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Nitrogen Source Differently Regulates Barley *(Hordeum vulgare)* Response to NaCl Stress at Seed Germination and Early Seedling Development Stages

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Nitrogen (N) acts as nutrient and signaling molecule in plants all over their development stages. The involvement of various N forms in the regulation of seed germination response to salt stress was assessed in the present work. Nitrogen sources (NO, NO_2^-, NO_3^-, NH_4^+, glutamine and glutamate) were added at 1 mM to the germination medium of barley (*Hordeum vulgare,* cv Ardhaoui) in combination or not with NaCl stress (14 $g.L^{-1}$). The application of nitrogen monoxide (NO) alleviated by about 20% the NaCl-induced germination capacity decrease. However, the addition of ammonium ions (NH_4^+) and glutamic acid (Glu) accentuated the inhibitory effects of NaCl, decreasing germination capacity by about 50% compared to the control. The levels of malondialdehyde (MDA), which is an indicator of membrane lipid peroxidation by stresses, were increased by salinity in seeds treated with nitrite (NO_2^-), NO_3^-, Glu and Gln. In N-free medium, NaCl stress induced a severe nitrate reductase activity (NR, EC 1.6.1.6) inhibition. Such an effect was alleviated by the application of N treatments. Glutamate dehydrogenase (GDH, EC 1.4.1.2) aminating activity (NADH-GDH) of seedlings was inhibited by NaCl stress in the presence of NO, Glu and Gln. Conversely, there was stimulation by salt stress of NADH-GDH activity in seedlings treated with NaCl and NH_4^+. Deaminating GDH activity (NAD-GDH) was found to be enhanced by salt stress in NO_2^- and NO_3^- treatments. The differential effects of applied N forms on germination and early seedling development processes in this grass probably underlines different regulatory actions within N mobilization and assimilation.

Keywords: barley, seed, germination, salinity, nitrogen source

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Introduction

Barley *(Hordeum vulgare)* is a cereal crop largely cultivated in over 90 countries (Doré and Varoquaux 2006). In Tunisia, it ranks second after wheat and is an important crop for human and animal feed. Approximately 35% of the national barley production is processed into pellets and malt, the remaining part is used for animal feed (Deghais et al. 1999). Barley and wheat occupies large areas devoted to annual plants, representing about a quarter of the Tunisian cereal production. However, the distribution of the surface used for barley cultures is remarkably disparate, with a concretion in the north and the north-west of the country. Barley cultivation in central and southern Tunisia remains constrained by low and irregular rainfall associated with uncontrolled irrigation (Sbei et al. 2012). In fact, the use of alternative sources of irrigation water, often salty, is one of the solutions undertaken by farmers to overcome fluctuations in rainfall. However, this practice is generally associated with a progressive soil salinisation and crop productivity decrease (Levigneron et al. 1995).

Improving cereal tolerance to salinity is one of the solutions being undertaken to promote agriculture and food production in arid and semi-arid regions (Munns et al. 2006). Numerous approaches have been assayed for cereal salt tolerance improvement, such as genetic modifications (Gorham et al. 1997; Gregorio et al. 2002), screening, and germplasm selection (Lindsay et al. 2004). However, breeding cultivars for salt tolerance experiments remain complex for farmers working on unpredictable field conditions (Barrett-Lennard 2003). Alternatively, optimizing plant establishment in saline conditions will be a simple and inexpensive method to improve crop tolerance (Farooq et al. 2012).

Seed fertilization and/or seed priming by soaking them with nitrogen compounds at pregerminative phase has been shown as an effective practice to enhance germination capacity (Pérez-Fernández and Rodríguez-Echeverría 2003; Atia et al. 2009). Nevertheless, the improvement of seed germination closely depends on nitrogen form and ratio. For instance, Li et al. (2005) showed that salt stress effects on *Suaeda salsa* seed germination were alleviated by nitrite but not by nitrate treatments. At growth stage, a controlled NO_3^- to NH_4^+ ratio alleviated the deleterious effect of salt stress by better nitrogen assimilation efficiency (Kant et al. 2007). Indeed, nitrogen is a nutrient and signal molecule of a great importance regulating metabolic pathways during plant development (Forde and Lorenzo 2001). Nitrogen assimilation occurred firstly by nitrate reduction via the nitrate reductase (NR) activity to form ammonium which will be incorporated into aminoacids. Nitrogen remobilization, which generally occurs during germination and senescence, involves catabolic pathway such as glutamate dehydrogenase (GDH) and proteolysis. For the first time, we investigated the involvement of different nitrogen sources (NO, NO_2^-, NO_3^-, NH_4^+, glutamine and glutamate) at low concentration (1 mM) in alleviating salt stress effects on seed germination and young seedling development of a local barley variety, "Ardhaoui-Médenine". Our aim was to identify a nitrogen source that could be used for barley seed priming to ameliorate its response to salinity.

Materials and Methods

Germination experiment

Barley *(Hordeum vulgare)* seeds of a local variety "Ardhaoui-Médenine" were kindly provided by the Institute of Arid Lands (IRA) of Médenine (Tunisia). Seeds were sterilized with H_2O_2 (10%) for 5 min and subsequently washed with distilled water to avoid fungus attack, then germinated at 25°C in Petri dishes containing two disks of Whatman No. 1 filter papers with 5 mL of distilled water (control) or salt water (NaCl, 14 $g.L^{-1}$). The effects of N sources on germination and seedling development under saline and non-saline conditions were investigated by the application of 1 mM solutions containing NO (sodium Nitroprussiate), NO_2^- (Na_2NO_2), NO_3^- (KNO_3), NH_4^+ $(NH_4)_2SO_2$, Gln (L-glutamine) and Glu (L-Glutamate) in germination medium. A completely randomized design was used in the experiment. For each treatment, three replicates of 25 seeds were used. After five days, the numbers of germinated seeds were recorded and the coleoptile lengths of obtained seedlings were measured. A seed was considered germinated when the emerging radical elongated to 2 mm. Germination capacity (GC) was calculated as $GC = (\Sigma\ ni)* 100/N$, with (Σ ni) the cumulative number of germinated seeds on day i and N the total number of seeds.

Malondialdehyde (MDA) contents

After five days in each germination medium, the levels of lipid peroxidation in barley seedlings were assessed in terms of malondialdehyde (MDA) content by thiobarbituric acid (TBA), as recommended by Heath and Packer (1968), with minor modifications following Dhindsa et al. (1981). Seedlings were homogenized in trichloroacetic acid (TCA) (0.1%, w/v). The homogenate was then centrifuged at 8,000 *g* for 15 min. After that, the supernatant (1 mL) was precipitated with 4 mL TCA (20%) containing TBA (0.5%, w/v). The mixture was heated in a water bath shaker at 95°C for 30 min and quickly cooled in an ice bath. The absorbance was measured at 532 nm after samples were centrifuged at 8,000 *g* for additional 10 min, and the value for non-specific absorption at 600 nm was subtracted. MDA content was calculated using its extinction coefficient $e = 155\ mM^{-1}.cm^{-1}$.

Enzymatic assays

Nitrate reductase (NR) activity

Frozen barley seedlings collected after five days from each germination medium were homogenized in chilled mortar and pestle with 100 mM potassium phosphate buffer (pH 7.4) containing 7.5 mM cysteine, 1 mM EDTA, and 1.5% (w/v) casein. The homogenate was centrifuged at 30,000 *g* for 15 min at 4 °C. NRA activity was determined according to the method described by Robin (1979). The extract was incubated in a reaction mixture containing 100 mM potassium phosphate buffer (pH 7.4), 10 mM EDTA, 0.15 mM NADH, and 0.1 M KNO_3 at 30 °C for 30 min. The reaction was stopped by 100 µL sulfanilamide (5.8 mM) then an equal volume of 0.8 mM N-naphthyl-ethylene-diamine-dichloride

(NNED) was added. The absorbance of the supernatant was determined at 540 nm after diazotation of nitrite ions with 5.8 mM sulfanilamide and 0.8 mM NNED.

Glutamate dehydrogenase (GDH) activity

Enzyme extraction was performed according to the method described by Magalhaes and Huber (1991). Frozen barley seedlings collected after five days from each germination medium were homogenized in cold mortar and pestle with 100 mM Tris-HCl (pH 7.5), 14 mM β-mercaptoethanol, and 1% (w/v) PVP. The extract was centrifuged at 12,000 *g* for 15 min at 4 °C. GDH activity was determined by following the absorbance changes at 340 nm (Loyala-Vergas and De Jimenez 1984).

Statistical analysis

Data were subjected to a variance analysis (one-way ANOVA) using SPSS 16.0 for Windows program and means were separated according to Tukey's test at $P = 0.05$.

Results

Salt stress

In distilled water, germination was at least 2-fold faster than in the presence of NaCl, reaching its maximum (96%) within 24 h (Fig. 1). For the same period, only 36% of the seeds germinated in saline medium. Moreover, the final germination percentage did not exceed 70% in salt-treated seeds.

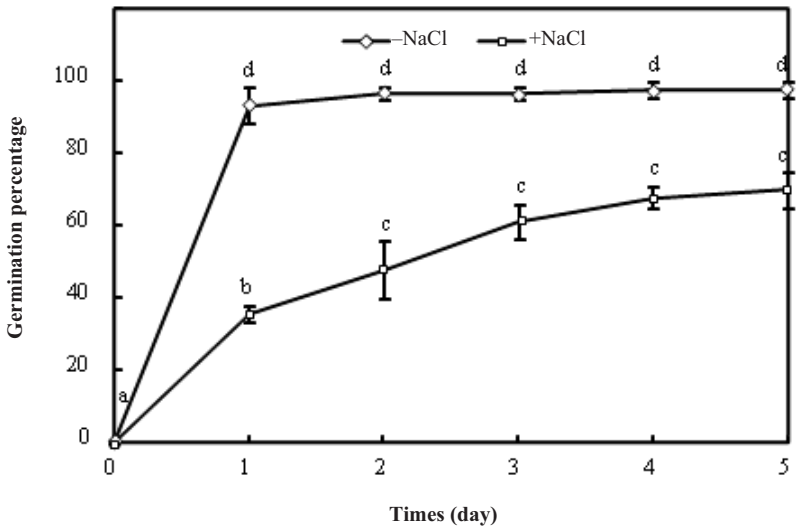

Figure 1. Germination rate in barley seeds under saline and non-saline conditions. Means of three replicates ± SE. Means labeled with different letters are significantly different according to Tukey's test at $P = 0.05$

Nitrogen treatments

Our results showed that the addition of nitrogen monoxide (NO) alleviated the inhibitory effect of salt on the germination process by about 20% (Fig. 2). The presence of nitrate (NO_3^-), nitrite (NO_2^-) and glutamine (Gln) in the germination medium did not improve seed responses to salt stress. However, ammonium (NH_4^+) and glutamic acid (Glu) addition accentuated the inhibitory effects of NaCl, inducing about 50% decrease in germination percentage compared to the control. In the absence of NaCl and regardless of nitrogen source, the length of emerged coleoptile was about 5 cm at the end of the treatment (Fig. 3). In the presence of NaCl, coleoptile length was blocked at an average of 1 cm. The addition of nitrogen compounds in the germination medium failed to significantly improve coleoptile elongation.

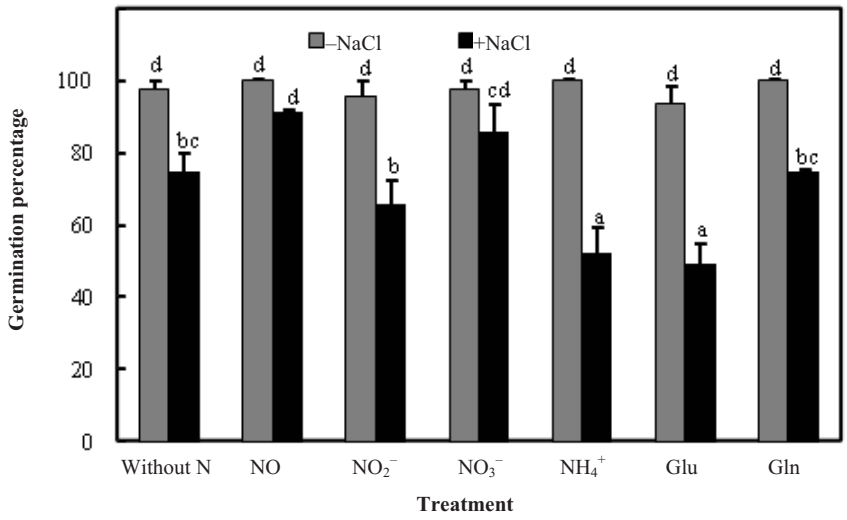

Figure 2. Germination percentages of barley seeds in response to several nitrogen sources under saline and non-saline conditions. Bars are means of three replicates ± SE. Bars labeled with different letters are significantly different according to Tukey's test at $P = 0.05$

MDA contents

MDA is a marker of oxidative stress that reflects the level of lipid peroxidation under stress, directly or indirectly by reactive oxygen species (ROS) (Sharma et al. 2012). Our results showed that salt treatment significantly reduced MDA concentration (Fig. 4). By contrast, NO, NH_4^+, Glu and Gln application under non-saline conditions resulted in increased concentrations of this compound, the highest value being recorded in Gln-treated seeds. The combined effects of salinity and NO, NO_2^-, NO_3^-, Glu or Gln induced also an increase in MDA level.

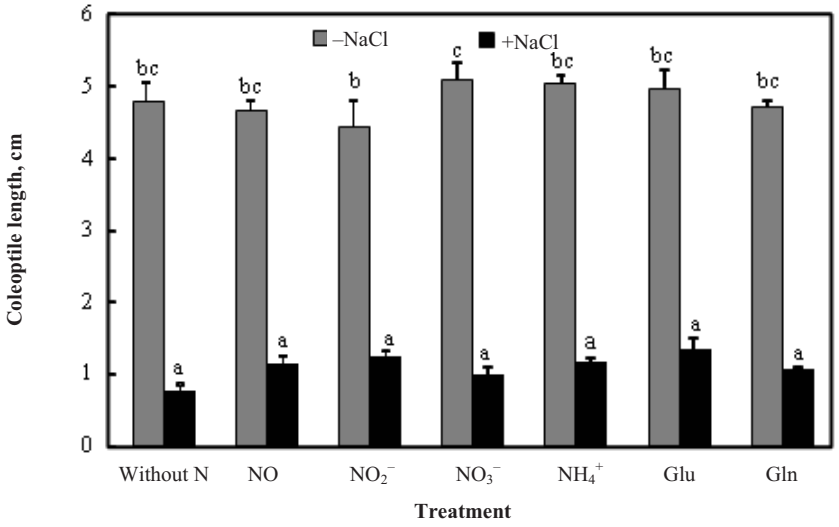

Figure 3. Coleoptile length of barley seedlings in response to several nitrogen sources under saline and non-saline conditions. Bars are means of nine replicates ± SE. Bars labeled with different letters are significantly different according to Tukey's test at $P = 0.05$

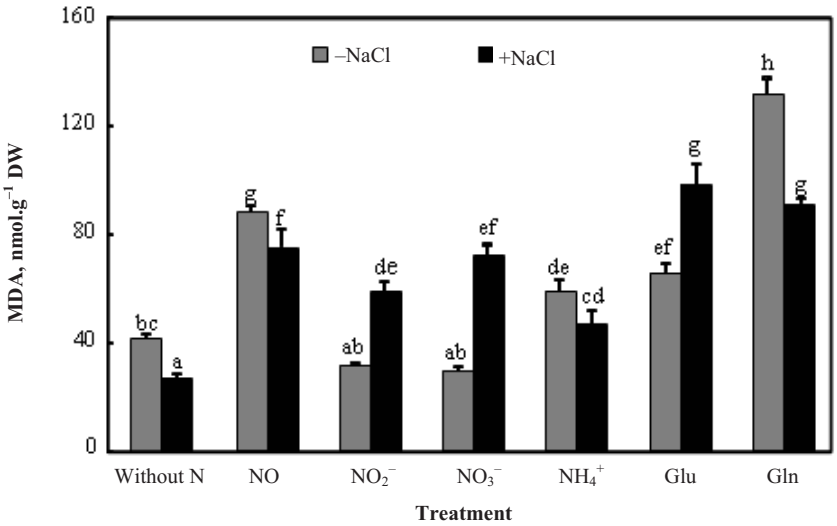

Figure 4. MDA concentration in barley seedlings in response to several nitrogen sources under saline and non-saline conditions. Bars are means of three replicates ± SE. Bars labeled with different letters are significantly different according to Tukey's test at $P = 0.05$

Nitrogen assimilation

In germination medium without nitrogen, NR activity was severely inhibited by NaCl stress (Fig. 5). Not only salinity reduced NR activity but also some nitrogen forms; NO_3^- induced no effect on this enzyme neither under saline conditions nor under non-saline conditions, whereas NH_4^+, Glu and Gln severely affected its activity regardless of salt concentration. The comparison of N form effects showed that as a whole, NR activity was higher in seeds treated with oxidized nitrogen forms (NO, NO_2^- and NO_3^-) compared to reduced ones. In particular, it seems that NR activity was enhanced by NO monoxide and NO_2^-.

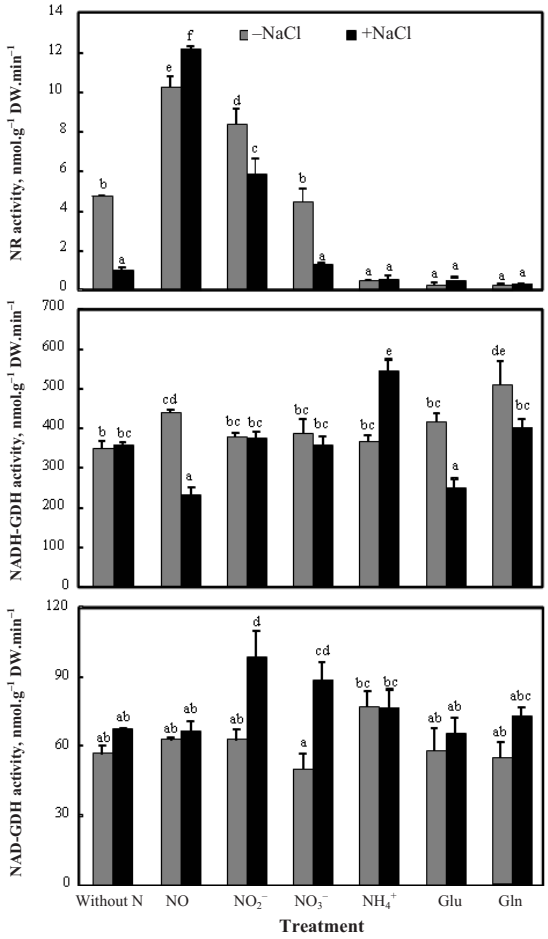

Figure 5. Changes in nitrate reductase (NR), aminating NADH-GDH and deaminating NAD-GDH glutamate dehydrogenase activities in barley seedlings in response to several nitrogen sources under saline and non-saline conditions. Bars are means of three replicates ± SE. Bars labeled with different letters are significantly different according to Tukey's test at $P = 0.05$

Glutamate dehydrogenase (GDH) is an enzyme which reversibly catalyses glutamate formation and catabolism in plants. During germination and/or senescence, released ammonium by catabolic reactions is re-assimilated by GDH to form glutamate (Glevarec et al. 2004). According to our results, GDH aminating activity (NADH-GDH) was not affected by salinity (Fig. 5). However, it experienced different responses to nitrogen forms and their interaction with salt treatment. Under non-saline conditions, only NO and Gln improved GDH aminating activity, whereas no detrimental effect was recorded. Under saline conditions, however, NO and Glu decreased this activity contrarily to NH_4^+ that elevated it to 155% of the control.

Similarly to GDH aminating activity (NADH-GDH), its deaminating activity (NAD-GDH) was not modified by salt treatment (Fig. 5). In addition, under non-saline conditions, N supply exhibited no significant effect regardless of its form. Under saline conditions, however, GDH deaminating activity was enhanced by 72 and 54%, respectively, in nitrite and nitrate-supplied seedlings.

Discussion

Based on obtained changes in physiological and biochemical germination parameters, we tried to give further insight into the involvement of N forms in salt response regulation of the Tunisian barley cultivar (Ardhaoui Medenine) at the germination and seedling stages.

The inhibitory action of salt stress on barley seeds was displayed by a lowering of seed germination capacity (Fig. 1). Similar effects of NaCl on other poaceae germination were often reported in literature (Li et al. 2010; Debouba et al. 2012). This effect may be related to osmotic and/or ionic effects. Indeed, NaCl excess in the germination medium increases the osmotic pressure, which restricts seed imbibition, delaying reserve mobilization and inhibiting the transport of hydrolysis products to the embryo (Voigt et al. 2009; Debez et al. 2012). Other studies showed that the action of salt stress on germination was related to the oxidative stress it induced (Wang et al. 2009).

In a second part of this work, we applied different nitrogen sources in attempt to improve the germination capacity of barley seeds in saline medium. In fact, the interaction between two or more environmental factors can induce additive (the sum of the individual effects), synergistic (higher than additive), or antagonistic (lower than additive) effects (Bansal et al. 2013). In this investigation, we found that the addition of nitrogen monoxide form was associated with promising mitigation of inhibitory effects of NaCl on barley seed germination (Fig. 2). The ameliorative effect of NO under salt stress conditions has been mentioned in some research studies (Shi et al. 2007; Manai et al. 2014), while its mechanism of action is still hypothetical. Some authors postulated that endogenous NO avoids toxic accumulation of salt ions by decreasing cell membrane permeability (Zhang et al. 2006). Zhang et al. (2004) showed also that the improvement of salt tolerance in maize *(Zea mays)* by NO is closely related to the increased activity of ATPase and pyrophosphatase Na^+/H^+ antiporter at the tonoplast. The activation of the Na^+/H^+ antiporter at the plasma membrane and tonoplast facilitates the efflux of Na^+ ions, reducing their toxic accumulation.

It should be noted that NO treatment of barley seeds was associated with an improvement in NR activity (Fig. 5), a lower deaminating activity of GDH and a higher peroxidation of membrane lipids (Fig. 4). Our results are in disagreement with some findings showing that the application of NO stimulated the antioxidant enzymes to protect plant cells from lipid peroxidation under salt stress conditions (Shi et al. 2007).

The inhibitory effect of NaCl on germination was severely accentuated by NH_4^+ (Fig. 2). Indeed, NH_4^+ is known as a toxic compound, whose accumulation induces toxicity in plant cells (Britto and Kronzucker 2002). It disrupts cellular respiration by altering membrane structures and canceling proton gradient. It acts also as uncoupler of phosphorylation in mitochondria. Ammonium accumulation under salt stress conditions was generally observed in salt sensitive species (Santos et al. 2004). The induction of aminating GDH activity (Fig. 5) appears to be an adaptive mechanism to avoid toxic ammonium levels in salt-stressed seeds.

The application of L-Glu also increased barley seed sensitivity to salt stress (Fig. 2). Besides, Glu treatment induced a greater accumulation of MDA in comparison with the control (Fig. 4). However, it seems that L-Glu effect on germination is closely dependent on plant species. In fact, it improved the germination capacity of cucumber seeds under saline conditions (Chang et al. 2010).

The addition of several nitrogen sources to barley seeds exposed to NaCl stress resulted in: (i) an alleviation of salt stress effects (case of NO), (ii) an aggravation of salt stress effects (cases of NH_4^+ and L-Glu) or (iii) no significant changes (cases of NO_2^-, NO_3^- and Gln). The differential actions of applied N forms on barley seeds may underline different regulatory actions within N mobilization and assimilation during germination. It would be interesting to follow their function as nutrient and signal on the key nitrogen metabolism steps (GS/GOGAT cycle) during barley seed germination and establishment under salt stress. All these data taken together showed that the applied nitrogen treatments changed germination capacity of barley seeds, but without recovering coleoptile elongation.

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