in controlling the activation of NR, taking into account the work of Morcuende et al. (1998), who concluded that at least some of the signals that modulate the post-translational regulation of NR are derived from the uptake or metabolism of sucrose, rather than glucose. In the present work, drought-induced increase in NR activation state in

sunflower roots was correlated with the concomitant accumulation of sucrose, but not hexose, in accordance with the hypothesis of sucrose having a predominant role in regulating NR. In apparent contradiction with that hypothesis, the activation of NR in lupin roots did not increase in response to soil drying (Fig. 8), although sucrose also accumulated in the roots of water-stressed plants of this species (Fig. 6). However, it is noteworthy that in the roots of well-watered white lupin plants sucrose levels were similar to those found in water-stressed roots of sunflower (Fig. 6), and NR was nearly fully activated in the absence of water deficits (Fig. 8). Hence drought-induced sucrose accumulation in lupin roots could not lead to any further increase in NR activation.

In the present study, drought- and nutrient deficiency-induced inhibition of NR_{act} (Fig. 9) were closely paralleled by decreased maximal extractable NR activity (Fig. 7), both in leaves and roots of sunflower and white lupin. Similar results have been previously reported following water deprivation in tobacco leaves (Ferrario-Méry et al. 1998), and in both leaves and roots of sunflower (Azedo-Silva et al. 2004). Since NR_{max} is thought to reflect NR protein content, the observed depression in enzyme activity might result from NR degradation, which has been reported to increase when excised oat leaves are dehydrated (Kenis et al. 1994). However, sugar signalling and NR phosphorylation have been found to influence the degradation of NR, protein stability being positively correlated with sugar availability and NR activation state (Kaiser and Huber 2001, Kaiser et al. 2002). In the present study, the losses in NR_{max} (Fig. 7) occurred in plants exhibiting the highest concentrations of soluble sugars (Figs. 5 and 6), and were not associated with decreased NR activation state (Fig. 8). Therefore, the observed drought-induced decrease in NR_{max} is not likely to result from increased rate of NR degradation, and probably results from

inhibition of NR gene transcription, as previously reported for water-stressed leaves of tobacco (Ferrario-Méry et al. 1998) and maize (Foyer et al. 1998).

The observed variations in foliar NR_{max} (Fig. 7) were strongly correlated with concomitant changes in tissue nitrate content (Fig. 2), regardless of changes in nitrate concentration resulting from soil drying or varying nutrient supply regime. Similar concurrent decreases in NR_{max} and tissue nitrate contents have been previously found in response to nitrate withdrawal (Li and Oaks 1993), and water deficits imposition (Foyer et al. 1998, Azedo-Silva et al. 2004). These results are in accordance with the recognized role of nitrate in the regulation of NR at the transcriptional level (Crawford 1995, Kaiser et al. 2002). At the level of sunflower roots, NR_{max} and nitrate pool also varied concurrently, but in this case drought-induced accumulation of amino acids also contributed to explain the observed NR_{max} depression, in accordance with the suggested role of organic products of nitrate assimilation as repressors of NR gene transcription (Scheible et al. 1997), namely at the root level (Sivasankar et al. 1997). The fact of amino acids accumulation in water-stressed sunflower plants having a contributory role to explain NR_{max} decreases in roots, but not in leaves, is conform to Sivasankar *et al.* (1997), who found that the negative effects of glutamine-feeding on the nitrate assimilatory system were more pronounced in the roots than in the shoot of maize seedlings.

In contrast with what occurred at the level of sunflower roots, no concurrent changes in NR_{max} (Fig. 7) and nitrate contents (Fig. 2) was found in lupin roots. A similar lack of correlation between NR activity and nitrate concentration was previously found in barley leaves by Man et al. (1999), who suggested that the measured total tissue nitrate concentrations are not representative of the concentrations into the cytosol where NR is located, and release of nitrate from storage pools may be responsible for the maintenance of NR activity under

conditions of limited nitrate availability. In accordance, van der Leij et al. (1998) reported the lack of change in cytosolic nitrate concentration, during the withdrawal of external nitrate supply to barley roots. This may contribute to explain why NR_{max} was unaffected in response to whole tissue nitrate depletion induced by limiting the amount of nutrients supplied to lupin roots. Conversely, it may be hypothesized that the existence of a positive correlation between NR_{max} and root nitrate contents in sunflower may reflect a lower homeostatic control of cytosolic nitrate content in this species. However, the depression in NR_{max} in droughted lupin roots (Fig. 7) occurred despite the maintenance of whole tissue nitrate pool (Fig. 2). These results are difficult to reconcile with the hypothesis of NR_{max} being controlled by cytosolic nitrate concentration, unless the ratio between cytosolic nitrate and vacuolar nitrate decreases in response to dehydration. The partitioning of nitrate between the metabolic and storage pool has been reported to vary between species and to depend on the rate of nitrate supply (Chen et al. 2004), but so far no experimental data exist as to whether it is also affected by water deficits.

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Fig. 1. Predawn leaf water potential (Ψ) of well-watered (WW), water-stressed (WS) and re-watered (RW) sunflower (A) and white lupin (B) plants supplied with solutions containing either an adequate (closed bars) or deficient (open bars) level of nutrients.

Fig. 2. Nitrate concentrations determined in leaves (A, B) and roots (C, D) of well-watered (WW), water-stressed (WS) and re-watered (RW) sunflower (A, C) and white lupin (B, D) plants supplied with solutions containing either an adequate (closed bars) or deficient (open bars) level of nutrients.

Fig. 3. Amino acids concentrations determined in leaves (A, B) and roots (C, D) of well-watered (WW), water-stressed (WS) and re-watered (RW) sunflower (A, C) and white lupin (B, D) plants supplied with solutions containing either an adequate (closed bars) or deficient (open bars) level of nutrients.

Fig. 4. Soluble protein concentrations determined in leaves (A, B) and roots (C, D) of well-watered (WW), water-stressed (WS) and re-watered (RW) sunflower (A, C) and white lupin (B, D) plants supplied with solutions containing either an adequate (closed bars) or deficient (open bars) level of nutrients.

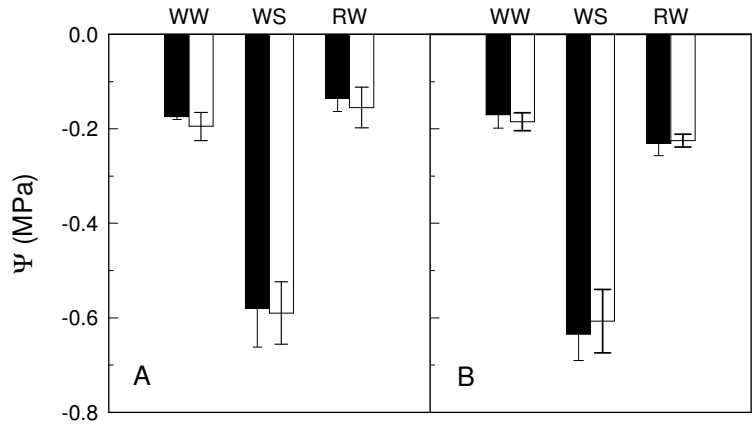
Fig. 5. Hexose concentrations determined in leaves (A, B) and roots (C, D) of well-watered (WW), water-stressed (WS) and re-watered (RW) sunflower (A, C) and white lupin (B, D) plants supplied with solutions containing either an adequate (closed bars) or deficient (open bars) level of nutrients.

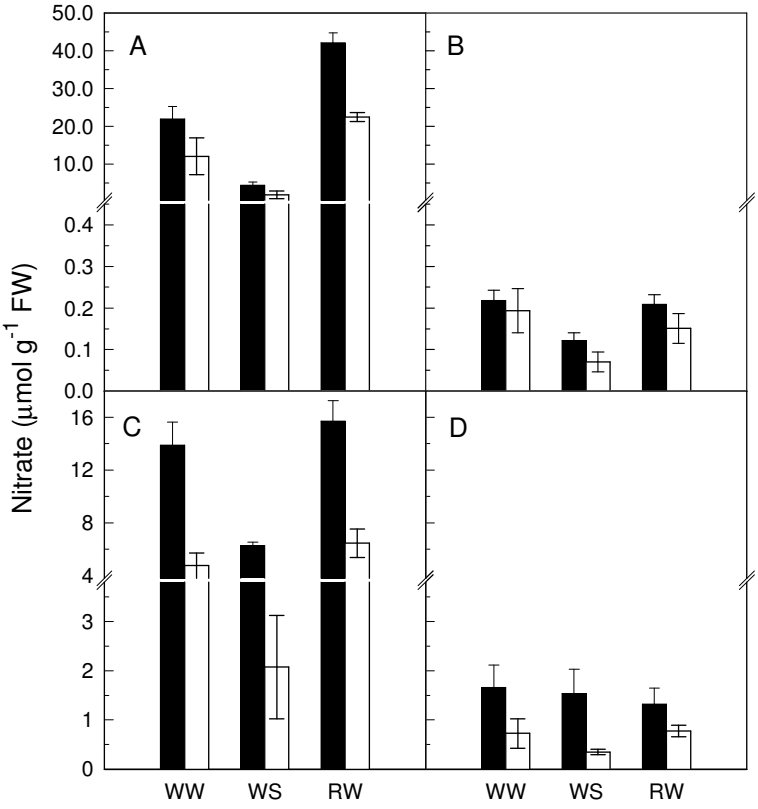
Fig. 6. Sucrose concentrations determined in leaves (A, B) and roots (C, D) of well-watered (WW), water-stressed (WS) and re-watered (RW) sunflower (A, C) and white lupin (B, D) plants supplied with solutions containing either an adequate (closed bars) or deficient (open bars) level of nutrients.

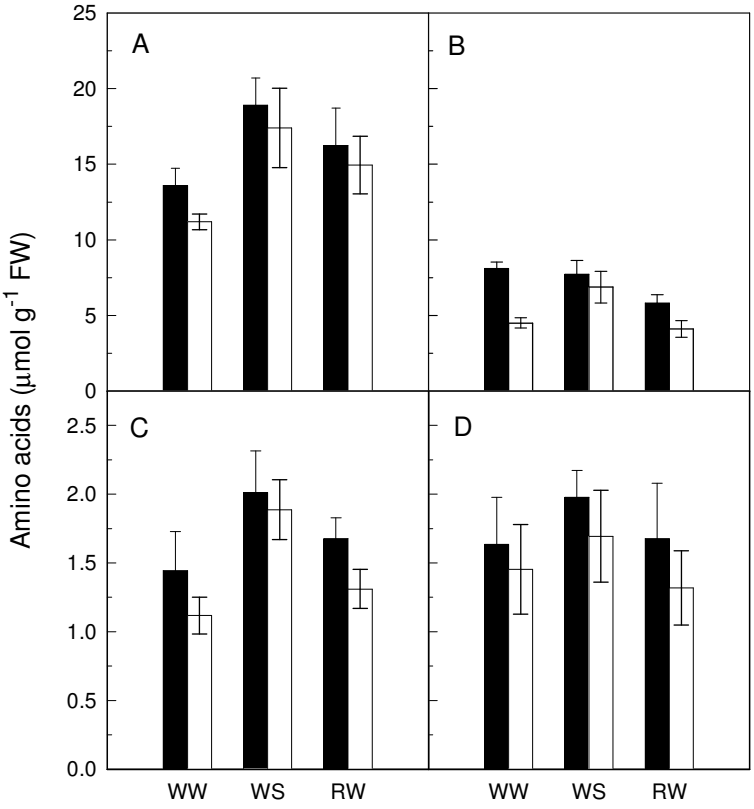
Fig. 7. Maximum NR activity determined in the presence of excess EDTA (NR_{max}) determined in leaves (A, B) and roots (C, D) of well-watered (WW), water-stressed (WS) and re-watered (RW) sunflower (A, C) and white lupin (B, D) plants supplied with solutions containing either an adequate (closed bars) or deficient (open bars) level of nutrients.

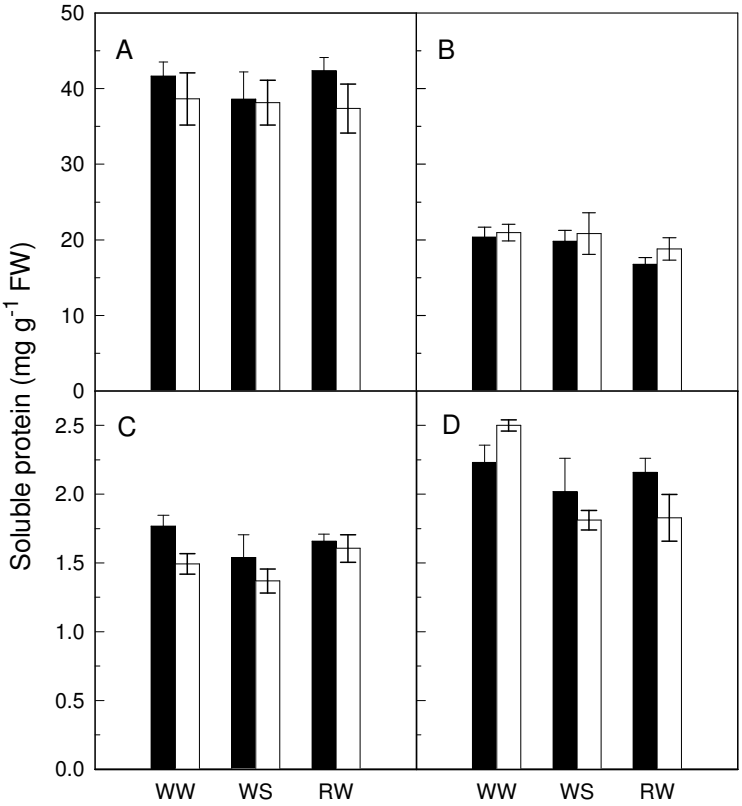
Fig. 8. Activation state of NR determined in leaves (A, B) and roots (C, D) of well-watered (WW), water-stressed (WS) and re-watered (RW) sunflower (A, C) and white lupin (B, D) plants supplied with solutions containing either an adequate (closed bars) or deficient (open bars) level of nutrients.

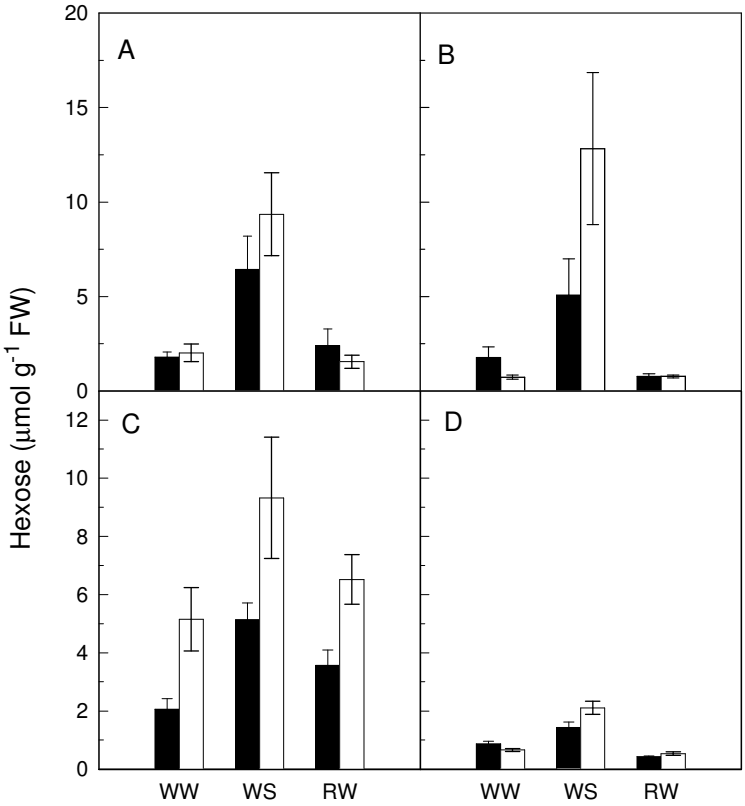
Fig. 9. Activity of the active NR unphosphorylated form determined in the presence of Mg^{2+} (NR_{act}) determined in leaves (A, B) and roots (C, D) of well-watered (WW), water-stressed (WS) and re-watered (RW) sunflower (A, C) and white lupin (B, D) plants supplied with solutions containing either an adequate (closed bars) or deficient (open bars) level of nutrients.

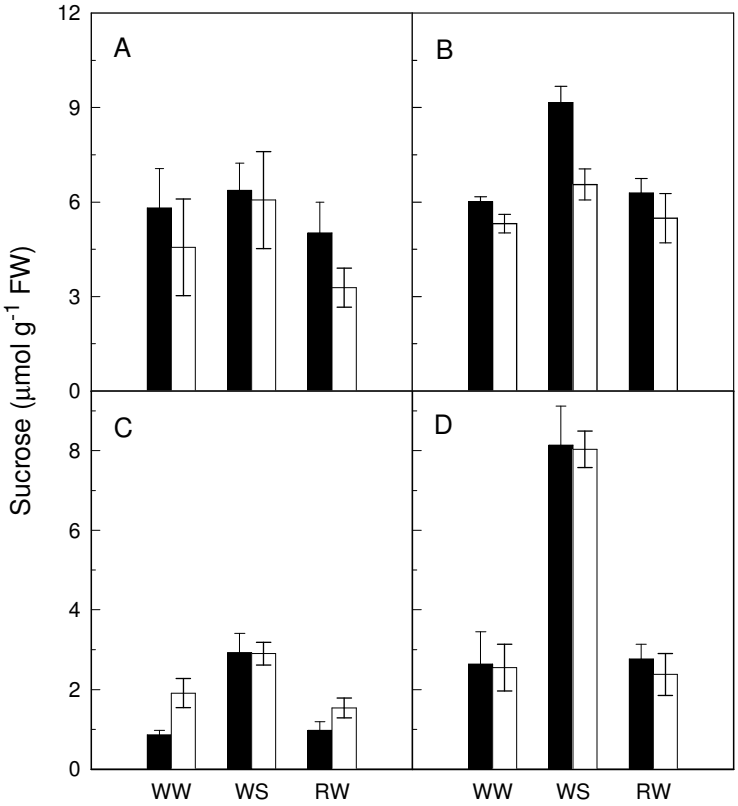

WW
WS
RW
WW
WS
RW
0.0
-0.2
-0.4
-0.6
-0.8
Ψ (MPa)
A
B

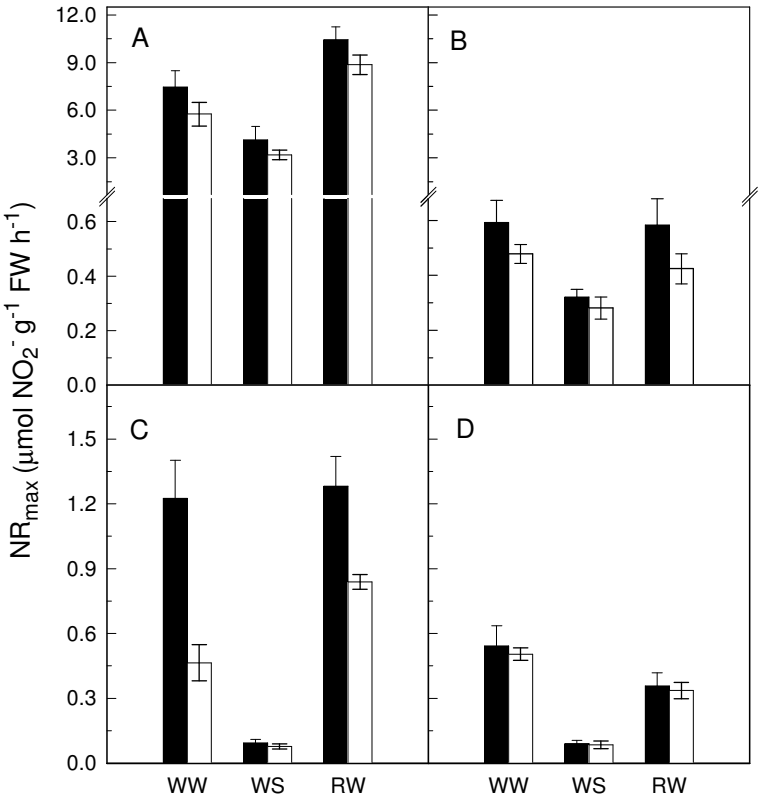

A
B
C
D
Nitrate (µmol g-1 FW)
50.0
40.0
30.0
20.0
10.0
0.4
0.3
0.2
0.1
0.0
16
12
8
4
3
2
1
0
WW
WS
RW
WW
WS
RW

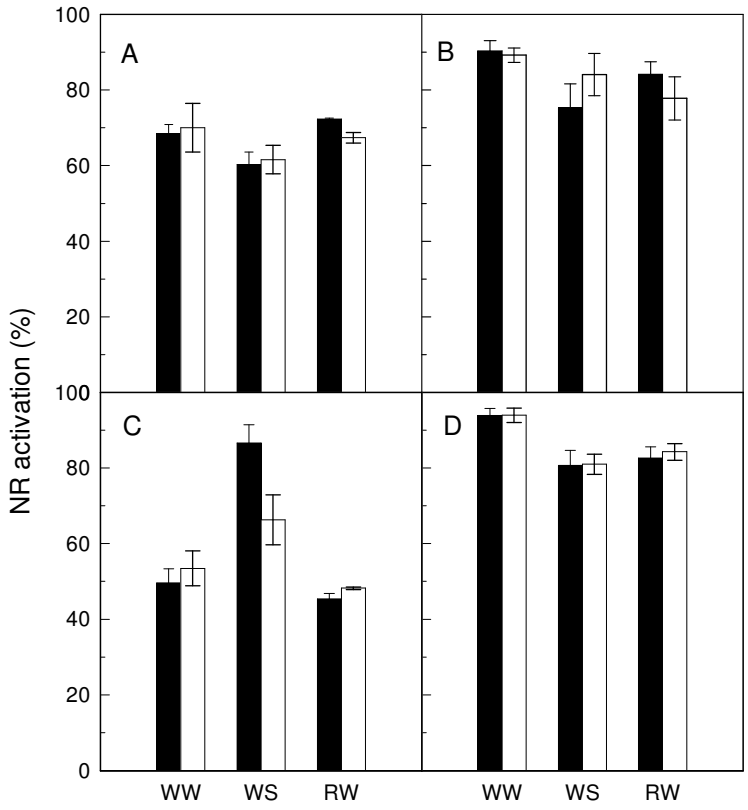

A
B
C
D
Amino acids (µmol g-1 FW)
25
20
15
10
5
0
2.5
2.0
1.5
1.0
0.5
0.0
WW
WS
RW
WW
WS
RW

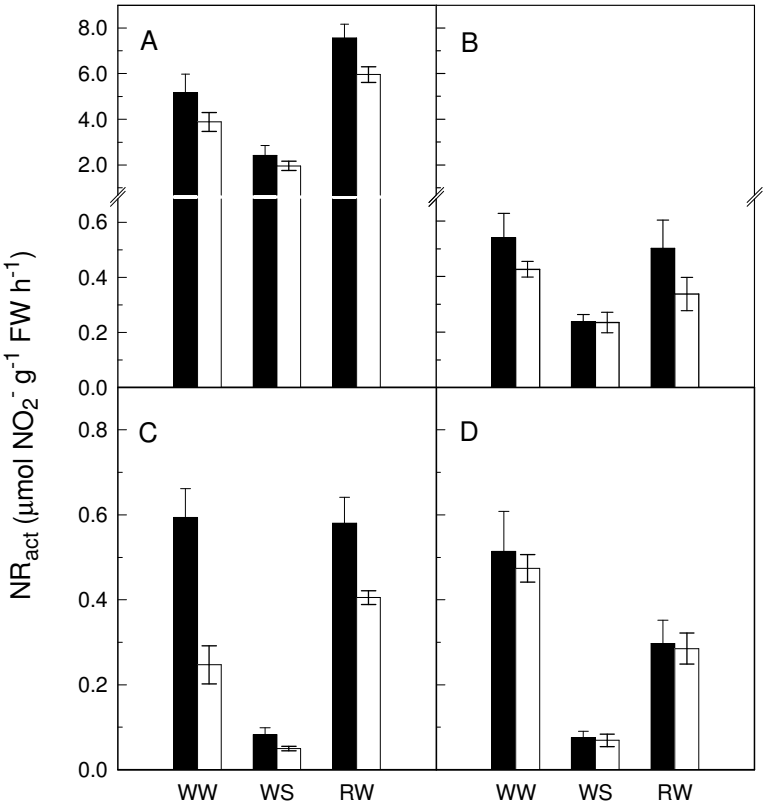

A
B
C
D
Soluble protein (mg g-1 FW)
50
40
30
20
10
0
2.5
2.0
1.5
1.0
0.5
0.0
WW
WS
RW
WW
WS
RW


A
B
C
D
Hexose (µmol g-1 FW)
0
5
10
15
20
0
2
4
6
8
10
12
WW
WS
RW
WW
WS
RW


A
B
C
D
Sucrose (µmol g-1 FW)
12
9
6
3
0
8
6
4
2
0
WW
WS
RW
WW
WS
RW


A
B
C
D
12.0
9.0
6.0
3.0
0.6
0.4
0.2
0.0
1.5
1.2
0.9
0.6
0.3
0.0
WW
WS
RW
WW
WS
RW
NRmax (µmol NO2- g-1 FW h-1)


NR activation (%)
100
80
60
40
20
100
80
60
40
20
0
A
B
C
D
WW
WS
RW
WW
WS
RW


A
B
C
D
8.0
6.0
4.0
2.0
0.6
0.4
0.2
0.0
0.8
0.6
0.4
0.2
0.0
WW
WS
RW
WW
WS
RW
NRact (µmol NO2- g-1 FW h-1)