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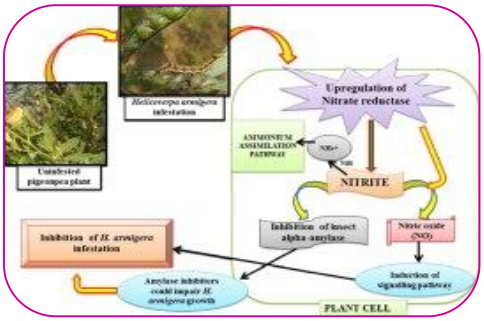
NITRATE REDUCTASE ACTIVITY IN BARLEY AS INFLUENCED BY LIGHT/DARK/LIGHT PERIODS

[1]Salwa A. Abdel-Latif and [2]Hanan M. Abou-Zeid
[1]Biology and Geology Department, Faculty of Education, Alexandria University, Egypt.
[2]Botany and Microbiology Department, Faculty of Science, Alexandria University, Egypt.



ABSTRACT

Barley (Hordeum vulgare L. cv. Giza 123) plants has NADH-specific and NAD (P)H-bispecific nitrate reductase isozymes. We examine the activities of both isozymes after the induction with nitrate in leaves and roots of hydroponically N-depleted grown seedlings in buffer containing either 5mM Mg^{+2} or 5mM EDTA. The two isozymes activities were studied under a light/dark cycle of 14: 10h and were correlated with total reduced nitrogen and nitrate contents. Our data indicate the activation state of NR in leaves accounted for 63% (light), 43% (dark), and 66% (light) during each of the three periods. In barley, nitrate induced both NADH and NAD (P)H-NR activities in roots, but only NADH-NR activity in leaves. Levels of NADH-NR activity in leaves were higher than in roots and showed higher NR_{max} activity when EDTA ions were present in the assay medium, while lower NR_{act} activity was detected in extracts with Mg^{+2} ions. During the light/dark/light periods NR_{act} is decreased by 37% (14h), 57% (10h) and 34% (38h) from NR_{max}. Total reduced N was greater in leaves than roots during the three periods. Accumulation of nitrate in leaf after N supply at 38h light period is coincident with the increase in nitrate reduction in leaf. The seedlings incubated with nitrate in the light were developed NR activity more rapidly than N-depleted seedlings. The benefit of quantifying NR activity of roots and shoots in vitro was that it allowed the assessment of the activities of both enzymes independently during light/dark cycle.

KEYWORDS: *Barley; Nitrate reductase isozymes; Light/dark; Total reduced nitrogen; Activation state.*

INTRODUCTION

Barley (*Hordeum vulgare* L.) is grown as a commercial crop in one hundred countries and considered one of the most important cereal crops in the world (Yousufinia *et al.*, 2013). Barley is an important agricultural crop for food, feed and also has been used virtually worldwide as a model plant for biological research since it ranks fourth among field crops in grain production in the world after wheat, maize and rice (FAO, 2008). In recent years, about two thirds of barley crop has been used for seed, one – third for malting and about 2% directly for food (Baik and Ullrich, 2008). In Egypt, barley is qualified to be cultivated in wide areas, especially in the north coast region and in the new cultivated because of its tolerance characteristics to salt and drought stresses. Nitrate (NO_3^-) is the major form of inorganic nitrogen available to plants in the environment must be reduced to nitrite and then to ammonium in reactions catalysed by the enzymes nitrate reductase and nitrite reductase, respectively (Bloom *et al.*, 1992; Wiencke and Bischof, 2012) prior to its assimilation into the amino acid pool via either the glutamine synthetase/ glutamate synthase cycle or the action of glutamate dehydrogenase (Lam *et al.*, 1995). Nitrate reductase is the first enzyme in the NO_3 assimilation pathway and probably represents the rate limiting step in this process and generates NO_2 in the

cytoplasm of a plant cell, which is translocated into the plastids for further reduction and metabolization (Sauer and Frébort, 2003, Cruz *et al.*, 2004; Mazid *et al.*, 2010).

The assimilation of nitrate is considered energetically more costly than ammonia because nitrate must first be reduced to nitrite and then to ammonia (Taiz and Zeiger, 2002). The NR exists in three forms: NADH-specific, NAD (P) H bispecific, and NADPH-specific. While the occurrence of nitrate-reducing activities in plant tissues has been known for more than a century (Irving and Hankison, 1908) their mechanisms and physiological roles (Campbell, 1999), genetics (Hirel *et al.,* 2001), modes of regulation (Lillo *et al.*, 2004; Lillo, 2008) and potential for improvement in the context of nitrogen use efficiency (Zhao *et al.*, 2013) have been the foci of ever increasing numbers of investigations. NR is a most interesting enzyme especially from the aspect of its complex regulation expression and control of catalytic activity. NR requires NADH as electron donor in most of higher plant species (Kaiser and Huber, 2001). NR activity is also regulated by a number of other factors, including plant growth regulators (Lu *et al.*, 1990, Zhang *et al.*, 2011), growth hormones, reduced nitrogen metabolites, drought, and light (Foyer *et al.*, 1998; Huber *et al.*, 1992b; Lillo, 1994). In light/dark studies, a drop in the activity of NR is usually observed in plants when transferred from light to darkness. The activity rises when plants are transferred back to light (Reins and Heldt, 1992). In plants, it is known that nitrate is the primary factor regulating NR induction (Cabello *et al.*, 1998). The NR activity is inducible by NO_3 (Kronzucker *et al.*, 1995).

Light is most often reported to have an enhancing effect on NR activity, and this enhancement may be either the direct result of light perception (Rajasekhar *et al.*, 1988), or through stimulation by the products of photosynthesis (Cheng *et al.*, 1992). In addition, light entrains the plant's circadian rhythm, which has been proposed to influence the cyclic accumulation of NR trancript in anticipation of daylight, and corresponding decrease as night approaches (Lillo and Ruoff, 1989; Deng *et al.*, 1990), though whether this modulation is necessarily integrated with the cell's central diurnal timekeeping function, termed the "central oscillator," has been called into question (Lillo *et al.*, 2001). Regulation of NR takes place at the transcriptional as well as posttranslational level. At both levels signals from the chloroplasts are involved in initiating an increase in NR activity levels, but these signals and their processing are still unknown (Jonassen *et al.*, 2008; Nunes-Nesi *et al.*, 2010).

Generally, the phosphorylated form of NR is thought to predominate over the dephosphorylated NR in darkness and vice versa. The expression of NR is induced by the application of nitrate, and the activity of the protein is altered in response to changes in environmental conditions, such as light, dark, anoxia, pH, and carbon dioxide concentration (Kaiser *et al.*, 1999). Evidence for the involvement of phosphorylation for inactivation of NR is reported for several species, *Nicotiana plumbaginifolia* (Nussaume *et al.*, 1995), maize *(Zea mays)* (Merlo *et al.*, 1995) and barley *(Hordeum vulgare)* (Lillo *et al.*, 1996a). NR is activated in the light and deactivated in the dark; therefore, light-to-dark transitions were used as the stimulus (Kaiser and Spill, 1991). NR activity is modulated post-translationally through regulatory phosphorylation (Su *et al.*, 1996) permitting the association of a 14-3-3 family protein that alters electron flow through the enzyme's modular structure (Lambeck *et al.*, 2012); this feature appears to be widely conserved among flowering plants (Bachman *et al.*, 1996c) and may have emerged prior to the divergence of Magnoliophyta (Nemie-Feyissa *et al.*, 2013).

In addition to 14-3-3 proteins, Mg^{+2}, Ca^{+2}, or polyamines have repeatedly been shown to be necessary for the inactivation of NR, and the effects of these ions on NR inactivation are complex (Provan *et al.*, 2000). The fundamental mechanism underlies the rapid modulation of NR activity in leaves in response to light/dark transitions. In detached spinach leaves in the dark, NR measured in the presence of Mg^{+2} was rapidly inactivated to a very low level (Kaiser and Huber, 1994a). Multiple levels and mechanisms of regulation have been reported to impact NR activity in plants. At the transcriptional level, promoter sequences and other functional elements associated with the *Arabidopsis* NR-encoding *NIA1* gene have been demonstrated to contribute qualitatively and quantitatively to nitrate-dependent induction (Lin *et al.*, 1994; Wang *et al.*, 2010; Konishi and Yanagisawa, 2011).

MATERIALS AND METHODS

Seed germination and plant growth

Seeds of barely (*Hordeum vulgare* L .cv. Giza 123) were soaked and subjected to surface sterilization

with 0.1% sodium hypochlorite solution for 5min and then rinsed several times with distilled water and placed on moistened paper in the dark for 24 h in an environmentally controlled room. After 24 h seeds were spread over a plastic net placed 3 cm above the surface of a 2 l of 0.5 mM $CaCl_2$. The seeds were covered with wet filter paper and kept in the dark. After 48 h the seeds, from which the endosperm were removed, were placed in hydroponic culture solution without nitrogen (pH 5.6) containing 0.2 mM $CaCl_2$, 0.2 mM $MgSO_4$, 2 mM KH_2PO_4, 0.025 mM Fe-EDTANa_2, and micro-elements as described by (Mae and Ohira, 1981). Seeds were germinated and grown with the same nutrient solution and were placed in growth chamber under a light-dark 14:10h cycle at 25°C as previously described (Sueyoshi *et al.*, 1999). Experimental treatment was started after this adaptation period. The seedlings were grown for 8 days hydroponically and at day 9 after germination, 2.5mM KNO_3^- were supplied with nutrient solution to the N-deficient seedlings and the nutrient solution was renewed every day. Leaves and roots were harvested at end of the light 14h (light), after 24h (light-dark) and 38h (light-dark-light) in the nutrient solution.

In vitro assay of NR activity

Extraction

Leaves and roots were harvested, weighted and immediately ground for assays NADH-NR and NAD(P)H-NR activities.The frozen organs were homogenized with an extraction buffer (2 ml /g fresh weight) containing 50 mM Hepes-KOH (pH 7.7), 25 mM NaF, 1 mM $Na_4P_2O_7$, 10 mM 2-mercaptoethanol, 10 µM leupeptin and 1 mM EDTA. The crude homogenate was then centrifuged at 15,000 rpm for 20 min and the resulting supernatant was used for the NR assay. In case of light, 1.5 g of the sample was extract with 3ml of the extracting buffer containing 15 µL of 200 µM saturosporin , a protein kinase inhibitor, to the sample (light sample) or 12 µL of 250 µM microcyctin, a phosphatase inhibitor, (dark sample). Both inhibitors were dissolved in 50 % DMSO with distilled water. NR activities were measured in leaves and roots for determination of NADH- specific and NAD(P)H- bispecific NRs in barley and in the presence either 5mM $MgCl_2$ or 5mM EDTA (Aslam *et al.,* 2001) and using enzyme extraction buffer.

Measurement of NR activity

The NADH-specific NR activity (NADH-NR; EC 1.6.6.1) was assayed in 450 µL of reaction mixture (50 mM Hepes-KOH (pH 7.7), 5 µM leupeptin, 2 mM KNO_3, 10 µM FAD, 1 mM EDTA and 0.2 mM ß-NADH) as described by (Dailey *et al.*, 1982b). In the case of the NAD(P)H-bispecific NR (NAD(P)H-NR; EC 1.6.6.2) assay, a reaction mixture containing 0.1 mM ß-NADPH, 0.1 mM ß-$NADP^+$ and 0.2 mM glucose-6-phosphate instead of ß-NADH was used, in order to allow rapid regeneration of NADPH (Savidov *et al.*, 1998). In the case of the sample harvested under light or darkness, the activity measured in a reaction mixture containing either 5mM $MgCl_2$ (for determination of the actual NR activity, NRA_{act}) or 5mM EDTA (for determination of the maximum NR activity, NRA_{max}).The reaction was carried out at 30°C for 15 min and stopped by the addition of 50µL of 1 M zinc acetate. After removal of the precipitate by centrifugation, equal volumes of 1% sulfanilamide and 0.02% *N*-(1-naphthyl) ethylene diamine dichlorohydrate were added to the supernatant, after which the absorbance was measured with a spectrophotometer at 540 nm. NR_{act} and NR_{max} were estimated according to the method of (Kaiser and spill, 1991).The calibration curve was prepared using sodium nitrite solution. NR activity state was defined by the ratio of the actual activity (Mg assay) to the total NR activity (EDTA assay) multiplied by 100 (MacKintosh *et al.*, 1995). NR activity is expressed in ($\mu mol.g.f.wt^{-1}.hr^{-1}$) in leaves and root extracts.

Determination of Nitrate

After sampling measure fresh weight of roots and leaves, then put the samples into appropriate envelope and heat it in microwave oven for 15 sec. to inactivate enzyme in plant. Nitrate was colorimetrically determined by nitration of salicylic acid using the method of (Cataldo *et al.*, 1975). Under a strong acidic solution nitrate and salicylic acid make a complex. When this complex was transferred into alkaline condition (> pH 12), yellow colure is observed. Nitrate was quantified by measuring absorbance at 410 nm. Ammonium, nitrite and chlorate do not disturb the absorbance. Nitrate extraction was performed in water (4mL/g dry weight). After homogenization and centrifugation, nitrate was measured in the supernatant by a spectrophotometric assay.

Extraction and determination of total reduced nitrogen

Leaves were washed, rinsed with distilled water and blotted by a paper towel. Fresh weight (FW) of leaves was recorded after washing. Leaves were packed in an envelope and dried in oven at 60°C for dry weight (DW) determination and for subsequent analysis. The dry leaves were ground into fine powder and extracted with distilled H_2O at 80°C for 20 min. After separation of the soluble and insoluble fractions by centrifugation, the insoluble fraction was re-extracted by the same procedure. Two soluble fractions were combined and distilled H_2O was added up to 25 mL. The small portion (100µL) of combined soluble fractions was used for nitrate determination. To the remaining soluble fractions, 0.25 mL of 36N H_2SO_4 and 2.5 mL of 30% H_2O_2 were added, after which the mixture was heated for 48 h at 100°C to eliminate the nitrate (and the nitrite, if any). The resulting soluble reduced N fraction was mixed with the insoluble fraction, which had been dried in oven at 60°C for 24 h in advance, and the total reduced N content in the mixture was determined by the modified Kjeldahl digestion method which converts organic N in the sample to NH_4^+-N by digestion with concentrated H_2SO_4 containing substances that promote oxidation of organic matter and conversion of organic N to NH_4^+-N. The modified method was described by (Ohyama *et al.*, 1991), and the indophenol method to determine total nitrogen in the Kjeldahl digestion solution. The indophenol method consists basically of a reaction of a sample containing NH_4^+ with phenol (actually phenate) and hypochlorite at pH to form a blue color, the intensity of which is proportional to the NH_4^+ concentration in the sample.

Statistical Analysis

Each treatment was analyzed with at least three replicate tissue samples. Results are the means of three replicates. SEs exceeding the symbol size is indicated by bars.

RESULTS

Accumulation of reduced N

Data demonstrated in in (Table 1a) showed that during the initial 14h light period the roots of barley seedlings represented about 38% of seedling mass whereas, the proportion of total reduced N accumulated in roots was 14%. Total reduced N was greater in leaves than roots during the whole light/dark/light treatment. However, much accumulation of reduced N in leaves occurred during the 10h dark period and the difference became smaller at 38h light period (30.81 versus 34.62 µ mol.plant^{-1} in 10h dark and 38h light, respectively) (Table 1b&c).

Nitrate accumulation

The accumulation of nitrate changed in roots and leaves at light/dark 14:10h cycle (Fig. 1). The root nitrate content was 0.65 and 1.68 µ mol. plant^{-1} respectively during the 14h light and 10h dark periods after the transfer to nutrient solution containing nitrate. In the dark nitrate content of both roots and leaves was elevated. However, the nitrate content decreased in roots and leaves during the 38h light period and significantly low nitrate contents were measured in roots and leaves and amounted to (1.12 and 0.53 µmol.plant^{-1}, respectively). On a whole plant basis, roots accounted for 45% (14h), 58% (24h) and 68% (38h) of the nitrate accumulation of the whole plant during light/dark/light period.

Nitrate reductase activity and NR activation state

In the present study, N-depleted seedlings (9-d-old) were treated with a nutrient solution containing 2.5mM KNO_3^- under a light-dark cycle of 14:10 h at 25ºC. Leaves were harvested after 14, 24, and 38h in the nutrient solution. Data represented in (Fig. 2a) indicated that NADPH-NR activity was undetectable in the plants leaves and only the NADH-NR is present. Leaves NADH-NR showed higher NR_{max} activity when EDTA ions were present in the assay medium, while lower NR_{act} activity was detected in extracts incubated with Mg^{+2} ions. During the light/dark/light periods NR_{act} is decreased by 37% (14h), 57% (10h) and 34% (38h) from NR_{max}. The NR_{act} was activated in the light, and inactivated during the dark period. The NR_{act} was increased from 10h dark to 38h light by 3-fold (from 0.91 to 2.79 µ mol.g^{-1}fw.h^{-1}). The values of extractable NR_{max} in the leaves of barley during the first 14h light period of the treatment and in the 10h dark were unchanged. However, at 38h light period the value of NR_{max} activity in leaves was 2-fold increases (from 2.1 to 4.25 µmol.g^{-1}. fw.h^{-1}) (Fig. 2a). The activation state of NR in leaves, given as the percentage ratio of NR_{act}

to NR_{max} increased rapidly in the light (Fig. 3b), it rose to a maximum 63% and 66% in the light and declined to 43% in the dark, respectively. The NR_{act} and NR_{max} activities of root NADH-NR during light/dark/light period are shown in (Fig. 3c) and only NR_{max} activities of root NAD(P)H-NR are shown in (Fig. 3d) . Data represented in (Fig. 3c, d) demonstrated that both NADH and NAD(P)H isozymes were induced in barley roots . NR activity was higher in leaves than in roots (Fig. 2, Fig. 3 c, d). Whereas, the lowest activity of NR_{act} was detected in extracts incubated with Mg^{+2} in the10h dark and at 38h light periods (Fig. 3c). Nearly equal low amounts of NR_{max} were detected in root NADH and NAD(P)H isozymes (Figure 3c,d) during the light/dark/light periods. The amount of root NADH-NR maximal activity was significantly low and accounted to (0.11 and 0.14 $\mu mol.g^{-1}.fw.h^{-1}$ in the 14h and 10h light/dark, respectively), and (0.13 $\mu mol.g^{-1}.fw.h^{-1}$) in the 38h light. However the amount of NAD(P)H actual activity was not detected in barley roots (Fig. 3d), whereas, the amount of maximal NAD(P)H-NR was determined and showed an increase from the10h dark to the second 38h light period by 43% (0.2 vs 0.14 $\mu mol.g^{-1}.fw.h^{-1}$). The activities of both NADH of NAD (P)H isozymes were below the limits of detection prior to nitrate induction in root or leaf extracts of barley seedlings (data not shown).

DISCUSSION

Total reduced N was greater in leaves than roots during the whole light/dark/light treatment. However, much accumulation of reduced N in leaves occurred during the 10h dark period and amounted to 30.81 $\mu mol.plant^{-1}$ (Table 1b). As a result, the difference became smaller at the 38h light period (30.81 versus 34.62 μ $mol.plant^{-1}$ in 10h dark and 38h light, respectively) (Table 1b&c). Thus the total reduced N was strongly dependent upon continuous supply of nitrate. Decrease accumulation of nitrate content in leaves after nitrate supply at in the second 38h light period (Fig. 1) in leaves than roots is roughly coincident with the increase in NADH-dependent NR activity in leaf than root as shown in (Figs. 2a & 3c). Similar observations were reported by (Abdel-Latif *et al.*, 2004; Oji *et al.*, 1989) working on barley. Gojon *et al.*, (1986) have reported a shift of nitrate assimilation from root to shoot in N-deficient barley and corn seedlings. Our results show that in barley leaves during the second 38h light period NRA increase to a maximum (Fig. 2a), nitrate decreases (Fig. 1), and total reduced nitrogen accumulated (Table 1c). Indeed, the higher nitrate content in leaves can be explained by its lower NRA. Light is often reported to enhance plant nitrate reductase (NR) activity. In fact, it has been reported NR activity in plant leaves drastically changes during light/dark transition (Oji *et al.*, 1989; Remmler and Campbell, 1986). On the other hand, Schoenbeck *et al.*, 2015 identified a context in which light strongly suppresses NR activity and the suppressive mechanism appears to act at a point after nitrate perception; tissues pre-incubated with nitrate in the light were potentiated and developed NR activity more rapidly than nitrate-induced tissues not so pre-exposed. Our results show that the activation state of NADH-NR in leaves extracted in darkness is generally inhibited by 57% (Fig. 2a). The present results are in agreement with those obtained by (MacKintosh and Meek, 2001; Abdel-latif and Abou-Zeid, 2014). Many authors (Kojima *et al.*, 1995; Su *et al.*, 1996; Man *et al.*, 1999) have reported that the NR activation state in leaves is mainly lower in the dark than in the light. In Barley nitrate induced NADH and NADPH NR activities in roots but only NADH-dependent NR activity in leaves. Since the NADH-specific NR isozyme has little activity with NADPH as an electron donor (Dailey *et al.*, 1982a), the presence of NADPH-NR activities in roots of barley is attributed to the NAD(P)H-bispecific NR isozyme. In barley, NADH-NR and NAD(P)H-NR are nitrate-inducible and are expressed in the roots, but only NADH-NR is detected in the leaves (Sueyoshi *et al.*,1995 ; Abdel-latif and Abou-Zeid, 2014).Several plant species, including barley, maize, rice, and soybean, carry both NADH-NR and NAD(P)H-NR (Warner and Kleinhofs, 1992). The two forms of NR differ in overall activity, distribution among organs, and biochemical characteristics. As indicated by the results in (Figs. 2a & 3a) the NR_{act} in the leaves and roots was declined to 57% and 82%, respectively from NR_{max} in the dark-grown plants. The inhibitory effect of Mg^{+2} on barley NADH-NR activity extracted from illuminated and darkened leaves and its protection by EDTA can be interpreted within the framework of the phosphorylation/dephosphorylation (Huber *et al.*, 2002) in terms of a different sensitivity of the dark-phosphorylated enzyme to Mg^{+2}. Phosphorylation enables a 110 kDa NR inhibitory protein to interact with the enzyme, and suppress its activity. Barley NR would be sensitive to Mg^{+2}; the inactivation could be manifest in the absence of added divalent cations. Release of the effector and-or hydrolysis of the phosphate groups from the enzyme by protein phosphatases present in the crude extracts could explain the reactivation of NR under

in-vitro assay conditions. The differential Mg^{+2}/EDTA assays, therefore, enable an efficient and precise way of calculation of activity state and also adjust for possible variations in total NR in each extract. Spinach NR protein is phosphorylated immediately after dark treatment at serine 543 which is located in the hing one region connecting the Cytochrome b domain with the molybdenum cofactors binding domain (Bachmann *et al.*, 1996; Sueyoshi *et al.*, 1998). Microcystin-LR is known inhibitor of the protein phosphatase and prevented light activation of NR in spinach (*Spinacia oleracea*) leaves and barley (*Hordeum vulgare*) protoplasts (MacKintosh, 1992; Lillo *et al.*, 1996a). Okadaic acid and microcystin also prevented activation of NR in extracts of leaves. The NADH-NR activity was higher in leaves than in roots (Fig. 2a, Fig. 3c). Many studies suggest that leaves account for the vast majority of nitrate reduction under normal conditions, with most of this reduction occurring in the light (Aslam and Huffaker, 1982). Data represented in (Fig. 2b) showed that the activation state of NR in leaves increased rapidly it rose to a maximum 63% and 66% in the light and declined to 43% in the dark, respectively in agree with the conclusions of many authors that the inhibition of shoot NO_3^- reduction in darkness is a well-known phenomenon, and has been attributed to a lack of reducing equivalents for both NR and NiR (Kojima *et al.*,1995; Man *et al.*, 1999; Lillo *et al.*, 2004), or to inactivation of NR by phosphorylation (Kaiser and Huber, 1994a). In whole plants, NADH-NR is responsible for 80 to 90% of the overall *in vitro* NR activity, while the activity of NAD (P)H-NR accounts for the remaining 10 to 20% (Warner and Huffaker, 1989). In the absence of nitrate, neither NADH nor NADPH-dependent NR activities were detected in root and leaf extracts of barley. No significant difference was observed in the amount of both isozymes NADH and NAD (P)H in barley roots during the whole treatment. Barley roots had nearly similar NADH and NADPH NR activities (Fig. 3c&d). The presence of greater NADH than NADPH NR activity in leaves of barley than in roots is an indication that both NR isozymes are present. In addition, (Kaiser and Huber, 2001) suggested that the expression of NR was induced by NO_3^- and the NR activity altered by the change in light and dark conditions, pH, CO_2 concentration and anoxia. The goal of future works will be to determine whether the light activation acts on NR protein abundance, or post-translational modulation of NR activity. In addition, as phosphorylation mediated suppression at the protein level is dependent upon the availability of Mg^{+2}, which suggests that NR regulated by phosphorylation. This has been shown to occur in various conditions in which photosynthesis is prevented (Huber *et al.*, 1992a; De Cires *et al.*, 1993). The employment of an *in vitro* assay to compare NR activity levels, with or without Mg^{+2} sequestration, may help to determine if light activation is mediated by post-translational modifications. In conclusion, we have provided evidence for a connection between plant light perception and a mechanism by which nitrate-induced NR activity is activated. It is important to explore the possible pathways of nitrogen nutrition and its regulation in plants. Efforts to improve plant nitrogen use efficiency have focused on different physiological functions. As such, researchers have been encouraged to adopt a systems biology approach that integrates the best understanding of these processes (Gutiérrez, 2012).

CONCLUSION

In this study, we examine the activities of both isozymes in barley after the induction with nitrate in leaves and roots of hydroponically N-depleted grown seedlings in buffer containing either 5mM Mg^{+2} or 5mM EDTA. The two isozymes activities were correlated with total reduced nitrogen and nitrate contents. Total reduced N was greater in leaves than roots during the whole light/dark/light treatment. However, much accumulation of reduced N in leaves occurred during the 10h dark and the difference became smaller at the 38h light period. Thus the total reduced N was strongly dependent upon continuous supply of nitrate. Our results also indicated that decrease accumulation of nitrate content in leaves after nitrate supply at in the second 38h light period in leaves than roots is roughly coincident with the increase in NADH-dependent NR activity in leaf than root.

Table 1: Fresh weight and total content of reduced N in barley seedlings after treatment with a N-medium. N-depleted seedlings (9-d-old) were treated with a nutrient solution containing 2.5mM KNO_3^- under a light-dark cycle of 14:10 h at 25ºC. Leaves and roots were harvested after 14h (a), 24h (b) and 38h (c) in the nutrient solution. Results are the means of three replicates (3 x 5 plants) ±SE.

(a)

Plant Part	Fresh weight ($g.plant^{-1}$) 14h (Light)	Reduced N (μ $mol.plant^{-1}$)
Roots	0.69 ± (0.13)	3.84 ± (0.07)
Leaves	1.16 ± (0.04)	23.63 ± (0.12)
Total plant	1.85 ± (0.16)	27.47 ± (0.17)
% of total Roots	37.92	13.97

(b)

Plant Part	Fresh weight ($g.plant^{-1}$) 24h (Light-Dark)	Reduced N (μ $mol.plant^{-1}$)
Roots	0.57 ± (0.07)	5.43 ± (0.04)
Leaves	1.44 ± (0.15)	30.81± (0.62)
Total plant	2.01± (0.09)	36.24 ± (0.61)
% of total Roots	28.36	14.98

(c)

Plant Part	Fresh weight ($g.plant^{-1}$) 38h (Light-Dark-Light)	Reduced N (μ $mol.plant^{-1}$)
Roots	0.80 ± (0.14)	6.99 ± (0.06)
Leaves	1.71± (0.19)	34.62± (0.37)
Total plant	2.51± (0.19)	41.61± (0.39)
% of total Roots	31.87	16.79

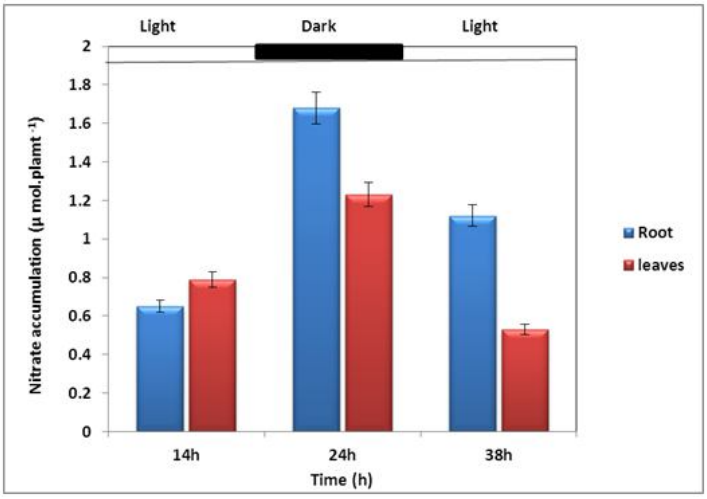
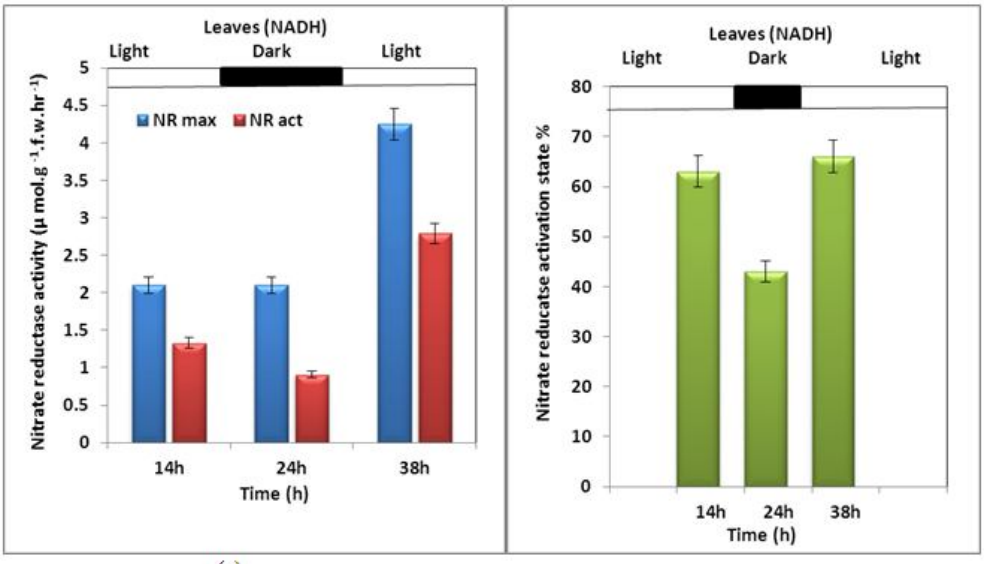
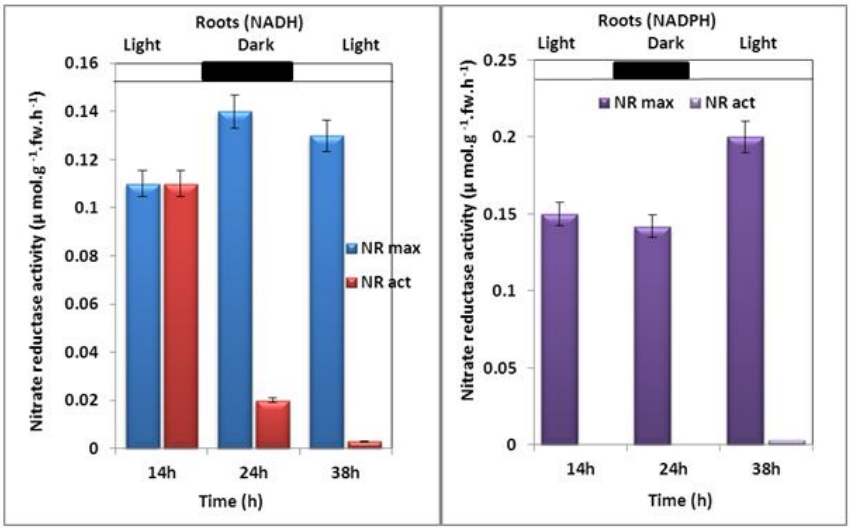


Fig. 1: Nitrate accumulation in roots and leaves of barley seedlings after treatment with a N-medium. N-depleted seedlings (9-d-old) were treated with a nutrient solution containing 2.5mM KNO_3^- under a light-dark cycle of 14:10 h at 25ºC. Leaves and roots were harvested after 14, 24, and 38h in the nutrient solution. Results are the means of three replicates (3 x 5 plants) ±SE.



(a) **(b)**

Fig. 2: N-depleted seedlings (9-d-old) were treated with a nutrient solution containing 2.5mM KNO_3^- under a light-dark cycle of 14:10 h at 25ºC. Leaves were harvested after 14, 24, and 38h in the nutrient solution. Leaves were harvested after 14, 24, and 38h in the nutrient solution. Activities of NR_{act}, NR_{max} (a) and activation state, which is NR_{act} x $100/NR_{max}$ (b) were determined in leaves. The enzyme activity was expressed as $\mu mol.g^{-1}.fw.h^{-1}$. Results are the means of three replicates ±SE. SEs exceeding the symbol size are indicated by bars.



(c) (d)

Fig. 3: N-depleted seedlings (9-d-old) were treated with a nutrient solution containing 2.5mM KNO_3^- under a light-dark cycle of 14:10 h at 25ºC. Roots were harvested after 14, 24, and 38h in the nutrient solution. Seedlings roots were harvested for determination of NR_{act} and NR_{max} for NADH-specific (c) and NAD (P)H-bispesific NRs in barley (d). The enzyme activity was expressed as µmol.g^{-1}.fw.h^{-1}. Results are the means of three replicates ±SE. SEs exceeding the symbol size are indicated by bars.

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