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Effect of varying concentrations of salinity on some biochemical parameters involved in nitrogen metabolism of four grass species

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ABSTRACT: Salinity adversely affects crop productivity and the quality of yield. The present work was carried out to estimate the changes in nitrogen metabolism under the influence of NaCl salinity in four selected grasses. All the species recorded a decrease in nitrate nitrogen content at the highest concentration of salinity stress (300 mM). Its maximum increase was 14 and 19% (100 mM) for *Cymbopogon nardus* and *Cynodon dactylon*, respectively. The maximum increase in nitrite nitrogen found was 11% (200 mM) in *Cymbopogon nardus*, 12% (100 mM) in *Cynodon dactylon*. The concentration of proline and amides in the leaves of all the experimental grasses showed a positive correlation with increasing concentrations of salinity. The maximum increase in proline content was 81, 88, 126, and 68% in *Cymbopogon nardus*, *Cynodon dactylon*, *Pennisetum alopecuroides*, and *Vetiveria zizanioides*, respectively, at 300 mM NaCl salinity. The concentration of free amino acids in the leaves of all the experimental grasses was considerably increased under saline condition and showed a positive correlation with increasing concentrations of salinity. Similar results were obtained in the case of amides. The concentration of nitrate reductase enzyme was elevated in *Cymbopogon nardus* and *Pennisetum alopecuroides* at lower salinity regime.

KEYWORDS: nitrate nitrogen, nitrite nitrogen, amino acids, amides, nitrate reductase, salinity stress

INTRODUCTION

Salinity is one of the major abiotic stresses that adversely affect crop productivity and quality[1,2] with increasing impact on the socio-economic fabric and health, especially of the farming communities. Plants under stressful conditions adapt very differently from one another, even from a plant living in the same area. A plant first line of defence against abiotic stress is in its roots. Even a low concentration of the contaminants typically alter plant metabolism, most commonly to reduce crop yields. Severe land degradation affects a significant portion of the earth arable lands, decreasing the wealth and economic development of nations.

Statistics about the extent of salt-affected areas vary according to authors, but estimates are close to one billion hectares, representing about 6% of the earth continents. Based on the Food and Agriculture Organization[3] soil map of the world, the total area of saline soils is 397 Mha and of sodic soils is 434 Mha. Of the current 230 Mha of irrigated land, 45 Mha are salt-affected soils (19.5%) and of the almost 1500 Mha of dryland agriculture, 32 Mha are salt-affected soils (2.1%) to varying degrees by human-induced processes[3].

Nitrogen is a component of many important structural, genetic, and metabolic compounds in plant cells. Nitrogen is a critical chemical element in both proteins and DNA. The key enzyme in nitrogen metabolism, nitrate reductase, is very sensitive to NaCl[4]. Proline is an α-amino acid and one of the twenty DNA-encoded amino acids, and occurs widely in higher plants and accumulates in large amounts compared to all other amino acids in salt stressed plants[2,5]. Proline may act as a signalling or regulatory molecule[6], able to activate multiple metabolic responses belonging to the adaptation process. Therefore, the present investigation was carried out to assess deleterious effects of NaCl salinity on the nitrogen and its key parameters in the four selected grasses.

MATERIALS AND METHODS

Seedlings of *Cymbopogon nardus* (L.) Rendle, *Pennisetum alopecuroides* (L.) Spreng var. Mourdy, and *Vetiveria zizanioides* (L.) Nash were collected from government nursery, Kagal (Kolhapur, Maharashtra) while those of *Cynodon dactylon* (L.) Pers. were collected from Shivaji University campus (Kolhapur, Maharashtra). The seedlings were uniformly cut to a minimum height of 15 cm required for their growth and were transplanted into the open (i.e., free draining) earthen pots (30 cm height with a narrow base) to establish and grow under normal conditions with proper irrigation. After four weeks of normal growth, salinity and heavy metal stresses were commenced. The plants were treated with increasing concentrations of NaCl, i.e., 25, 50, 100, 200, and 300 mM. Every alternate day, they were watered with a double amount of water to maintain the uniform salt concentration by leaching and drainage out of excess water in the pots and to cope up with the loss of water by evaporation from the soil surface and by transpiration from the plant surface.

Nitrate (NO_3^-) nitrogen concentration in soil was estimated using the method of Kolhoff and Noponen[7]. Nitrite content was determined following the method by Jaworski[8]. Proline concentration was estimated from the oven-dried leaves, following the method described by Bates et al[9]. The concentration of free amino acids was estimated following the method by Moore and Stein[10] using leucine as reference standard. Amides were estimated following the same procedure described for free amino acids except that asparagine was used in place of leucine as standard. In vivo activity of nitrate reductase (EC 1.6.6.2) enzyme was determined following the method by Jaworski[8].

Statistical analysis of the data was carried out by using GraphPad software. Tukey-Cramer multiple comparison test was carried out and, based on P values, data was represented as significant ($P = 0.01$–0.05), highly significant ($P = 0.001$–0.01), and very highly significant ($P < 0.001$).

RESULTS

Nitrate nitrogen

The nitrate nitrogen concentration in different parts of the experimental grasses under the influence of NaCl salinity is shown in Table 1. It is also clear from results that the nitrate nitrogen was accumulated at the higher concentrations of salinity. It was increased by a maximum of 14 and 19% (100 mM), respectively, for *Cymbopogon nardus* and *Cynodon dactylon* while comparable figures were 44 and 36% for *Pennisetum alopecuroides* and *Vetiveria zizanioides*, respectively. Nitrate nitrogen concentration decreased at the highest concentration of salinity stress (300 mM).

Table 1 Effect of NaCl on nitrate nitrogen concentration (mg/100 g dry matter) of the leaves of four grass species.

Species	NaCl (mM)					
	Control	25	50	100	200	300
Cymbopogon nardus	1142 ± 13 (0)[a]	1206^{*} ± 18 (+6)	1268^{**} ± 16 (+11)	1303^{*} ± 15 (+14)	1260^{***} ± 16 (+10)	1099 ± 28 (−4)
Cynodon dactylon	1076 ± 10 (0)[a]	1105 ± 9 (+3)	1239^{***} ± 11 (+15)	1283^{***} ± 5 (+19)	952^{**} ± 18 (−12)	745^{**} ± 33 (−31)
Pennisetum alopecuroides	758 ± 14 (0)[a]	733 ± 8 (−3)	860^{*} ± 18 (+14)	975^{***} ± 9 (+29)	1088^{***} ± 19 (+44)	639^{***} ± 23 (−16)
Vetiveria zizanioides	973 ± 11 (0)[a]	1036^{**} ± 9 (+6)	1152^{**} ± 16 (+19)	1254^{*} ± 5 (+29)	1321^{*} ± 9 (+36)	705^{***} ± 35 (−28)

[a] Values in parentheses indicate percentage variations relative to control values, values are mean of three experiments ± SD, significantly different from the control at $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ by one-way ANOVA with Tukey-Kramer multiple-comparison test.

Table 2 Effect of NaCl on nitrite nitrogen concentration (µg/100 g dry matter) of the leaves of four grass species.

Species	NaCl (mM)					
	Control	25	50	100	200	300
Cymbopogon nardus	2.27 (0)[a]	1.86^{***} (−18)	2.18^{***} (−4)	2.34^{**} (+3)	2.52^{***} (+11)	1.51^{***} (−34)
Cynodon dactylon	1.01 (0)[a]	1.04 (+3)	1.05 (+4)	1.13^{***} (+12)	0.83^{***} (−18)	0.61^{***} (−40)
Pennisetum alopecuroides	1.22 (0)[a]	1.30^{**} (+7)	1.38^{***} (+13)	1.11^{***} (−9)	1.02^{***} (−16)	0.78^{***} (−36)
Vetiveria zizanioides	2.02 (0)[a]	2.13^{***} (+6)	2.22^{***} (+10)	2.34^{***} (+16)	2.38^{***} (+18)	1.90^{***} (−6)

[a] Values in parentheses indicate percentage variations relative to control values, significantly different from the control at $^{*}P < 0.05$, $**P < 0.01$, $^{***}P < 0.001$ by one-way ANOVA with Tukey-Kramer multiple-comparisons test.

Nitrite nitrogen

Nitrite nitrogen content was elevated under lower concentrations of salinity and decreased under the highest (300 mM). The maximum increase was 11% (200 mM) in *Cymbopogon nardus*, 12% (100 mM) in *Cynodon dactylon*, 13% (50 mM) in *Pennisetum alopecuroides*, while it was 18% (200 mM) in *Vetiveria zizanioides* (Table 2).

Table 3 Effect of NaCl on proline concentration (mg/100 g dry matter) of the leaves of four grass species.

Species	NaCl (mM)					
	Control	25	50	100	200	300
Cymbopogon nardus	16.7 ± 1.5 (0)[a]	16.0 ± 1.6 (−4)	18.9 ± 1.5 (+14)	19.5 ± 1.5 (+17)	21.1*** ± 1.5 (+45)	30.2*** ± 1.2 (+81)
Cynodon dactylon	24.9 ± 1.5 (0)[a]	23.4 ± 1.3 (−6)	28.3 ± 1.3 (+14)	36.2*** ± 1.7 (+46)	42.9*** ± 1.6 (+73)	46.8 ± 1.6 (+88)
Pennisetum alopecuroides	19.5 ± 1.5 (0)[a]	18.6 ± 1.5 (−4)	21.5 ± 1.6 (+10)	23.5 ± 1.6 (+21)	34.2*** ± 1.5 (+75)	44.0*** ± 1.6 (+126)
Vetiveria zizanioides	12.5 ± 1.6 (0)[a]	11.8 ± 1.6 (−6)	10.8 ± 1.5 (−4)	13.0 ± 1.3 (+4)	16.4 ± 1.5 (+32)	20.9*** ± 1.3 (+68)

[a] Values in parentheses indicate percentage variations relative to control values, values are mean of three experiments ± SD, significantly different from the control at $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ by one-way ANOVA with Tukey-Kramer multiple-comparisons test.

Table 4 Effect of NaCl on free amino acids concentration (mg/100 g dry matter) of the leaves of four grass species.

Species	NaCl (mM)					
	Control	25	50	100	200	300
Cymbopogon nardus	396 ± 6 (0)[a]	425 ± 13 (+7)	518*** ± 11 (+30)	603*** ± 28 (+52)	735*** ± 28 (+86)	520*** ± 25 (+31)
Cynodon dactylon	679 ± 13 (0)[a]	735* ± 9 (+8)	792*** ± 16 (+17)	939*** ± 22 (+38)	601** ± 37 (−11)	526*** ± 15 (−23)
Pennisetum alopecuroides	575 ± 9 (0)[a]	585 ± 12 (+2)	658** ± 37 (+15)	823*** ± 24 (+43)	878*** ± 12 (+53)	951*** ± 16 (+65)
Vetiveria zizanioides	353 ± 18 (0)[a]	374 ± 15 (+6)	465*** ± 23 (+32)	443** ± 13 (+25)	518*** ± 24 (+47)	607*** ± 25 (+72)

[a] Values in parentheses indicate percentage variations relative to control values, values are mean of three experiments ± SD, significantly different from the control at $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ by one-way ANOVA with Tukey-Kramer multiple-comparisons test.

Proline

The concentration of proline in the leaves of all the experimental grasses was increased under saline conditions and showed a positive correlation with increasing concentrations of salinity. Maximum percent increase in proline content was found as 81, 88, 126, and 68%, respectively, in *Cymbopogon nardus*, *Cynodon dactylon*, *Pennisetum alopecuroides*, and *Vetiveria zizanioides* at 300 mM NaCl salinity (Table 3).

Free amino acids

The concentration of free amino acids in the leaves of all the experimental grasses was considerably increased under saline conditions stress and showed a positive correlation with increasing concentrations of salinity. Maximum percent increase was found as 86% (200 mM), 38% (100 mM), 65% (300 mM), and 72% (300 mM), respectively, in *Cymbopogon nardus*, *Cynodon dactylon*, *Pennisetum alopecuroides* and *Vetiveria zizanioides* (Table 4).

Amides

The concentration of amides in the leaves of all grasses increased under salinity stress and showed a positive correlation with increasing concentrations of salinity (Table 5). Maximum percent increase was found to be 87% (200 mM), 38% (100 mM), 72% (300 mM), and 73% (300 mM), respectively, in *Cymbopogon nardus*, *Cynodon dactylon*, *Pennisetum alopecuroides*, and *Vetiveria zizanioides*. It can also be noted that the concentration of amides in the leaves was increased linearly with increasing the concentration of salinity in the rooting medium of all grasses except *Cynodon dactylon* in which it declined when the salinity concentration exceeded 100 mM NaCl.

Table 5 Effect of NaCl on amides concentration (mg/100 g dry matter) of the leaves of four grass species.

Species	NaCl (mM)					
	Control	25	50	100	200	300
Cymbopogon nardus	582 ± 14 (0)[a]	619 ± 30 (+6)	759*** ± 18 (+31)	890*** ± 41 (+53)	1085*** ± 41 (+87)	767*** ± 36 (+32)
Cynodon dactylon	1002 ± 19 (0)[a]	1084 ± 16 (+8)	1169*** ± 24 (+17)	1385*** ± 32 (+38)	887** ± 54 (−11)	776*** ± 23 (−23)
Pennisetum alopecuroides	815 ± 28 (0)[a]	862 ± 26 (+6)	971 ± 54 (+19)	1214 ± 36 (+49)	1295*** ± 18 (+59)	1403*** ± 24 (+72)
Vetiveria zizanioides	522 ± 27 (0)[a]	552 ± 23 (+6)	687*** ± 34 (+32)	723*** ± 18 (+39)	764*** ± 36 (+47)	900 ± 42 (+73)

[a] Values in parentheses indicate percentage variations relative to control values, values are mean of three experiments ± SD, significantly different from the control at $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ by one-way ANOVA with Tukey-Kramer multiple-comparison test.

Nitrate reductase

The activity (expressed as µg of NO_2^- liberated per h per mg protein) of nitrate reductase was increased

Table 6 Effect of NaCl on nitrate reductase activity (µg of NO_2^- liberated per h per mg protein) of the leaves of four grass species.

Species	NaCl (mM)					
	Control	25	50	100	200	300
Cymbopogon nardus	0.30 ± 0.01 (0)[a]	0.32 ± 0.02 (+11)	0.68*** ± 0.02 (+130)	0.39** ± 0.02 (+37)	0.19** ± 0.03 (−35)	0.11*** ± 0.02 (−63)
Cynodon dactylon	0.31 ± 0.01 (0)[a]	0.46*** ± 0.01 (+49)	0.48*** ± 0.01 (+54)	0.38*** ± 0.01 (+21)	0.42*** ± 0.02 (+36)	0.41*** ± 0.01 (+34)
Pennisetum alopecuroides	0.22 ± 0.01 (0)[a]	0.28 ± 0.01 (+26)	0.29** ± 0.02 (+31)	0.23 ± 0.01 (+2)	0.18 ± 0.02 (−19)	0.12*** ± 0.02 (−44)
Vetiveria zizanioides	0.41 ± 0.02 (0)[a]	0.44 ± 0.03 (+5)	0.48 ± 0.02 (+16)	0.58** ± 0.03 (+40)	0.95*** ± 0.04 (+130)	0.62*** ± 0.06 (+50)

[a] Values in parentheses indicate percentage variations relative to control values, values are mean of three experiments ± SD, significantly different from the control at $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ by one-way ANOVA with Tukey-Kramer multiple-comparison test.

at all salt concentrations in *Cynodon dactylon* and *Vetiveria zizanioides* over the control (Table 6). However, the concentration of this enzyme was elevated in *Cymbopogon nardus* and *Pennisetum alopecuroides* only at lower salinity regime, i.e., up to 100 mM NaCl concentrations and was considerably declined with further increase in the salt concentration. The changes in activities might be due to varying levels of defence shown by the grasses. The highest percent increase over the control was 130, 54 (50 mM), 31 (50 mM), and 130% (200 mM) in *Cymbopogon nardus*, *Cynodon dactylon*, *Pennisetum alopecuroides*, and *Vetiveria zizanioides*, respectively.

DISCUSSION

Nitrate is a natural product formed from the oxidation of organic nitrogenous compounds and is the major source of inorganic nitrogen for plants in cultivated soil. Nitrate content of the leaves and roots of salt tolerant legume *Prosopis alba* decreases at high concentration of NaCl in the growth medium[11]. Similarly, a decrease in nitrate content due to NaCl salinity has been reported in *Allenrolfea occidentalis*[12] and in *Bruguiera parviflora*[13]. In the present study, nitrate nitrogen was accumulated in the grass species grown under saline conditions especially at 50 and 100 mM NaCl as compared to control values. It might be due to an adaptation mechanism developed by the plants to overcome osmotic stress caused by salinity while further decrease in nitrate nitrogen might be related to the antagonistic relation between toxic Cl^- and NO_3^- [11,14].

As per the study by Toro[14], the assimilation of nitrite was not affected by salinity stress in cv. CO-5 but it disrupted in cv. SIC-1 of *Setaria italica*. The accumulation of nitrite in horsegram seems to be due to shortage of reductive energy[15]. From the present results it appears that in general the accumulation of nitrite nitrogen is inhibited in *Cymbopogon* and *Vetiveria*, perhaps indicating resistance to salinity stress in these grasses. The accumulation of nitrite nitrogen at elevated concentrations of NaCl in *Cynodon* and *Pennisetum* might be due to shortage of energy necessary for its reduction.

Proline occurs widely in higher plants and accumulates in larger amounts than other amino acids[16]. Proline production decreases the osmotic potential of cells, which can increase the uptake of water[17]. The effect of salt stress on proline accumulation has been reported in many plant species including *Oryza sativa*, *Morus alba*[18], and *Phaseolus mungo*[19]. Proline may act as a signalling or regulatory molecule and able to activate multiple responses that are component of the adaptation process[6]. To maintain the ionic balance in the vacuoles, cytoplasm accumulates low molecular mass compounds, called compatible solutes, as they do not interfere with normal biochemical reactions. From the foregoing discussions it is evident that in general proline accumulates in plants exposed to various environmental stresses and is not an exception to salinity stress. The increased concentrations of proline in the leaves of the experimental grasses showed a positive correlation with increasing concentrations of salinity and an increase in its concentration might be able to maintain osmoregulation and stabilization of proteins.

Amino acids are the building blocks of proteins, which form the chemical basis necessary for life and have a variety of roles in metabolism. Glutamate, glutamine, serine, alanine, and aspartate are the most abundant free amino acids in spinach under salt stress, while the pool of other amino acids like valine, leucine, isoleucine, lysine, tryptophan, and proline comprises approximately 1% of the overall amount with only traces of glycinebetaine[20]. Amino acids such as proline, asparagine, and aminobutyric acid can play an important role in the osmotic adjustment of the plant to saline conditions[21]. Total free amino acids in the leaves have been reported to be higher in salt tolerant than in salt sensitive lines of sunflower[22]. The free amino acids did accumulate in the four grasses under different concentrations of salinity, suggesting they might play an important role in osmoregulation under salinity[21] and probably as feature of selected experimental grasses to adapt under NaCl salinity stress.

Amides are the most stable of all the carbonyl functional groups. The amide content of roots of *Setaria italica* cv. SIC-1 is barely affected by salinity but that of stem and leaves is reduced, suggesting that amides do not play any significant role in osmotic adjustment under saline conditions[14]. However, in Durum wheat seedlings under salinity stress, asparagine functions as osmolyte to balance water potential within the cells, especially when nitrogen availability exceeds the need for growth[23]. An increased accumulation of asparagine and glutamine was reported to accumulate in *Salvadora persica* under saline stress[24]. From the present results it appears that an accumulation of amides under the saline environment in the grasses might be an adaptive response to tolerate NaCl salinity by acting as an osmoprotectant.

Nitrate reductase (NR), a metaflavoprotein catalysing the reduction of nitrate to nitrite, is considered as the limiting step for conversion of nitrate-N to amino acids and so for protein synthesis. NR activity in wheat plants is affected more than the nitrogen concentration under the influence of salinity stress[25]. The decrease in NR activity has been reported in lentil, pea, and chickpea[26] under saline conditions. Decrease in NR activity in *Prosopis alba* may be associated with a substantial reduction in nitrate content[11]. The decrease in nitrate reductase activity, inhibition of photosystem II and chlorophyll breakdown are all associated with increased Na^+ concentrations[27]. From the present investigation it is clear that a decrease in NR activity in the leaves of grasses at higher salinity concentrations shows a negative correlation with nitrate concentrations. That means that the substrate NO_3^- seems to be the limiting factor for the decreased activity of NR the in leaves of the grasses and the factors like dissociation of enzyme protein, unavailability of reducing power, increased Na^+ concentration in the leaves or photosynthetic products, or decrease in the enzyme synthesis might be responsible for the decline in the activity of this enzyme at higher salinity concentrations.

In the present study, it was seen that the selected parameters of nitrogen metabolism are very sensitive to NaCl salinity. Accumulation of nitrate nitrogen at lower levels in the grass species indicates an adaptation mechanism by the grasses to overcome osmotic stress. In contrast, accumulation of toxic nitrite nitrogen at elevated concentrations of the NaCl definitely related with shortage of energy necessary for its reduction and reveals a toxicity of NaCl. Increased levels of proline might play an important role to maintain osmoregulation similarly free amino acids and amides are not an exception to a role of osmoprotectant. It is also important to note that substrate NO_3^- seems to be the limiting factor for the decreased activity of nitrate reductase and the overall damage caused by NaCl salinity. The tolerance level to NaCl salinity differs within the grasses and even slightly higher concentration than the tolerance level may impair the studied parameters involved in nitrogen metabolism.

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