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Nitrate reductase in durum wheat seedlings as affected by nitrate nutrition and salinity

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Abstract. The combined effects of nitrate (0, 0.1, 1, 10 mM) and salt (0, 100 mM NaCl) on nitrogen metabolism in durum wheat seedlings were investigated by analysis of nitrate reductase (NR) expression and activity, and metabolite content. High salinity (100 mM NaCl) reduced shoot growth more than root growth. The effect was independent of nitrate concentration. NR mRNA was present at a low level in both leaves and roots of plants grown in a nitrogen-free medium. NaCl increased NR mRNA at low nitrate, suggesting that chloride can mimic nitrate as a signal molecule to induce transcription in both roots and leaves. However, the level of NR protein remained low in salt-stressed plants, indicating an inhibitory effect of salt on translation of NR mRNA or an increase in protein degradation. The lower activity of nitrate reductase in leaves of high-nitrate treated plants under salinity suggested a restriction of NO_3^- transport to the shoot under salinity. Salt treatment promoted photorespiration, inhibiting carbohydrate accumulation in plants grown on low nitrate media. Under salinity free amino acids, in particular proline and asparagine, and glycine betaine could function as osmolytes to balance water potential within the cell, especially when nitrogen availability exceeded the need for growth.

Keywords: compatible solutes, glycine betaine, nitrogen metabolism, nitrate reductase, salt stress, *Triticum durum*.

Introduction

Salinity can greatly reduce crop productivity in glycophytic plants, which usually tolerate only relatively low salt concentrations. Salt stress reduces plant growth by inhibiting many physiological and biochemical processes such as nutrient uptake and assimilation (Munns 2002). High salt concentrations, usually sodium chloride, cause osmotic stress by decreasing water potential within the cells, and ionic stress due to specific inhibition of metabolic processes. Plants respond to salinity by sequestering toxic ions in the vacuoles and accumulation of compatible solutes in the cytoplasm to balance the decrease of water potential (Hasegawa *et al.* 2000; Di Martino *et al.* 2003). These substances are rapidly synthesised under osmotic stress and rapidly removed when no longer required. Many plants synthesise nitrogen-containing compatible osmolytes such as amino acids, amines or betaines (Abd-El Baki *et al.* 2000; Mansour 2000). The accumulation of compatible solutes, which are non-toxic at high concentrations, allows turgor maintenance and / or protection of macromolecular structures against the destabilising effect of the decrease in water activity (Sairam *et al.* 2002).

Nitrate uptake and transport are particularly affected by salinity (Gouia *et al.* 1994), since chloride competes with nitrate for uptake and translocation within the plants by nitrate transporter proteins (Campbell 1999). The sensitivity of different species to salt is related to the chloride sensitivity of their nitrate uptake systems (Leidi and Lips 1990). The loading of nitrate into the root xylem is also thought to be a highly salt-sensitive step (Peuke *et al.* 1996; Tischner 2000). Salinity may strongly affect the overall nitrate assimilation process because nitrate is required to induce nitrate reductase (NR; EC 1.6.6.1), the key enzyme of the overall assimilation process (Campbell 1999). NR activity in leaves is largely dependent on nitrate flux from roots (Ferrario-Méry *et al*. 1998; Foyer *et al*. 1998) and is severely affected by NaCl salt stress (Rao and Gnanam 1990; Abd-El Baki *et al.* 2000; Silveira *et al.* 2001).

Nitrate reductase is regulated at transcriptional, translational and post-translational levels (Tischner 2000). With the exception of nitrate, sugars and amino acids can also control NR expression (Stitt *et al.* 2002; Iglesias-Bartolomé *et al*. 2004; Lillo *et al.* 2004). The enzyme itself is regulated at the post-translational level

Abbreviations used: FW, fresh weight; NR, nitrate reductase; RT–PCR, reverse transcriptase–polymerase chain reaction; RWC, relative water content.

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by reversible protein phosphorylation on a serine residue. The phosphorylated enzyme binds to 14-3-3 proteins, inhibiting enzyme activity and making it more susceptible to proteolytic degradation (Kaiser *et al.* 1999).

Phosphorylation is involved in the response of the enzyme to light and dark conditions, stomatal closure and oxygen supply in the roots (Botrel and Kaiser 1997). In addition, the nitrate concentration in the cytosol, as well as triggering NR synthesis, seems to protect the enzyme against proteolytic degradation (Campbell 1999). Therefore, any impact of salinity on nitrate levels might be expected to affect NR transcription, expression, activity and activation state, as well as protein turnover (Kaiser *et al.* 1999, 2002).

In this paper we studied the combined effects of different concentrations of nitrate (0, 0.1, 1, 10 mM) and salt (0, 100 mM) on nitrogen assimilation in durum wheat seedlings (*Triticum durum* Desf. cv. Ofanto) by analysis of nitrate reductase. Effects at different levels of regulation were investigated by measurements of NR mRNA, protein, enzyme activity, activation state in roots and leaves. Ion and metabolite levels were also measured to provide further information on possible factors affecting not only NR expression and activity, but also more global changes such as nitrogen metabolism and its relations to photosynthesis, photorespiration and ultimately growth.

A systematic study of the physiological and biochemical responses to salt stress in different nitrogen conditions is very important in understanding how plants operate under salt stress. Such knowledge will assist in development of methods to detect cultivars more resistant to salinity, and selection of salt-tolerant cultivars through new methods such as genetic engineering (Lindsay *et al.* 2004).

Materials and methods

Plant material and growth conditions

Durum wheat (*Triticum durum* Desf. cv. Ofanto) seeds, supplied by the 'Istituto Sperimentale per la Cerealicoltura, Foggia', were germinated in the dark at 21°C on filter paper moistened with deionised water. After 3 d, when the primary root was emerging from the seed, individual seedlings were transferred to eight 4-L pots with perforated plastic tops (60 plants per pot) containing aerated distilled water. After 3 d of hydroponic culture the water was replaced with a modified (nitrogen-free) Hoagland's solution as described by Rigano *et al.* (1996). Plants were kept under controlled conditions (16 h photoperiod, 300 μmol m^{-2} s^{-1} PAR, thermoperiod 25 / 20°C day / night, 65% relative humidity).

Experimental design for nitrate and salt treatment

On day six of hydroponic culture, the plants were divided into four groups (two pots in each group), and each group was transferred to Hoagland's solution containing a different concentration of KNO_3 (0, 0.1, 1 or 10 mM). The K^+ concentration in the culture media ranged between 5 and 15 mM from low to high nitrate treatments, respectively, K^+ being a component of the salts used to prepare the culture Hoagland's solution. The nutrient solution was replaced every 3 d. Starting from day 10 of culture, one pot from each group was subjected to salt stress by supplementing the hydroponic medium with 50 mM NaCl, increased to 100 mM NaCl on the day 11. The NaCl concentration was increased gradually in this way to reduce the salt shock to the plants. The control plants in the other pot from each group were grown without supplemented NaCl. The lengths of the leaves and roots were measured every 5 d.

Measurements of fresh and dry weight

Six replicate plants from each treatment were harvested on the 10th and 20th days of hydroponic culture and weighed immediately to give the fresh weight. They were then oven-dried at 70°C to constant weight (48 h) and the dry weight was recorded. The water content was calculated as the difference between the fresh and dry weights.

Plant analysis

After 20 d of hydroponic culture, plants from each pot were harvested 4 h after the beginning of the light period, divided into shoot and root, ground in liquid nitrogen and either used immediately for assays or stored at –80°C.

Protein determinations

Soluble and total protein content were quantified by the method described by Bradford (1976) with bovine serum albumin as standard as described by Augusti *et al.* (1999).

Solute analysis

Na^+, Cl^-, K^+ and NO_3^- were extracted from 50 mg of dried tissue. The tissue was ground to a fine powder and suspended in 5 mL of 10 mM glycine solution, pH 6.1, then subjected to three freeze–thaw cycles by freezing in liquid nitrogen and thawing at 40°C for 20 min. Samples were centrifuged (4218 D, ALC, Cologno Monzese, Italy) at 4000 *g* for 10 min and the clear supernatants were assayed in a 100 mM MES–Tris, pH 7, ionic strength adjustor with continuous stirring using Na^+- (Orion 86-11, Orion Research, Boston, MA), K^+- (Orion 9719BN), NO_3^-- (Orion 9307BN) and Cl^-- (Orion 9417SC) specific electrodes connected to a CR10X data logger (CRX10, Campbell Scientific, North Logan, UT). A calibration curve was constructed with standard solutions containing 0.1, 1.0 and 10 mM K^+, Cl^-, Na^+ or NO_3^-. Measurements were taken in triplicate.

Amino acids were extracted by mixing aliquots of 50 mg fresh weight with 1 mL of ethanol : water (40 : 60 v : v), incubated overnight at 4°C and centrifuged (5417 R, Eppendorf, Hamburg, Germany) at 14 000 *g* (5 min). The extraction procedure was repeated on the pellet. Preliminary experiments showed that 93% of the total amino acids were recovered in the first extraction. The supernatants were pooled and used for the analyses. The primary amino acids were determined by HPLC after pre-column derivatisation by *o*-phthaldialdehyde (OPA). The OPA reagent mixture was prepared by mixing 550 μL of 0.8 mM Na–borate buffer, pH 10.4, 200 μL of OPA dissolved in methanol (50 mg mL^{-1}) and 55 μL of pure β-mercaptoethanol. The extract (20 μL) was derivatised with OPA reagent (20 μL) for 3 min in the autosampler needle, injected onto the column (reverse phase 5 μm ultrasphere C18, 250 × 4.6 mm internal diameter; Beckman Coulter, Fullerton, CA) and eluted at a flow rate of 1 mL min^{-1} at 25°C with a discontinuous gradient. Solvent A was a mixture of 50 mM sodium acetate, adjusted to pH 6.4 with acetic acid, plus 2% (v / v) tetrahydrofuran. Solvent B was pure methanol. The gradient program was as follows: 0 min 0% B, 1 min 0%B, 11 min 3% B, 13 min 7.2% B, 17 min 7.2% B, 32 min 10% B, 38 min 18% B, 48 min 38% B, 57 min 60% B, 60 min 80% B, 62 min 95% B, 72 min 100% B, 80 min 0% B. The amino acid-OPA derivatives were detected by their fluorescence with excitation at 330 nm and emission at 450 nm. Proline was extracted and estimated by the procedure by Bates (1973). Sugars and starch were extracted and determined according to Pietrini *et al.* (1999). Starch was expressed as glucose equivalents.

Glycine betaine was extracted and assayed according to Bessieres *et al.* (1999). Powdered leaf and root samples (50 mg) were mixed with 1250 mL of distilled water, incubated at 4°C overnight, and then

centrifuged for 5 min at 14 000 *g*. The extraction procedure was repeated on the pellet. More than 95% of total glycine betaine was recovered in the first extraction. The pooled supernatants were passed through a cationic exchange resin (AG1 8X resin, 200–400 mesh, OH^- form, Bio-Rad, Hercules, CA). A 5-mL syringe type column was loaded with 2 mL of resin and centrifuged at 300 *g*, 4°C, for 3 min. An aliquot of the soluble tissue extract (125 µL) was applied to the column, followed by 875 µL of distilled water. The eluate was collected by centrifugation (300 *g*, 4°C, for 3 min) and either analysed immediately or stored at –20°C. Glycine betaine was determined by isocratic HPLC on a Spherisorb 5 ODS2 C_{18} column (250 × 4.6 mm internal diameter). The solvent (13 mM sodium heptane sulphonate, 5 mM Na_2SO_4 in deionised water, adjusted to pH 3.7 with H_2SO_4) was delivered in isocratic conditions and the flow rate 0.8 mL min^{-1}. The eluted glycine betaine was detected by measuring absorbance at 200 nm using a diode array spectrophotometer (HP 8452A, Agilent Technologies, Palo Alto, CA) and quantified by comparison with standard solutions eluted in the same conditions.

Nitrate reductase extraction and assay

Nitrate reductase activity was extracted and assayed as described by Gibon *et al.* (2004).

Western blot analysis

Nitrogen reductase protein level in root and leaf extracts was analysed by immunoblotting. Root and leaf tissues were extracted according to Botrel and Kaiser (1997) and Abd-El Baki *et al.* (2000), respectively. Samples containing 5 µg protein were submitted to SDS–PAGE (10% separating gel, 6% stacking gel), and the separated proteins were blotted onto nitrocellulose membranes (Protran®, Schleicher and Schuell, Germany). After blotting, the membranes were blocked by incubating with 5% non-fat milk powder in PBS (10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, 137 mM NaCl, 2.7 mM KCl, pH 7.4) containing 0.1% (v/v) Tween 20 for 1 h at room temperature. Immunodetection of NR was conducted with rabbit antiserum raised against purified NR from maize (Vector Laboratories, DBA Italia SRL, Milan, Italy). The secondary antibody was peroxidase-linked anti-rabbit IgG. The membrane was incubated for 1 min in the detection reagent (1.25 mM luminol, 6.8 mM coumaric acid, 3% hydrogen peroxide) and then exposed to X-ray film (X-OMAT S, Kodak, Rochester, NY) for 10 min at room temperature. NR bands were identified by co-electrophoresis with purified maize NR (Sigma, St Louis, MO).

Analysis of NR mRNA

Total RNA from roots and leaves of durum wheat seedlings was extracted using Trizol® reagent (Gibco BRL), based on the phenol–guanidium thiocyanate procedure (Chomczynski and Sacchi 1987). To determine the level of mRNA for nitrate reductase an aliquot of total RNA was reverse transcribed and the resulting NR-cDNA was detected by PCR, using a SuperScript One-Step RT–PCR kit with Platinum® Taq, (Gibco BRL) according to the manufacturer's instructions. This assay allowed both cDNA synthesis and PCR amplification in a single tube using gene-specific primers and, as target, total RNA. The reaction mixtures (50 µL) contained 1 µg of total RNA, 1 µM of each primer, 1.6 mM $MgSO_4$ and 0.2 mM of each dNTP.

NR-specific primers were designed by comparison of maize and barley genes (Gen-Bank accession number AF153448 and X57845, respectively): *Triticum*-NR-forward (5′-ACGTACCACA CACCTTGAGCCTGTC-3′) and *Triticum*-NR-reverse (5′-AGCA CGTCCACGCCGTTCATGAACACC-3′). The cDNA of the IVS1 region of two ribosomal genes was used as an internal standard in the same reaction. The primers bind to three regions of the 18S and 5.8S rDNA respectively: *IVS1*-AS (5′-ATCCTGCAATTCACACCAAGTA TCG-3′), *IVS1*-S (5′-GGAGAAGTCGTAACAAGGTTTCCG-3′). The lengths of the PCR products were 900 and 600 base pairs for NR and rDNA, respectively.

Reactions were conducted with the Gene Amp PCR system 9700 (PE Biosystems, Branchburg, NJ) under the following conditions: 30 min at 50°C, 2 min at 94°C, followed by 30 cycles of 1 min at 94°C, 1.5 min at 50°C, 1 min at 72°C and 10 min of final extension at 72°C.

The resulting PCR products were resolved by electrophoretic separation through a 1% agarose gel in 1 × TBE buffer and directly visualised with ethidium bromide staining and ultraviolet illumination. The NR signal was normalised to the rRNA signals.

Statistical analysis

The experiment was repeated three times. In each experiment the measures were repeated on at least four different samples for each treatment. The results are given as mean ± SD of replications within each experiment.

Results

Plant growth

The shoot length of wheat seedlings increased between days 1 and 20 of hydroponic culture in control plants (Fig. 1*A*). Nitrate nutrition strongly affected shoot growth. The growth rate for seedlings grown with 10 mM NO_3^- was 2–3-fold higher than in plants grown without nitrate in both the first and second 10-d-periods of the study. In contrast, the rate of root elongation was higher in plants grown without nitrate between days 10 and 20, remaining higher in roots than in leaves (Fig. 1*C*).

At day 20 the fresh shoot weight was highest in the high nitrate plants. It varied from 0.21 ± 0.04 g to 0.81 ± 0.16 g per plant from nitrogen-free and 10 mM NO_3^- culture, respectively (Table 1). The dry shoot weight (about 10% of the fresh weight) showed a similar pattern. The fresh and dry root weights were not significantly different in low and high nitrate grown plants (Table 1).

During the 10 d of salt addition shoot and root elongation rates were reduced to the same value, ∼0.25 and ∼1.75 cm d^{-1}, respectively, independently of nitrate nutrition (Fig. 1*B–D*).

Fresh shoot weight decreased with salinity by 25 and 40% of controls in 0 and 10 NO_3^- grown plants, respectively. The fresh root weight decreased to 65% of the controls at all nitrate concentrations. There was no significant difference, in relation to salinity, in the water content of either shoots and roots, at day 20 (Table 1).

Inorganic ion content of leaves and roots in salt treated plants

The concentration of chloride in Hoagland's solution without added NaCl was 2 mM. The chloride content of roots from controls plants decreased as the nitrate concentration in the culture medium was increased (Fig. 2*A*). Conversely, Cl^- concentration in leaves was independent of nitrate nutrition. In the salt stressed plants, root chloride concentration increased most when nitrate was lower than 10 mM in the culture medium. The Cl^- content of the leaves increased at all NO_3^- concentrations, reaching about 400 µmol g^{-1} FW,

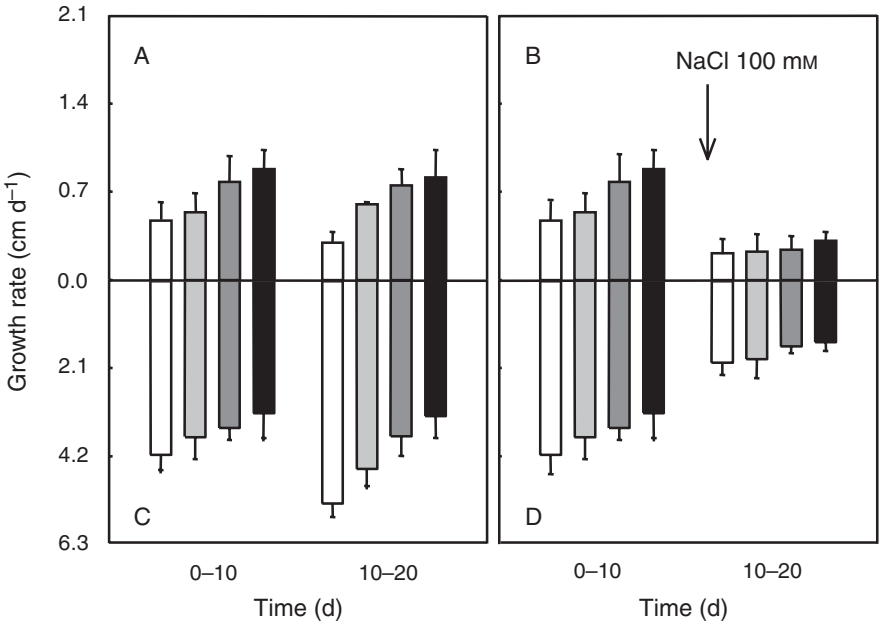

Fig. 1. Comparative growth rates of shoot (*A*, control; *B*, + salt) and root (*C*, control; *D*, + salt) length growth in durum wheat. Six replicate plants of each treatment were measured on days 10 and 20 of hydroponic culture. Nitrate was added on day five: white bars, 0 mM KNO_3; pale grey bars, 0.1 mM KNO_3, dark grey bars, 1 mM KNO_3, black bars, 10 mM KNO_3. 100 mM NaCl was added from day 10. The values are means ± SD ($n = 6$).

Table 1. Growth parameters, relative water content (RWC), glycine betaine and protein content of shoots and roots of durum wheat

Plants were harvested after 20 d of hydroponic culture and 10 d after the beginning of salt treatment, 4 h into the light period. Water content is given as percentage of water per FW. The values are means ± SD ($n = 4$–6)

	Control		NaCl (100 mM)	
Growth parameter	NO_3^- (0 mM)	NO_3^- (10 mM)	NO_3^- (0 mM)	NO_3^- (10 mM)
Shoot				
FW (g)	0.21 ± 0.04	0.81 ± 0.16	0.16 ± 0.01	0.49 ± 0.07
DW (mg)	46.4 ± 7.8	93.2 ± 20.3	29.5 ± 1.7	78.7 ± 8.8
Maximum length (cm)	14.6 ± 1.7	19.5 ± 1.6	13.3 ± 2.0	17.9 ± 1.5
RWC (% water)	78.1 ± 1.2	88.5 ± 0.6	81.4 ± 0.8	83.8 ± 1.0
Glycine betaine (μmol g^{-1} FW)	0.66 ± 0.09	3.55 ± 0.15	1.86 ± 0.14	8.83 ± 0.38
Soluble protein (mg g^{-1} FW)	5.66 ± 1.29	13.69 ± 3.06	4.86 ± 0.22	14.67 ± 1.76
Total protein (mg g^{-1} FW)	15.27 ± 1.90	18.88 ± 1.68	11.93 ± 1.85	21.85 ± 0.29
Root				
FW (g)	0.60 ± 0.16	0.84 ± 0.08	0.43 ± 0.04	0.57 ± 0.01
DW (mg)	52.1 ± 6.5	58.0 ± 8.2	45.7 ± 6.7	46.3 ± 4.3
Maximum length (cm)	61.3 ± 8.8	43.1 ± 5.9	43.3 ± 4.1	33.6 ± 5.3
RWC (% water)	90.1 ± 0.8	93.1 ± 0.4	89.3 ± 0.9	91.9 ± 0.4
Glycine betaine (μmol g^{-1} FW)	0.27 ± 0.03	0.55 ± 0.09	1.18 ± 0.05	4.29 ± 0.80
Soluble protein (mg g^{-1} FW)	1.25 ± 0.15	2.33 ± 0.44	1.03 ± 0.15	2.54 ± 0.23
Total protein (mg g^{-1} FW)	3.20 ± 0.88	5.11 ± 1.12	5.61 ± 0.22	8.38 ± 0.30

in all except the 10 mM nitrate grown plants, in which chloride content was about 300 μmol g^{-1} FW (Fig. 2*A*).

The sodium content of roots and leaves of plants grown in Hoagland medium were similar, ranging between 30 ± 4 and 10 ± 2 μmol g^{-1} FW in plants grown on low nitrate and 10 mM nitrate, respectively (Fig. 2*B*). In plants submitted to salt treatment the root Na^+ content increased with nitrate nutrition; it was 60 ± 10 and 120 ± 15 μmol g^{-1} FW in 0 and 10 mM NO_3^- grown plants, respectively. The leaf sodium content was fairly constant at approximately

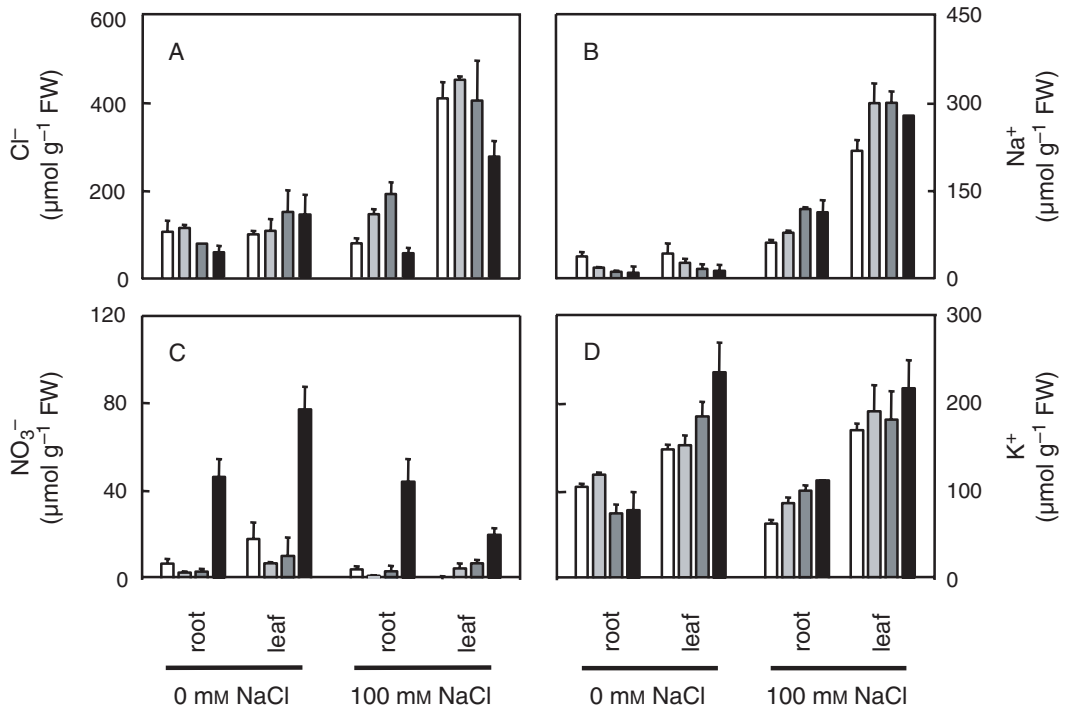

Fig. 2. Effect of nitrate and salinity on cation and anion concentrations in roots and leaves of durum wheat after 20 d of hydroponic culture. Nitrate was added on day five of hydroponic culture: bar colours are as in Fig. 1. 100 mM NaCl was added from day 10 of hydroponic culture. The values are means ± SD ($n = 4$–6).

300 ± 40 μmol g^{-1} FW in nitrate grown plants and was 220 ± 20 μmol g^{-1} FW only in plants grown in nitrate free medium (Fig. 2*B*).

The nitrate concentration of roots and leaves on a fresh weight basis always exceeded that of the nutrient solution (Fig. 2*C*). Root nitrate content was not significantly affected by the salt treatment, being similar to that in control plants. In contrast, leaf nitrate content was always lower in salt-treated plants than in controls. In plants grown with high nitrate, in particular, the salt treatment reduced leaf NO_3^- content to about 30% of the controls.

Potassium content was not significantly dependent on nitrate or salt treatment; the highest value of K^+ was found in leaves of plants grown with 10 mM NO_3^- (Fig. 2*D*).

Protein and amino acid content

The soluble and total protein content of both leaves and roots increased more when plants were grown in high-nitrate medium compared with nitrate-free medium. Leaf protein content was only mildly affected by the salt treatment, but the root total protein content was 70% higher in salt-treated plants grown on both low- and high-nitrate medium (Table 1).

The total free amino acid concentration in roots and leaves was directly dependent on nitrate nutrition, and was higher in leaves than in roots. Glutamate (Fig. 3*C*), glutamine (Fig. 3*E*), serine (Fig. 3*D*) and proline (Fig. 3*F*) were quantitatively the major amino acids.

The pool of total amino acids strongly increased in both organs in salt treated plants. This was mainly due to serine, asparagine and proline, which increased 2.5-, 7- and 10-fold, respectively. Glutamine and glutamate, which have both been implicated in regulation of NR expression and activity (Abd-El Baki *et al.* 2000), were both increased by salt treatment in leaves, independently of nitrate concentration, but they were relatively unaffected in roots, except in the plants grown with 10 mM nitrate.

Glycine betaine

In control plants, the glycine betaine content was higher in the leaves than in the roots, and was dependent mainly on the nitrate concentration in the media (Table 1). Under salinity glycine betaine concentration increased by about 4–5-fold in both roots and leaves of 10 mM nitrate grown plants compared with those grown without nitrogen in the culture medium.

Starch, sucrose and reducing sugar content

Starch content was strongly affected by nitrate nutrition in control plants. The amount of starch in leaves of plants grown without nitrate (213 ± 28 μmol glucose equivalents g^{-1} FW) was about 40-fold higher than in 10 mM NO_3^- cultured plants (5 ± 1 μmol glucose equivalents g^{-1} FW, Fig. 4*A*).

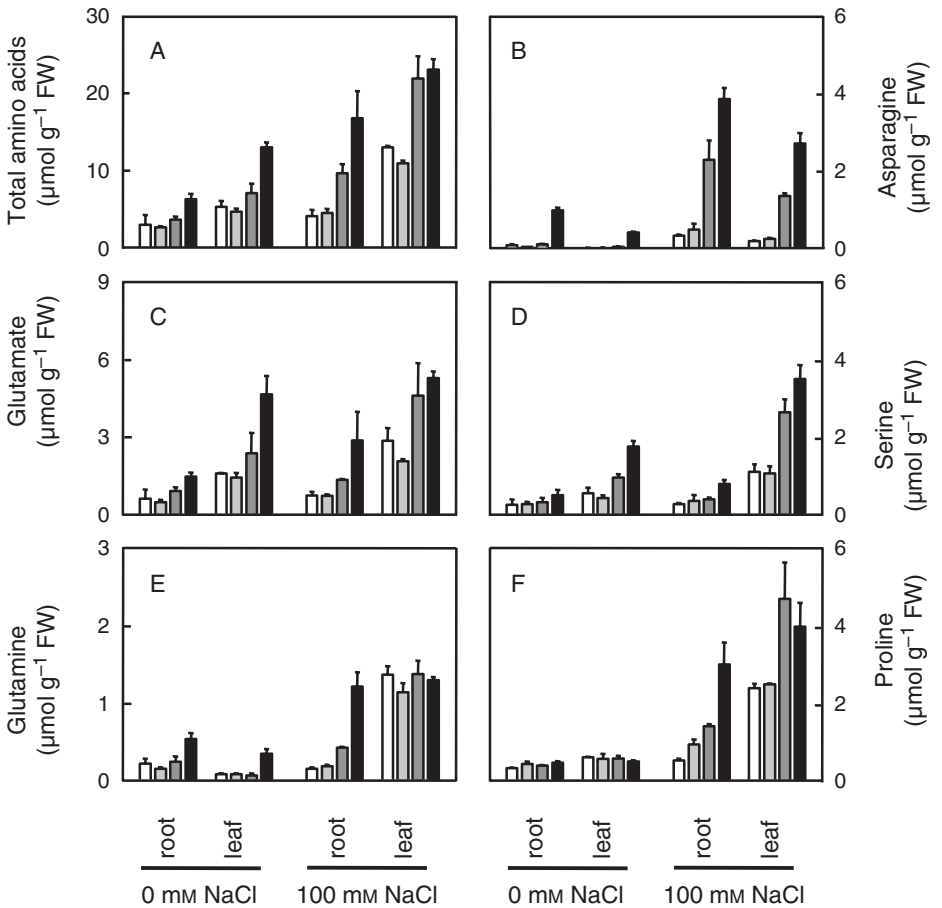

Fig. 3. Effect of nitrate on amino acid content under salinity in plants harvested after 20 d of hydroponic culture. Nitrate was added on day five of hydroponic culture: bar colours are as in Fig. 1. 100 mM NaCl was added from day 10 of hydroponic culture. The values are means ± SD ($n = 4$).

Root starch content was also influenced by nitrate nutrition, but to a lesser extent.

Salt treatment strongly reduced the starch content in leaves of plants grown in nitrate-free medium. Leaf starch content was also decreased by salt treatment in plants grown with low and intermediate levels of nitrate, but to a lesser extent. However, leaf starch content was increased slightly in plants grown with high nitrate.

In contrast, salt treatment increased the starch content of the roots by about 2-fold, regardless of the nitrate concentration in the culture medium.

Sucrose concentrations showed a similar trend to starch in leaves, but salt treatment did not significantly affect the sucrose concentration in the roots. The hexose (glucose and fructose) concentration in the roots decreased slightly in response to nitrate concentration in the culture medium, but it was not significantly affected by the salt treatment. Leaf hexose content was unaffected by nitrate or salt treatment.

Nitrate reductase transcription, expression and activity

Nitrate reductase-mRNA was present at low level also in leaves and roots of plants grown in a nitrogen-free medium (Fig. 7*A–B*). In figures 7*C* and *D*, the data were normalised to the rRNA signals. Salt induced an increase in the NR mRNA in roots at low nitrate concentration and in leaves at 0.1 mM nitrate, suggesting an inductive effect of chloride on NR. At high nitrate and salt concentration the effect was reversed, and the level of NR mRNA decreased.

In the roots (Fig. 8*A*) and leaves (Fig. 8*B*), NR protein content increased with nitrate concentration in the medium. Salt treatment decreased NR protein in the roots especially at low nitrate

Nitrate reductase activity was assayed in roots and leaves of durum wheat 4 h after the beginning of the light period (Fig. 8*C–D*). In control plants leaf NR activity varied during the light period reaching a maximum value 4 h after the beginning of the light period (Fig. 5). NR

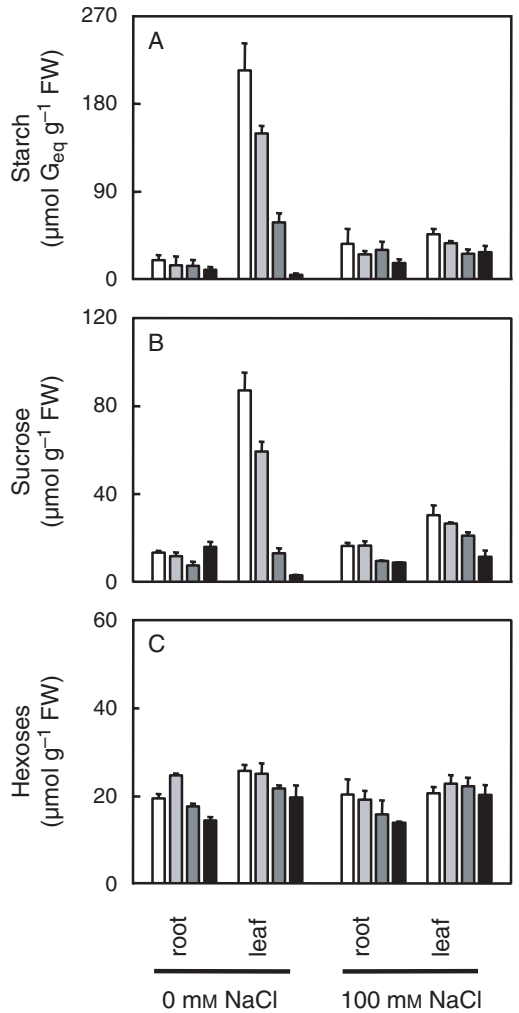

Fig. 4. Effect of nitrate on carbohydrate content under salinity of plants harvested after 20 d of hydroponic culture. Starch (*A*) is expressed as glucose equivalents. Nitrate was added on day five of hydroponic culture: bar colours are as in Fig. 1. 100 mM NaCl was added from day 10 of hydroponic culture. The values are means ± SD ($n = 4$).

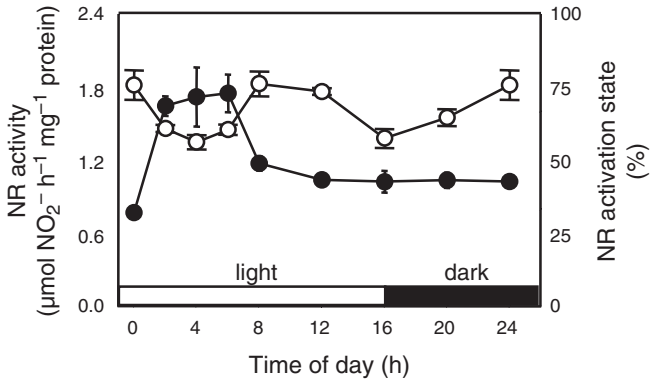

Fig. 5. Nitrate reductase activity (●) and activation state (◯) in leaf extracts from control plants after 20 d of hydroponic culture. 10 mM nitrate was added on day five of hydroponic culture. The values are means ± SD ($n = 4$).

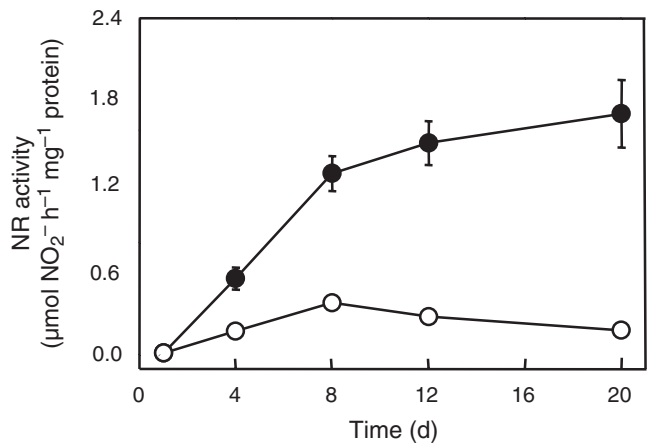

Fig. 6. Nitrate reductase activity in leaf extracts from plants grown in 10 (●) and 1 (◯) mM NO_3^-. Leaves were harvested on days 1, 4, 8, 12 and 20 of hydroponic culture. The values are means ± SD ($n = 4$).

activity was dependent on nitrate nutrition (Figs 6, 8). In roots of nitrate free grown plants the basal NR activity was about 1.8 µmol NO_2^- h^{-1} mg^{-1} protein, while in those from 10 mM NO_3^- medium the activity was 1.5-fold higher (Fig. 8*C*). In leaves of 0 and 10 mM NO_3^- grown plants, NR activity was about 0.06 and 1.72 µmol NO_2^- h^{-1} mg^{-1} protein, respectively, representing an increase of about 30-fold (Fig. 8*D*).

Salt treatment reduced leaf NR activity in 10 mM NO_3^- grown plants to about 50% of the control, but in 0.1 and 1.0 mM NO_3^- treatments it increased by about 3-fold compared with controls. In contrast, salt stress reduced the NR activity in the roots to about 50% of the controls, but only in plants grown on media containing less than 1.0 mM NO_3^-.

The activation state of NR in leaves was also dependent on nitrate; increasing from around 32% in plants grown without NO_3^- to 55% in 10 mM NO_3^- grown plants (Fig. 8*F*). The activation state of NR in the roots was fairly constant at around 90% and independent of the nitrate concentration in the culture medium (Fig. 8*E*).

Salt treatment reduced the activation state of the leaf nitrate reductase to 50% of controls but only in plants cultured with nitrate lower than 10 mM. It had no effect on the NR activation state of the roots, which remained at ~90%.

Discussion

The growth of durum wheat was very sensitive to nitrate treatment. The growth rate of 10 mM NO_3^- cultured plants was about 2.5-fold higher than in plants grown without nitrate (Fig. 1). Between days 10 and 20 leaf fresh weight was highest in high nitrate plants, being about 3-fold higher compared with plants grown without nitrogen. The leaf dry

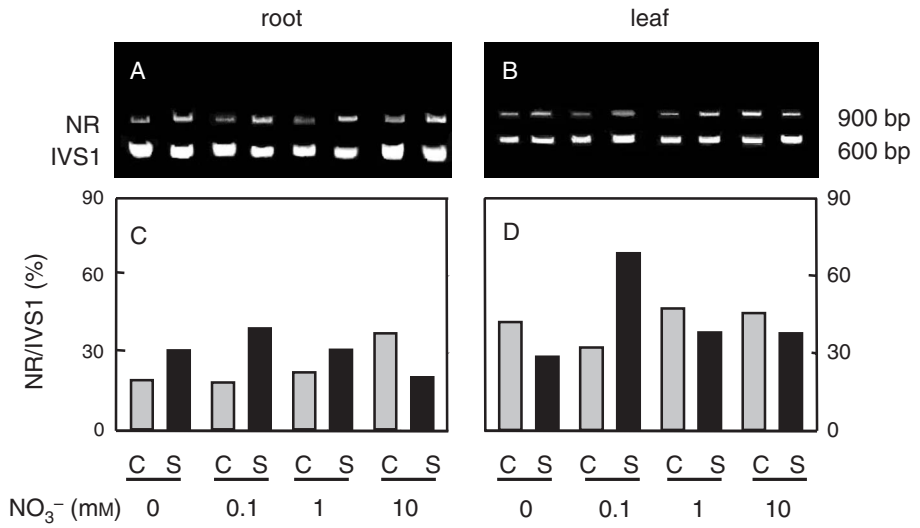

Fig. 7. Nitrate reductase mRNA content determined by RT–PCR from roots (*A*, *C*) and leaves (*B*, *D*) of non-treated (C) and 100 mM NaCl treated plants (S). (*A*, *B*) Ethidium bromide stained RT–PCR products; (*C*, *D*) densitometric quantification of NR RT–PCR products normalised to the values obtained for IVS1-rRNA. 100% represents the highest mRNA level. Nitrate was added on day five of hydroponic culture. 100 mM NaCl was added from day 10 of hydroponic culture. Plants were harvested after 20 d of hydroponic culture.

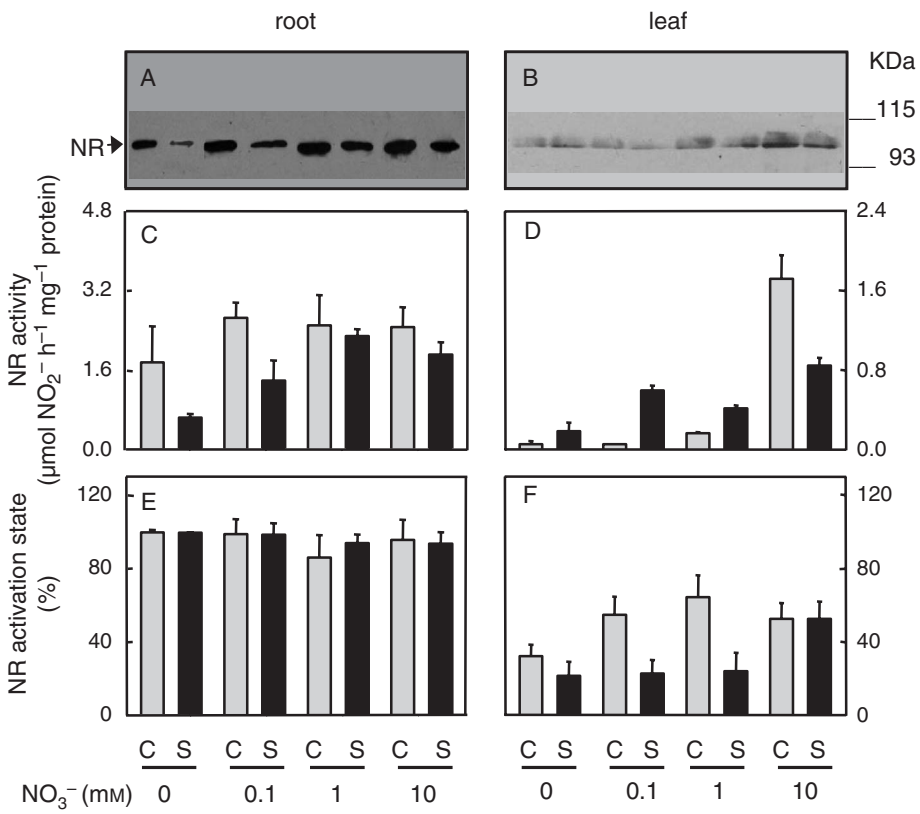

Fig. 8. Effects of nitrate and salt on NR protein, activity and activation state in R western blot, NR total activity and NR activation state in root (*A*, *C*, *E*) and leaf (*B*, *D*, *F*) extracts from control (C) and 100 mM NaCl-treated durum wheat plants (S). (*A*, *B*) Western blot; (*C*, *D*) NR activity; (*E*, *F*) NR activation state. Nitrate was added on day five of hydroponic culture: grey bars, 1 mM KNO_3; black bars, 10 mM KNO_3. 100 mM NaCl was added from day 10 of hydroponic culture. Plants were harvested after 20 d of hydroponic culture. The values are means ± SD ($n = 4$).

weight showed a similar pattern (Table 1). Salinity (100 mM NaCl) strongly reduced the growth of durum wheat seedlings, especially in 10 mM nitrate grown plants, while at low nitrate, nitrogen limited growth more than salt.

Nitrate reductase in durum wheat showed changes in activity and activation state (Fig. 5), depending on photoperiod and consistent with regulation by phosphorylation as reported in others plant species (Kaiser *et al.* 1999; Tischner 2000). NR activity was also induced by nitrate (Figs 6, 8). The level of the NR transcript in roots, but not in leaves, was correlated with nitrate concentration in the nutrient solution (Fig. 7), as also reported in *Zea mays* (Sivasankar and Oaks 1995). However, there was no correlation between the level of NR transcript and NR protein or activity (Figs 7, 8), suggesting that post-transcriptional processes, such as protein translation and degradation were more important in determining amount of protein and activity (Tischner 2000). Although NO_3^- induced NR protein and activity, it is important to note that, NR transcript was present in roots and leaves of plants grown in nitrate free medium (Fig. 7*A–B*).

Salinity reduced nitrate reductase activity in roots and leaves of durum wheat grown in high nitrate media. The lower activity was correlated with a decrease in nitrate concentration in the salt stressed high nitrate plants compared with control plants (Figs 2*C*, 8*C–D*), suggesting a restriction of NO_3^- transport to the leaves. The level of NR protein also decreased in both roots and leaves, suggesting that salinity might affect the translation of NR mRNA process or degradation of NR protein (Fig. 8*A–B*).

Nitrate concentration in the roots did not change significantly under salinity, but NO_3^- concentration in the leaves decreased (Fig. 2*C*); suggesting that chloride caused an inhibition of nitrate loading into the root xylem, as reported for salt stressed tomato plants (Cramer and Lips 1995). Alternatively, NO_3^- and Cl^- could compete for uptake also at plasmalemma or tonoplast of leaf cell. A NO_3^--dependent inhibition of chloride uptake was also suggested by considering that high nitrate reduced chloride accumulation especially in root tissues (Fig. 2*A–C*).

The leaf potassium content was not affected by salinity (Fig. 2*B–D*), therefore the K^+ / Na^+ selectivity was preserved, even although the cultivar studied was considered not to contain the sodium exclusion trait for salt tolerance described by Lindsay *et al.* (2004).

Although protein levels were mostly unaffected by salt (Table 1), total free amino acids increased both in roots and leaves at all nitrate concentrations (Fig. 3*A*). There were very large increases in leaf and root contents of glutamine, glycine (data not shown), serine, asparagine and proline, particularly in the latter two amino acids (7- and 10-fold, respectively; Fig. 3). The large increase in asparagine and proline suggests that durum wheat uses both of these amino acids as compatible solutes. Proline is well known as compatible solute in plants under salinity or drought (Nanjo *et al.* 1999), but amides such as glutamine and asparagine have also been reported to accumulate in plants subject to salt stress (Dubey 1997; Mansour 2000). Among the other nitrogen-containing compounds, durum wheat accumulated also glycine betaine as compatible solute (Table 1). The large increase of nitrogen-containing compounds under salinity suggests that they can contribute as osmolytes to balance water potential within the cell, mainly when nitrogen availability exceeds that needed for growth. A significant increase of free amino acids occurred also in control plants grown in high-nitrate media, in which photosynthates were turned from starch and sucrose to carbon skeletons for the synthesis of amino acids (Figs 3, 4).

Salinity strongly inhibited photosynthesis independently of nitrate nutrition (data not shown), probably by reduction of mesophyll and stomatal conductance as reported in spinach leaves (Delfine *et al.* 1998). Starch and sucrose content was strongly reduced (20% of control) in low nitrate and high salt (Fig. 4). In such case part of carbohydrates could be converted in fructans and retained in leaves to act as osmolytes, which have been reported to increase under stress conditions, particularly when nitrogen was limiting for growth (Pérez *et al.* 2001).

Under salinity, in high as in low nitrate media, the increase in serine (Fig. 3) and glycine was consistent with an increase of photorespiration (Di Martino *et al.* 2003). This could also account for the accumulation of glycine betaine whose pathway starts from serine.

Most of the sodium, chloride and nitrate was probably compartmentalised in the vacuoles, as reported in other plants (Speer and Kaiser 1991; Di Martino *et al.* 2003). Free amino acids, in particular proline and asparagine, as well as glycine betaine can contribute to the osmotic balance of cell organelles with respect to the vacuole.

In conclusion, salt stress (100 mM NaCl) strongly inhibited the growth of durum wheat seedlings, which was not significantly restored by high nitrate nutrition. This suggests that the bottleneck for growth was not significantly affected by nitrate assimilation, confirming and extending previous findings in *Zea mays* (Abd-El Baki *et al.* 2000). However, salt inhibited nitrate transport to leaves mainly because of nitrate / chloride competition and consequently affected nitrate reductase, supporting the view that also in durum wheat nitrate regulates NR transcription, translation, post-translation and protein degradation.

NaCl increased NR mRNA at low nitrate, suggesting that chloride can mimic nitrate as a signal molecule to induce transcription in both roots and leaves. This could explain why NR mRNA was also present at a low level in plants grown in a nitrogen-free Hoagland's medium with a chloride concentration of at least 5 mM. The level of NR protein, however, remained low in salt stressed plants, indicating that salt (probably Na^+) can inhibit NR mRNA translation or activate NR-protein degradation.

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