

Plant growth, metabolism and adaptation in relation to stress conditions: Further studies supporting nullification of harmful effects of salinity in lettuce plants by urea treatment

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ABSTRACT

Foliar application of urea to lettuce plants induced pronounced changes in the total amount and in the relative composition of the nitrogen pool. As compared with untreated lettuce plants, urease (UR), nitrate reductase (NR), asparaginase (AS) and glutamine synthetase (GS) activities were, in general, increased with an increase in the concentration of urea. On the other hand, salinization of lettuce plants with NaCl induced a significant decrease in the activities of UR, NR, AS and GS, at vegetative and adult growth stages. In general, treatment of lettuce plants with increasing concentrations of urea fertilizer in combination with each of the levels of salinity resulted in significant increases in all enzyme activities. Treatment with increasing concentrations of urea fertilizer induced significant decreases both in glycine and proline contents below control levels. On the other hand, salinization of lettuce plants with NaCl induced significant pronounced increases in the contents of the two amino acids. Further increments in glycine and proline contents were observed in differently salinized lettuce plants foliary sprayed with increasing concentrations of urea at vegetative and adult growth stages. Salinity solely enhanced the occurrence of novel proteins that were detected neither in the water control nor in the urea-treated plants. Protein banding patterns of lettuce plants treated with urea either alone or in combination with NaCl showed different *de novo* protein bands with different molecular weights, induced by urea and/or NaCl at vegetative and adult growth stages.

Keywords: *Lactuca sativa* L.; NaCl; urea; nitrogen content; amino acids; nitrogen enzymes; protein patterns

Saline soils contain a high percentage of soluble salts and one or more of these salt components are usually present in excess. Salts in the external medium are supposed to adversely affect growth and metabolism of glycophytic higher plants (Greenway and Munns 1980, Younis et al. 2008). Foliar fertilization represents an alternative means of applying supplementary nitrogen (N) during periods of rapid plant growth and increased N demand, or at times of critical physiological stress. Urea sprays often constitute a convenient method for correction as well as for maintaining green colour and nitrogen levels in plant tissues, and for sustaining growth (Sirko and Brodzik 2000).

In order to establish the metabolic status of the plants, Lorenzo et al. (2001) choose NR and GS, the first enzymes in the nitrate and ammonium assimilation pathways, respectively. It has been

reported that GS activity may facilitate the production of glutamine, proline and other organic solutes. Of interest in this connection, osmotic adjustment, protection of cellular macromolecules, storage form of nitrogen, maintaining cellular pH, detoxification of cells and scavenging of free radicals are proposed functions of these compounds under stress conditions (Venekamp 1989, Mansour 2000). To understand better the mechanisms by which plants can respond to salt stress, one looks for proteins that are specifically accumulated after exposure of plants to NaCl (Lopez et al. 1996). This was exploited in a number of studies conducted to identify polypeptides involved in the salt-stress response either in cell cultures (Ben-Hayyim et al. 1993) or in whole plants (Hurkman et al. 1991).

In a previous communication (Younis et al. 2008), foliar application of urea to NaCl-stressed lettuce

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plants appeared to counteract the stress-induced damage maintained in growth and photosynthetic parameters as well as in carbohydrate constituents and in activities of antioxidant enzymes. This report describes the modifications maintained in the total amount and in the relative composition of nitrogen pool including amino acids, changes in the activities of the nitrogen-related enzymes and protein patterns induced by salinity in lettuce plants, and the possible reversal of the inhibition of these parameters with foliar application of urea.

MATERIAL AND METHODS

Time course of experiments. Transplants of lettuce (*Lactuca sativa* L. cv. Baladi), of homogeneous appearance and structure, were used. The details of experimental set-up as well as procedures of transplantation, treatment and growing of plants were essentially those described by El-Bialy (2005) and Younis et al. (2008). The transplants (25-day-old) were washed thoroughly with tap water and then transplanted in a mixture of clay-loamy soil (2:1, v/v) in pots (30 × 28 × 26 cm), each pot containing 8 kg of homogeneous soil. The experiments were carried out outdoor under normal day and light conditions. In all cases, treatments of lettuce transplants with urea and/or NaCl were carried out after one week from the date of transplantation. Salinity stress was induced by NaCl (3, 5 and 7 mmhos; with irrigation water) via the rooting medium. Urea was applied to lettuce plants as a foliar spray. All pots were irrigated with tap water every three days to maintain soil at the field capacity throughout the whole experimental period.

An appropriate number of treatments representing all possible combinations of urea and salinity levels were replicated twice in a completely randomized design. Samples for determination of nitrogen content, amino acids and protein banding patterns and activities of nitrogen-related enzymes were taken from plants after 20 and 35 days from the date of transplantation to examine the vegetative and adult growth stages, respectively. An analysis of variance was performed on the data using the *F*-ratio test. Comparison among means, from duplicate determinations and quadruplicate samples, was carried out by calculating the least significant difference (LSD) at 5% probability level.

Estimation of nitrogenous constituents. The extraction method by Yemm and Willis (1956) was adopted. The extract was used for estimation of amino acids as well as nitrate-, ammonia-,

amide-, urea- and total soluble-N (TSN) fractions. Subtracting the summation of the above mentioned fractions from the TSN gave the residual-N fraction mainly considered as peptide-N (Younis et al. 1971) in the present work. Total nitrogen (TN) was determined directly by using the powdered tissue. Subtracting the TSN from TN gave the value of protein-N.

Nitrate-, ammonia- and amide-N were determined by the methods adopted by Younis et al. (1971). Urea-N was determined according to the method of Marsch et al. (1965), using urea Kits. The TSN and TN were determined by the conventional semi-micromodification of Kjeldahl method as adopted by Younis et al. (1971). For amino acids, glycine was determined by the method of Muting and Kaiser (1963). Proline concentration was determined by means of a rapid coloumetric technique developed for plant tissues by Bates et al. (1973).

Determination of nitrogen-related enzyme activities. The methods used for extraction and determination of enzyme activities in lettuce plants were those adopted by Joy and Ireland (1990) for asparaginase, by El-Saht et al. (1994) for nitrate reductase, by Shelp and Ireland (1985) for urease and by Lea et al. (1990) for glutamine synthetase. All enzyme extraction procedures were carried out at 4°C and enzyme assays were carried out at 30°C.

Determination of protein-banding patterns. Lettuce plants were subjected to protein analysis, according to their molecular weights by denatured sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) as described by Laemmli (1970). For full details of the methods of determination of enzyme activities as well as protein-banding patterns, the M.Sc. thesis of El-Bialy (2005) should be consulted.

RESULTS AND DISCUSSION

Changes in nitrogen content

The obtained results (Figure 1) indicate that the total amount and the relative composition of nitrogen pools were altered under the effect of urea fertilizer either alone or in combination with low, medium or high level of salinity applied to lettuce plants, at vegetative and adult growth stages. This appeared to be a function of (a) the kind of treatment used, (b) the level of urea or NaCl used and (c) the age of the treated plants.

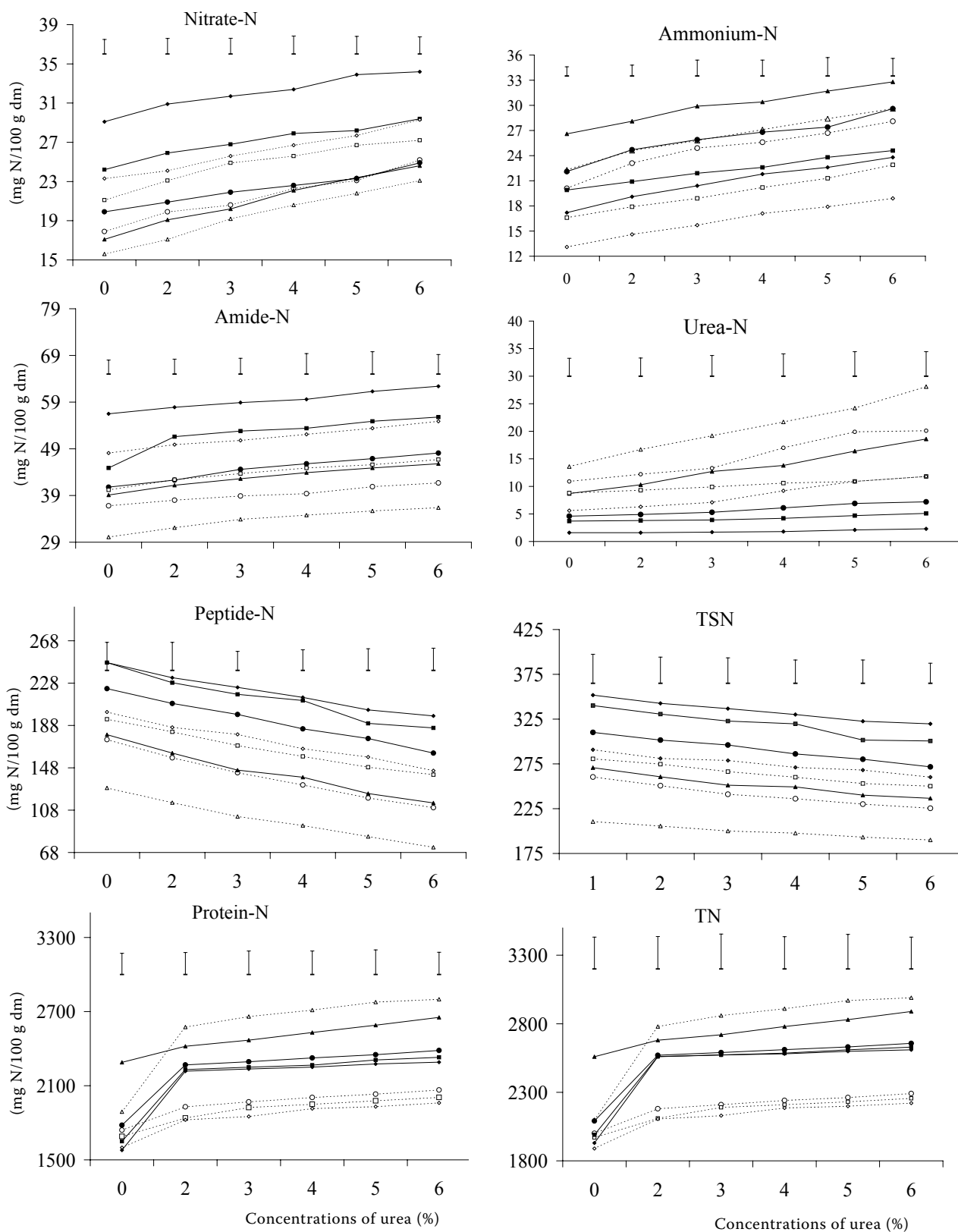


Figure 1. The effect of increasing concentrations of urea fertilizer either alone or in combination with low, medium or high concentrations of NaCl on nitrogen content of lettuce plants, at vegetative (V) and adult (A) growth stages. Vertical bars represent the LSD at 5% level. Dotted lines represent the vegetative growth stage and solid lines represent the adult growth stage [---△--- urea only (V); —▲— urea only (A); ---○--- urea + 3 mmhos NaCl (V); —●— urea + 3 mmhos NaCl (A); ---□--- urea + 5 mmhos NaCl (V); —■— urea + 5 mmhos NaCl (A); ---◇--- urea + 7 mmhos NaCl (V); —◆— urea + 7 mmhos NaCl (A)]

Treatment of lettuce plants with increasing concentrations of urea fertilizer led to significant increases in nitrate-, ammonia-, amide-, urea-, protein- and total-N contents. On the other hand, a significant decrease was observed in the contents of peptide- and TSN, at vegetative and adult growth stages, as compared with the levels of water control plants. Salinization of lettuce plants with low, medium or high concentration of NaCl induced increases in nitrate-, amide-, peptide- and TSN, as compared with water control plants; on the other hand, ammonia-, urea-, protein-, and total-N contents significantly decreased below the contents of control plants throughout the experimental period (Figure 1).

Foliar application of urea fertilizer to the salinized lettuce plants appeared to induce positive favourable responses in contents of different nitrogen fractions; the pattern of changes maintained in urea treated plants was comparable with the pattern of changes maintained in urea + NaCl-treated plants, in relation to the levels in control salinized plants. It is also clear that the percent recovery (improvement) calculated according to Younis et al. (2008), for the total nitrogen determined in differently salinized lettuce plants, showed a progressively greater significant increase with an increase in concentration of urea applied to the respective plants, throughout the entire period of the experiment.

As expected, nitrogen metabolism in lettuce plants is influenced by urea fertilizer and/or salinity used in the present study, because their site of action involves the synthesis of amino acids and/or proteins which are required in large amounts for growth as well as for osmoregulation. The above mentioned changes in soluble- and insoluble-N fractions, may be attributed mainly to the uptake of adequate amounts of urea, assimilated as such or hydrolyzed into CO₂, and ammonia which is assimilated within the lettuce plant and/or to the activities of enzymes controlling the assimilation of nitrogen (Ray et al. 2003, Medici et al. 2004) in response to foliar application of urea, either alone or in combination with NaCl. Ammonia is considered to be the unit of nitrogen metabolism from which different amino acids are produced, these being further incorporated into the protein synthesis. For its role in nitrogen metabolism and protein biosynthesis, ammonia is considered very important for plant survival if it can be utilized by the plant cell. Conversely, its accumulation without beneficial using would be harmful to the plant tissues.

To support this conclusion, El-Saht (1995) stated that the application of urea fertilizer led to progressive significant increases in ureide-N content of soybean plants with an increase in urea concentrations, whereas the amide-N content of such tissues was progressively and significantly decreased in relation to the control values of soybean plants. Similarly, Badawy (1989) reported significant decreases in TN, TSN and amino-N contents of *Vicia faba* plants stressed with NaCl. Fertilization with urea appeared to increase TN, TSN and protein-N contents in NaCl-treated plants as well as in water controls.

Changes in nitrogen-related enzyme activities

Careful examination of Figure 2 revealed the following main points:

- (a) UR and NR activities of variously treated lettuce plants showed a progressive significant increase, above the respective control levels, throughout the entire period of the experiment;
- (b) GS and AS enzyme activities of urea-treated lettuce plants were found either to increase significantly (with 2%, 3% and 4% urea), or to decrease significantly (with 5% and 6% urea), as compared with control levels. On the other hand, a significant decrease in GS and AS enzyme activities were observed in variously salinized lettuce plants, in relation to water control levels. Foliar application of urea fertilizer to the salinized lettuce plants induced progressive significant increases in the activities of GS and AS at 2%, 3%, 4% and 5%, whereas at 6% urea these activities were found either to decrease significantly (with 3 and 7 mmhos NaCl), or remain unaltered (with 5 mmhos NaCl);
- (c) the calculated percent improvements in UR, NR, AS and GS activities were in general, significantly increased in response to the application of urea to the variously salinized lettuce plants.

In accordance with the present results, Bi et al. (2003) reported an increase in the activities of GS and AS of lettuce plants treated with increasing concentrations of urea fertilizer. Also, Lorenzo et al. (2001) and Burman et al. (2004), experimenting with higher plants treated with salinity and nitrogen fertilization, found that NR and GS enzyme activities were decreased in saline-treated plants but the integration of nitrogen fertilization with

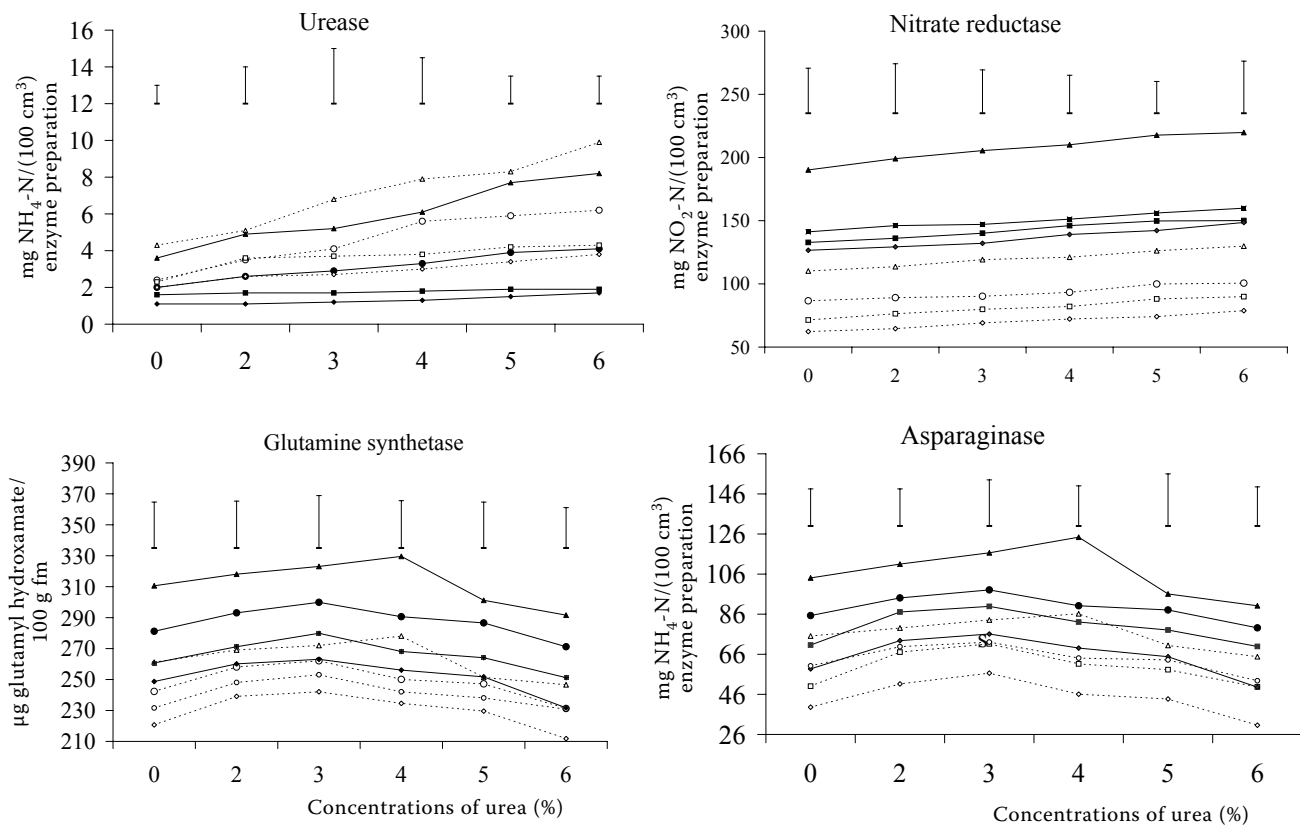


Figure 2. The effect of increasing concentrations of urea fertilizer either alone or in combination with low, medium or high concentrations of NaCl on nitrogen-related enzyme activities of lettuce plants, at vegetative (V) and adult (A) growth stages. Vertical bars represent the LSD at 5% level. Dotted lines represent the vegetative growth stage and solid lines represent the adult growth stage [---△--- urea only (V); —▲— urea only (A); ---○--- urea + 3 mmhos NaCl (V); —●— urea + 3 mmhos NaCl (A); ---□--- urea + 5 mmhos NaCl (V); —■— urea + 5 mmhos NaCl (A); ---◇--- urea + 7 mmhos NaCl (V); —◆— urea + 7 mmhos NaCl (A)]

saline-treated plants showed an improvement in the activities of such enzymes. Furthermore, several reports have indicated that salinity can cause different effects on NR activity depending on the part of the plant analyzed (Curry et al. 1992). In plants, UR is the only enzyme that is able to recapture nitrogen from urea (Sirko and Brodzik 2000). Fertilization with urea through leaves could be an efficient method of plant feeding and any modification leading to increased UR activity in leaves could result in more effective assimilation of this fertilizer. Thus, such an increase in UR activity under stress conditions might have a positive impact on the nitrogen metabolism in plants since more ammonia would be available for assimilation via glutamine into a variety of nitrogenous compounds (Sirko and Brodzik 2000).

It has been reported that NR, GS and AS can be used as physiological markers to establish plant health under stress conditions in rose cultivars (Lorenzo et al. 2001). These authors observed an increase with increasing urea fertilizer either

alone or in combination with low, medium or high NaCl. Thus, GS and AS activities are reported to facilitate the production of glutamine, asparagine, proline and other organic solutes characteristic for osmotic and pH adjustments under stress conditions (Venekamp 1989, Lorenzo et al. 2001). Our results with amino acids and enzyme activities strongly support these conclusions.

Changes in amino acids

With an increase in concentration of urea, a progressively greater significant decrease of proline and glycine contents below the control levels was observed (Figure 3). In relation to water control levels, salinization of lettuce plants induced significant increases in both proline and glycine contents, which appeared to be progressively higher with an increase in the salinity level. Foliar application of urea fertilizer to the differently salinized lettuce plants at vegetative and adult stages induced sig-

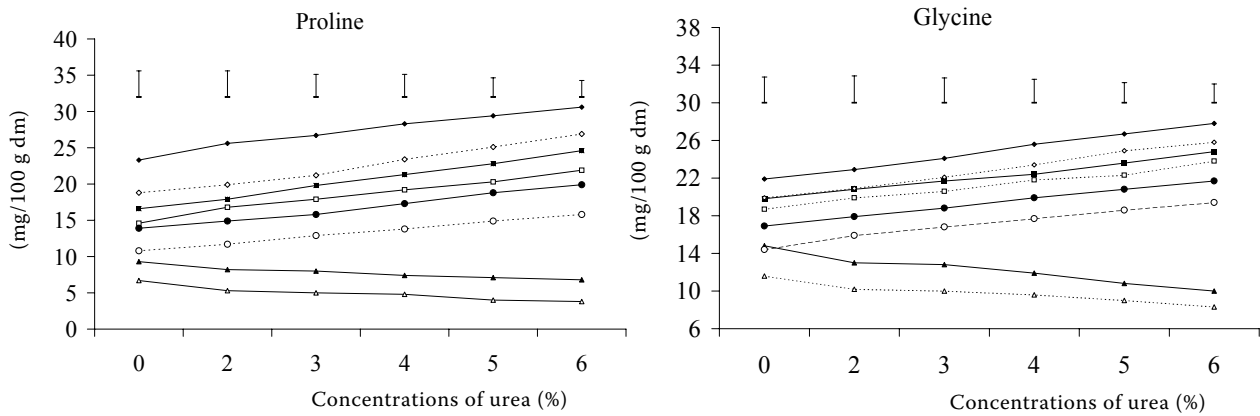


Figure 3. The effects of increasing concentrations of urea fertilizer either alone or in combination with low, medium or high concentrations of NaCl on glycine and proline contents of lettuce plants, at vegetative (V) and adult (A) growth stages. Vertical bars represent the LSD at 5% level. Dotted lines represent the vegetative growth stage and solid lines represent the adult growth stage [--- Δ --- urea only (V); --- \blacktriangle --- urea only (A); --- \circ --- urea + 3 mmhos NaCl (V); --- \bullet --- urea + 3 mmhos NaCl (A); --- \square --- urea + 5 mmhos NaCl (V); --- \blacksquare --- urea + 5 mmhos NaCl (A); --- \diamond --- urea + 7 mmhos NaCl (V); --- \blacklozenge --- urea + 7 mmhos NaCl (A)]

nificant progressive increases in the contents of both proline and glycine with an increase in urea concentration (Figure 3). Again, the higher was the salt concentration used in combination with urea, the higher was the accumulation of proline and glycine contents.

Careful examination of Figure 3 indicate that although the contents of proline and glycine were lowered in response to treatment with urea and were markedly increased in response to salinization, combined treatment of lettuce plants with different concentrations of NaCl + urea at different concentrations, not only counteracted the decrease maintained in response to urea but also induced additive increments above the levels attained in response to salinity. An appropriate explanation for these changes can be obtained if we consider the changes in the activities of UR, NR, AS and GS (Figure 2); all activities being increased under both fertilization and salinity. Here we should refer to an interactive effect between urea and salinity; each factor appeared to reinforce the action of the other.

Moreover, El-Saht (1995) working on soybean showed significant variable changes in a great number of amino acids including proline and glycine, in response to treatment with different concentrations of urea. Furthermore, in some plants including lettuce, salt tolerance appeared to associate with the capacity of a species to accumulate proline and glycine, which act as compatible solutes involved in osmotic adjustment at the plant cell level (Tarakcioglu and Inal 2002). Proline as well as glycine accumulation can help

plants to withstand osmotic stress (Ghoulan et al. 2002); they can also protect plants by maintaining protein structure or increasing scavenging of active oxygen species (Tester and Davenport 2003).

Changes in protein patterns

Careful examination of Table 1 revealed the following main points:

- treatment with different levels of urea led to positive changes in the number and intensity of protein electrophoretic patterns. The magnitude of response was most pronounced with 2–5% urea, and the least response was maintained in controls and 6% urea-treated lettuce plants;
- treatment with different levels of salinity induced varied changes in the total number of proteins detected, in the disappearance of certain protein bands and in appearance of new characteristic bands with different molecular weights. As apparent from Table 1, the higher was the concentration of NaCl, the stronger was the response for the above-mentioned changes. Thus, there was a gradual degradation in all polypeptide bands with a progressive increase in NaCl concentration;
- treatment of lettuce plants with different levels of urea, in combination with each of the three used levels of NaCl, also induced changes in the total number of proteins and in both sets of proteins that show disappearance and appearance. In accordance with the present results,

Table 1. Protein profile of native PAGE of *Lactuca sativa* plants treated with increasing concentrations of urea fertilizer either alone or in combination with low, medium or high concentration of NaCl, at vegetative and adult growth stages

Treatment	Vegetative (V)				Adult (A)				Total summation	
	total no. of protein bands	molecular weights (kDa)	no. of proteins disappeared	no. of new proteins	total no. of protein bands	molecular weights (kDa)	no. of proteins disappeared	no. of new proteins	total no. of protein bands	total no. of new proteins
0% urea (control water)	3	30.4, 29.4, 18.0	-	-	1	21.6	-	-	4	-
2% urea	5	30.4, 29.4, 18.0, 14.6, 12.4	-	2	1	21.6	-	-	6	2
3% urea	5	30.4, 29.4, 18.0, 14.6, 12.4	-	2	1	21.6	-	-	6	2
4% urea	5	30.4, 29.4, 18.0, 14.6, 12.4	-	2	2	17.0, 18.0	1	2	7	4
5% urea	5	30.4, 29.4, 18.0, 14.6, 12.4	-	2	2	17.0, 18.0	1	2	7	4
6% urea	3	18.0, 14.6, 12.4	2	2	2	17.0, 18.0	1	2	5	3
3 mmhos NaCl	5	30.4, 29.4, 18.0, 14.6, 12.4	-	2	2	17.0, 18.0	1	2	7	4
2% urea + 3 mmhos NaCl	3	30.4, 29.4, 18.0	-	-	2	17.0, 18.0	1	2	5	2
3% urea + 3 mmhos NaCl	4	30.4, 29.4, 18.0, 16.2	-	1	2	17.0, 18.0	1	2	6	3
4% urea + 3 mmhos NaCl	4	30.4, 29.4, 18.0, 16.2	-	1	2	17.0, 18.0	1	2	6	3
5% urea + 3 mmhos NaCl	4	30.4, 29.4, 18.0, 16.2	-	1	2	17.0, 18.0	1	2	6	3
6% urea + 3 mmhos NaCl	3	30.4, 29.4, 16.2	1	1	2	17.0, 18.0	1	2	5	2
5 mmhos NaCl	3	30.4, 29.4, 20.0	1	1	2	25.0, 21.0	-	1	5	2
2% urea + 5 mmhos NaCl	3	30.4, 29.4, 20.0	1	1	2	25.0, 21.0	-	1	5	2
3% urea + 5 mmhos NaCl	3	30.4, 29.4, 20.0	1	1	2	25.0, 21.0	-	1	5	2
4% urea + 5 mmhos NaCl	3	30.4, 29.4, 20.0	1	1	2	25.0, 21.0	-	1	5	2
5% urea + 5 mmhos NaCl	2	30.4, 29.4	1	-	2	25.0, 21.0	-	1	4	1
6% urea + 5 mmhos NaCl	2	30.4, 29.4	1	-	2	25.0, 21.0	-	1	4	1
7 mmhos NaCl	1	56.0	3	1	3	25.0, 38.68, 17.44	1	3	4	4
2% urea + 7 mmhos NaCl	1	56.0	3	1	3	25.0, 38.68, 17.44	1	3	4	4
3% urea + 7 mmhos NaCl	1	56.0	3	1	3	25.0, 38.68, 17.44	1	3	4	4
4% urea + 7 mmhos NaCl	1	56.0	3	1	3	25.0, 38.68, 17.44	1	3	4	4
5% urea + 7 mmhos NaCl	1	56.0	3	1	3	25.0, 38.68, 19.44	1	3	4	4
6% urea + 7 mmhos NaCl	1	20.0	3	1	1	25.0	1	1	2	2

Younis et al. (1999) working on bean plants, Khedr et al. (2003) working on *Pancreaticum maritimum* L. and Bahrman et al. (2003) working on wheat demonstrated that salt-stress upregulated several dehydrin proteins, even in non-stressed plants. They added that external addition of nitrogen improves the salt-tolerance of the used plants by protecting the protein turnover machinery against stress-damage and upregulating stress protective proteins;

- (d) the observed changes in protein profiles revealed the presence of different groups of salt-stress proteins; their exact identification requires amino acid sequencing which awaits further investigation;
- (e) salinized plants, recovered in response to the treatment with urea as foliar spray, showed lower levels or nearly complete absence of the major salt stress-induced proteins (SSPs). Chourey et al. (2003) showed proteins that accumulated during the salinity-triggered growth arrest of young Bura Rata rice seedlings are mobilized during the recovery of seedlings from salinity stress.

One of the most important abiotic factors limiting plant productivity is water stress imported by drought or salinity. Salt stress results in several alterations in plant metabolism including reduced water potential, ion imbalance and toxicity and reduction of CO₂ assimilation (Greenway and Munns 1980). Plants exposed to salt stress must undergo changes in gene expression to adapt to the altered environment (Lopez et al. 1996). The protein-banding pattern of an organism represents a biochemical genetic fingerprint of that organism. Hussein and Salam (1985) stated that each band in the protein-banding pattern of an organism reflects a separated transcriptional event. Furthermore, electrophoretic analysis of the protein provides information concerning the structural genes and their regulatory systems that control its biosynthetic pathways.

Considering the above presented detailed results as well as Pearson's correlation coefficients made between the changes in the different growth, metabolite and enzymatic parameters (Younis et al. 2008), we can state that foliar spray with urea at 2–4% appeared to be beneficial to growth and metabolic activities of lettuce plants; the magnitude of response being the most pronounced at 4% urea. The higher concentrations (5% and 6%), however, appeared harmful for reasons previously mentioned in a sporadic manner (Pew et al. 1984, Puttanna et al. 2001, Younis et al. 2008).

Supplemental foliar spray of urea to the variously salinized lettuce plants appeared to partially counteract the deleterious effects of salinity; the magnitude of response being the most pronounced with 3–4% urea.

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