

Sulfur and Nitrogen Co-ordinately Improve Photosynthetic Efficiency, Growth and Proline Accumulation in Two Cultivars of Mustard Under Salt Stress

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Abstract

The potential of applied nitrogen (N) or sulfur (S) at 100 mg.kg⁻¹ soil individually or in combination in the alleviation of 100 mM NaCl stress was studied in mustard (*Brassica juncea* L.) cvs. Alankar and Chutki. In general, salt stress decreased photosynthetic efficiency, nitrate reductase activity, N content and growth in both the cultivars, but the effect was greater in Chutki compared to Alankar. In contrast, proline accumulation increased under salt stress but to a greater extent in Alankar. The individual application of N and S protected the plants from salt stress, but combined N and S treatment was more efficacious in alleviating salt stress and more conspicuously in Alankar by limiting chlorophyll degradation, increasing N assimilation and proline accumulation. It was concluded that the cultivar Alankar was more responsive to individual and combined N and S treatments. However, the combined N and S application resulted in greater proline accumulation and alleviated salt stress effects on photosynthetic efficiency and growth.

Keywords: Nitrogen; Sulfur; Salt stress; Proline

Introduction

Soil salinity is one of the environmental stresses limiting agricultural productivity worldwide [1,2]. There are two main sources that contribute to the soil salinity: primary or natural cause resulting from weathering of minerals and soil derived from saline parent rocks [3], and secondary source of salinization is use of saline irrigation water, excessive application of chemical fertilizers, deforestation, overgrazing, or intensive cropping [3-5]. At present, its extent throughout the world is increasing regularly [6] and has now become a serious threat to sustainable agriculture [7-9]. According to an estimate by Food and Agriculture Organisation [10] over 6% of the world's land is salt affected. In addition, out of 230 million hectares of irrigated land, 45 million hectares (~20%) are salt affected [11]. It is expected that increased salinization of arable land to have global detrimental effects that may result in 30% land loss within the next 25 years and up to 50% by the year 2050 [12].

Salinity reduces plant growth by the presence of excessive amounts of Na⁺ and Cl⁻ ions, osmotic effects and nutrients imbalance [13,14]. Salt stress adversely affects nutrients uptake, carbon and nitrogen (N) metabolism, chlorophyll biosynthesis and photosynthesis and growth of plants [15,16]. The decline of photosynthesis in the presence of salt stress has been attributed to the sensitivity of photosynthetic apparatus [17]. The salt-inhibited photosynthetic activity might be due to PSII photo inhibition. Chlorophyll (Chl) *a* fluorescence measurement is a rapid tool used to identify the damage to PSII and has been widely used to screen cultivars for salinity tolerance.

Plants develop several mechanisms to induce tolerance to overcome salinity effects. The osmotic adjustment by the increased synthesis of osmolytes such as proline is considered of great significance [18]. The role of proline in cell osmotic adjustment, membrane stabilization and detoxification of toxic ions in plants exposed to salt stress has been reported [5]. The osmolytes in chloroplasts protect the PS II and chloroplast proteins from high NaCl concentration [19]. Additionally, management of mineral nutrients availability can be promotive in osmolytes synthesis for efficient defense system.

The assimilatory pathways of S and N have been considered functionally convergent and well coordinated [20,21]. The effects of one element are positively influenced by the other. It is, therefore, assumed that the effects of N in inducing proline synthesis will be greater in the presence of S, and the adequate supply of S and N may result in reducing the negative effects of salinity stress. Moreover, the cultivar with high N assimilation capacity if supplemented with adequate S can be a better ameliorator of salt stress. In the present study, the ameliorative effect of individual and combined application of N and S in salinity stress in Alankar and Chutki cultivars of mustard (*Brassica juncea* L. Czern & Coss.) with different N assimilation capacity was investigated.

Material and Methods

Plant material and treatments

Seeds of Alankar and Chutki cultivars of mustard (*Brassica juncea* L. Czern & Coss.) were surface sterilized and were sown in 23 cm diameter earthen pots containing soil composed of peat and compost (4:1, w/w) mixed with sand (3:1, w/w). The pots were kept in green house of the Botany Department, Aligarh Muslim University, Aligarh under natural day/night conditions with day/night temperatures of 22/12 ± 3°C, photo synthetically active radiation (PAR), 900 ± 25 μmol m⁻²s⁻¹) and relative humidity of 65 ± 5%. Two plants per pot were maintained after germination, and were treated with 100 mM NaCl individually or combined with N or/and S given at 100 mg.kg⁻¹ soil. In addition, the treatment with no salt and no N or S was considered

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as control. The native amount of N and S present in the soil was 100 mg.kg⁻¹ soils each. The experimental layout was completely randomized included five treatments and four replicates for each treatment. At 30 d after sowing (DAS), measurements were done and care was taken to select same age of leaves for the determinations.

Chlorophyll fluorescence and content, and percent pheophytin

Chlorophyll fluorescence of fully expanded second leaf from top was measured *in vivo* using chlorophyll fluorometer (Os-30P, USA). After 30 min dark adaptation of leaves, minimum chlorophyll fluorescence yield (F_0) and maximum chlorophyll fluorescence yield (F_M) were measured. The F_0 was obtained with modulated low light and F_M was determined by saturating light pulse. The variable fluorescence (F_V) was determined by the difference between F_0 and F_M , and the F_V/F_M ratio was calculated. F_V/F_M ratio represents the relative state of PSII and gives information on the functional damage of photosynthetic apparatus in plants.

Total chlorophyll of the same leaves was extracted using the method of Hiscox and Israelstam [22] using dimethyl sulphoxide (DMSO) as an extraction medium. The leaves of about 1 cm² were placed in 10 ml DMSO for 30 min for complete discoloration. The absorbance of the extract was read at 645 and 663 nm on UV-VIS spectrophotometer [SL164, Elico, Hyderabad, India] and chlorophyll content was calculated using the formula of Arnon [23].

For measuring pheophytin, per cent degradation of Chl, the method of Bowler et al. [24] was followed. Fresh leaf tissue was ground with sufficient amount of 80% acetone and centrifuge at 10,000 rpm for 5 min and the increase in the absorbance of the extract was recorded at 553 and 665 nm.

Plant growth

The plants were uprooted and washed under running tap water and dried in hot air oven at 80°C till constant weight. The samples were weighed to record plant dry mass. Leaf area of plant was measured using leaf area meter (LA211, Systronics, New Delhi, India).

Nitrate reductase (NR) activity and N content

Nitrate reductase (EC 1.6.6.1) activity in leaves was measured by preparing an enzyme extract using the method of Kuo et al. [25]. Leaf tissue (1.0 g) was frozen in liquid N₂, ground to a powder with a chilled mortar and pestle, and then stored at -80°C. The powder was thawed for 10 min at 4°C and was homogenized in a blender in 250 mM Tris-HCl buffer, pH 8.5, containing 10 mM cysteine, 1 mM EDTA, 20 μM FAD, 1 mM DTT, and 10% (v/v) glycerol. The homogenate was centrifuged at 10,000 × g for 30 min at 4°C. Nitrate reductase activity was assayed as the rate of nitrite production at 28°C adopting the procedure of Nakagawa et al. [26]. The assay mixture contained 10 mM KNO₃, 0.065 M HEPES (pH 7.0), 0.5 mM NADH in 0.04 M phosphate buffer (pH 7.2) and enzyme in a final volume of 1.5 ml. The reaction was initiated by adding NADH. After 15 min the reaction was terminated by adding 1 ml of 1 N HCl solution containing 1% sulfanilamide followed by the addition of 1 ml of 0.02% aqueous N-1-naphthylethylene-di-amine-dihydrochloride (NED). The absorbance was read at 540 nm using a spectrophotometer (SL164 Elico, New Delhi, India) after 10 min.

Leaf N content was estimated by the Kjeldahl digestion method as described by Lindner [27].

Proline content

Leaf proline content was estimated following the procedure of Bates et al. [28]. Fresh leaf sample (0.5 g) was homogenized in a mortar and pestle with 5 ml of 3% sulphosalicylic acid (3 g of sulphosalicylic acid was dissolved in sufficient DDW and final volume was maintained to 100 ml with DDW). The homogenate was filtered and collected in a test tube with two washing with 5 ml of sulphosalicylic acid. 2.0 ml of the filtrate and 2 ml each of glacial acetic acid and acid ninhydrin (1.25 g of ninhydrin was dissolved in a mixture of warm, 30 ml of glacial acetic acid and 6 M phosphoric acid (pH 1.0) with agitation till it got dissolved. It was stored at 4°C and used within 24 h. The 6 M phosphoric acid was prepared by mixing 11.8 ml of phosphoric acid with 8.2 ml of DDW) were taken in a test tube. This mixture was heated in boiling water for 1 hour. The reaction was terminated by transferring the test tubes to the ice bath. Four ml of toluene was mixed to the reaction mixture with vigorous shaking for 20-30 seconds. The toluene layer was aspirated and warmed to room temperature. The absorbance of red colour was read at 520 nm against a reagent blank. The amount of proline in the sample was calculated by using a standard curve prepared from pure proline (range 0.1-36 μ mol) and expressed on fresh mass basis of the sample.

Statistical analysis

Data were analyzed statistically using analysis of variance (ANOVA) and presented as treatment mean ± SE of four measurements. Least significant difference (LSD) was calculated for the significant data at $p < 0.05$. Bars with the same letter are not significantly different by LSD test at $p < 0.05$.

Results

Chlorophyll fluorescence

Salinity stress decreased the Chl fluorescence of both the cultivars, but the decrease was higher in Chutki than Alankar. The decrease in Chl fluorescence of Alankar was 18.7% and of Chutki was 34.3% in comparison to control (Figure 1).

The individual or combined application of N and S resulted in an increase in Chl fluorescence of both the cultivars in salt stressed plants. The individual application of N and S to salt-stressed plants increased Chl fluorescence by 5.1% and 7.6% in Alankar and 2.9% and 4.5% in Chutki, respectively in comparison to control. However, the combined application of N and S proved more effective in increasing Chl fluorescence of salt-stressed plants by 12.7% in Alankar and 11.9% in Chutki in comparison to control (Figure 1).

Chlorophyll content and pheophytin

A higher decrease in Chl in Chutki than Alankar resulted from salt treatment compared to control. The decrease in chlorophyll content was about two-times in Alankar and three-times in Chutki compared to control (Figure 2). Individual or combined application of N and S increased Chl content of both the cultivars in salt-stressed plants. The individual dose of N and S increased Chl content by 14.9% and 17.9% in Alankar and 8.3% and 11.8% in Chutki, respectively in comparison to control, but the combined application of N and S increased Chl by 43.7% in Alankar and 28.5% in Chutki in comparison to control in salt-stressed plants (Figure 2).

The degradation of Chl increased in salt treatment resulting in a significant increase in percent pheophytin of both the cultivars. The cultivar Alankar showed greater tolerance to NaCl treatment than Chutki and thus there was much lesser increase in per cent pheophytin

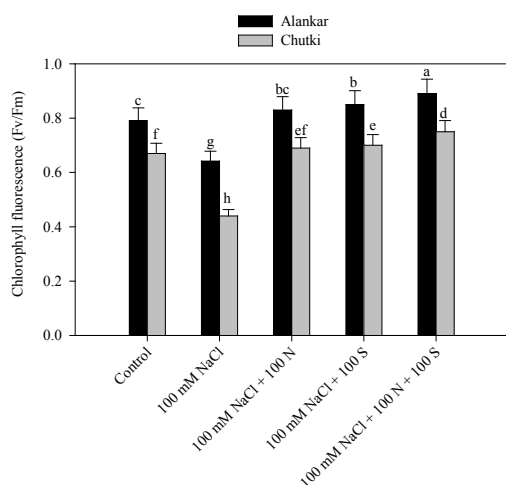


Figure 1: Chlorophyll fluorescence of leaves of Alankar and Chutki cultivars of mustard (*Brassica juncea* L.) treated with 100 mM NaCl individually or combined with N or/and S given at 100 mg kg⁻¹ soil.

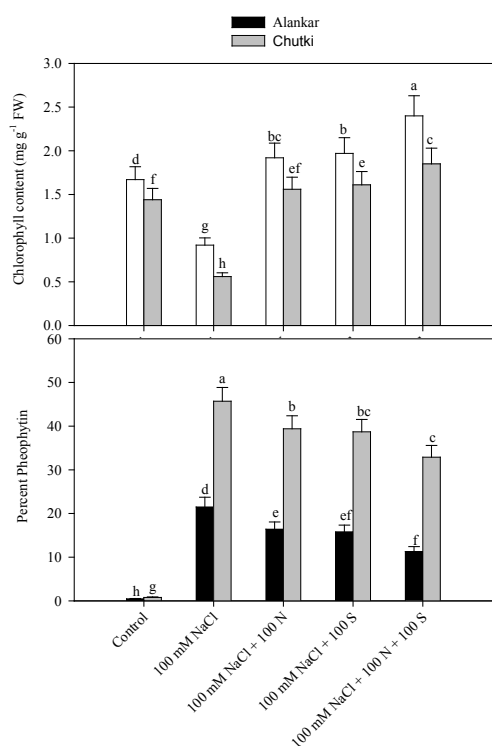


Figure 2: Chlorophyll content and percent pheophytin of Alankar and Chutki cultivars of mustard (*Brassica juncea* L.) treated with 100 mM NaCl individually or combined with N or/and S given at 100 mg kg⁻¹ soil.

in Alankar than Chutki. Individual application of N and S to salt-treated plants reduced the degradation of Chl compared to salt-treated plants. However, combined treatment of N and S on NaCl treated plants proved to be more effective in reducing the degradation of Chl and formation of pheophytin (Figure 2).

Plant growth

Plant dry mass and leaf area of both the cultivars were reduced

with salt treatment compared to control. Differences in values of plant dry mass and leaf area were large and significant depending on the tolerance capacity of the cultivars to salt stress. The cultivar Alankar showed greater tolerance to NaCl treatment than Chutki. The decrease in plant dry mass and leaf area due to NaCl in Alankar was 45.6% and 28.2%, whereas a higher decrease in plant dry mass and leaf area of 61.2% and 40.1% was recorded in Chutki in comparison with the control (Figure 3).

Treatment of salt-stressed plants with 100 mg N/kg soil or 100 mg S.kg⁻¹ soil alone or in combination resulted in the nullifying the negative effects of salt stress and subsequently promoted growth (plant dry mass and leaf area) of both the cultivars in comparison to control. The individual dose of N or S proved effective in alleviating salt-stressed decrease in plant dry mass and leaf area, but the combined dose of N and S showed more pronounced increase in plant dry mass and leaf of both the cultivars. The effect of combined application of N and S was more conspicuous in Alankar. The increase in plant dry mass due to 100 mg.N.kg⁻¹ soil, 100 mg S.kg⁻¹ soil and 100 mg.N.kg⁻¹ soil+100 mg.S.kg⁻¹ soil was 31.7%, 35.4% and 64.6% in Alankar and 17.6%, 23.5% and 47.1% in Chutki compared to control. These treatments increased leaf area by 26.4%, 30.5% and 50.1% in Alankar and 17.8%, 19.7% and 39.5% in Chutki in comparison to control (Figure 3).

Nitrate reductase activity and N content

The two cultivars differed in N assimilation capacity and showed differential effects of salt stress on NR activity and N content. These two cultivars responded differentially to individual N and S or combined N and S application in alleviation of salt stress. The activity of NR in salt-treated plants of Alankar was 42.8% lower than control, and there was

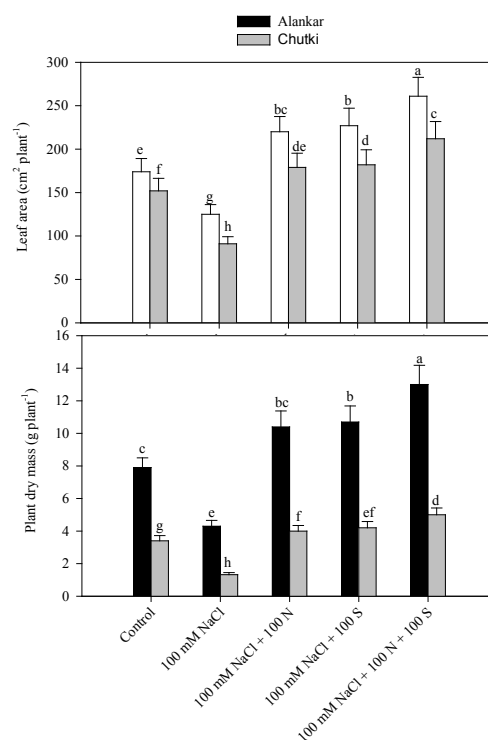


Figure 3: Leaf area and plant dry mass of Alankar and Chutki cultivars of mustard (*Brassica juncea* L.) treated with 100 mM NaCl individually or combined with N or/and S given at 100 mg.kg⁻¹ soil.

much higher decrease of 52.9% in NR activity of Chutki compared to control (Figure 4).

Application of N or S to salt-stressed plants increased NR activity of both the cultivars compared to control, but combined dose of N and S proved more effective and increased NR activity by 40.5% in Alankar and by 33.8% in Chutki in comparison to control in salt-stressed plants (Figure 4).

The treatment of plants with NaCl decreased leaf N content of both the cultivars. However, treatment of salt stressed plants with individual dose of N and S resulted in a significant increase in N content, but the effect was more pronounced in Alankar than Chutki. The N content of Alankar was increased by 11.3% with 100 mg.N.kg⁻¹ soil and 12.5% with 100 mg S.kg⁻¹ soil, whereas these treatments could increase N content by 5.5% and 7.0% in Chutki, respectively in comparison to control. Moreover, the follow up treatment with combined application resulted in significant improvement in N content of both the cultivars but to a greater extent in Alankar. The increase in N content of Alankar and Chutki was 41.7% and 23.0%, respectively in comparison to control.

Proline content

The content of proline increased in response to NaCl stress as well as N and S treatments, and was more pronounced in Alankar than Chutki. Individual application of N or S increased proline content compared to control, but lesser than combined application of N and S under salt stress. Nitrogen and S alone increased proline content by 62.3% and 65.0% in Alankar, and 27.5% and 32.6% in Chutki, respectively compared to control. The combined dose of N and S resulted in an increase in proline content by 80.5% in Alankar and 62.8% in Chutki in comparison to control in salt-stressed plants (Figure 5).

Discussion

Salinity stress negatively affects plant growth and photosynthetic functions of crop plants [2,29,30]. The plant species differ in their tolerance to salinity stress. Differences in salt tolerance exist not only among different genera and species, but also within the different organs of the same [31,32]. Response of cultivars of one species to salinity provides a convenient and useful tool for unveiling the basic mechanism involved in salt tolerance. The aim of the present study was to understand the physiological basis of difference in the tolerance of two cultivars of mustard to salt stress, and to study how these cultivars respond to the individual and combined application of N and S in the alleviation of salt stress.

Salt stress decreased growth of Chutki plants more conspicuously than Alankar. Plant dry mass and leaf area were reduced by 61.2% and 40.0% in Chutki, and 45.6% and 28.2% in Alankar due to salt treatment. The combined application of N and S was more effective in reducing the negative effects of salt stress than their individual application. The reduction in growth of plants is attributed to their differential capacity of N assimilation and proline synthesis under salt stress, which influenced photosynthetic efficiency of plants differently. Under salt stress, there appeared to be greater impairment in the biosynthesis of chlorophyll in Chutki resulting in the greater loss of chlorophyll and higher formation of pheophytin than Alankar. The quantum yield efficiency of PSII was also greatly reduced in Chutki. This cumulatively resulted in lesser growth in Chutki than Alankar. Khan [33] reported inhibition in chlorophyll biosynthesis intermediates under salt stress in wheat.

Application of N or S to salt-stressed plants increased salt tolerance, more conspicuously with combined application of N and S. The

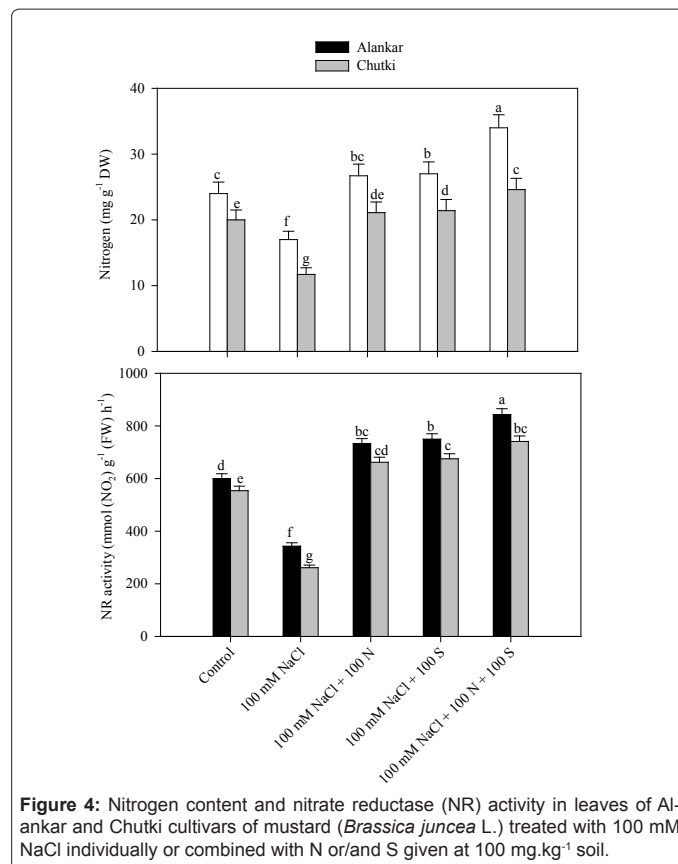


Figure 4: Nitrogen content and nitrate reductase (NR) activity in leaves of Alankar and Chutki cultivars of mustard (*Brassica juncea* L.) treated with 100 mM NaCl individually or combined with N or/and S given at 100 mg.kg⁻¹ soil.

individual role of N in the alleviation of salt stress by increasing N assimilation and osmolyte formation has been reported [34]. However, S may also regulate the formation of osmolytes by its influence on nitrate reductase activity and N assimilation as the importance of S in maintaining the tertiary structure of proteins is well documented [35]. Khan et al. [36] have reported that S supply improved photosynthesis and growth through regulating N assimilation. The availability of S regulates the activity of nitrate reductase and the accumulation of N [37]. The combined application of N and S improved N assimilation more than their individual application suggesting that these two nutrients worked co-ordinately in enhancing N assimilation resulting in protection of chlorophyll degradation and photosynthetic efficiency and growth of plants. Anjum et al. [38] have shown that sufficient-S supply improved the photosynthetic efficiency of *Brassica campestris* because S maintained higher cell redox state responsible for reducing environment in the cell. This helped in maintaining the appropriate structure and activity of protein molecules by avoiding inhibition of the formation of intermolecular disulfide bridges [39]. These results indicate a possible involvement of S in the nitrate-regulated growth response under salt conditions. Sulfur and N availabilities closely interact with S and N management by the plant [40]. Positive interaction between S and N has been reported to be beneficial for various aspects of oilseed brassicas including tolerance to various stress factors [41,42] have shown using field-grown oilseed rape that S deficiency can reduce N-use efficiency and that N deficiency can also reduce S-use efficiency. Thus, it may be said that the cultivars with high N assimilation capacity supplemented with adequate S can be a better ameliorator of salt stress.

In response to NaCl stress plants tend to accumulate large amounts of compatible solutes particularly proline [3,43-48]. It has been

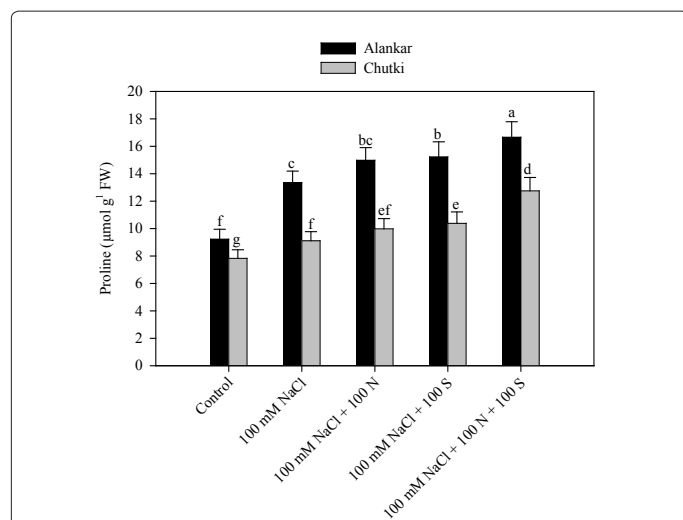


Figure 5: Proline content in leaves of Alankar and Chutki cultivars of mustard (*Brassica juncea* L.) treated with 100 mM NaCl individually or combined with N or/and S given at 100 mg.kg⁻¹ soil.

reported that *Brassica juncea* uses proline as compatible solutes and as an osmoprotectant [49]. Its main function appears not to be an osmotic, but also protective (protection of proteins, membranes etc.). P5CS is a novel bifunctional enzyme that catalyses the first two steps of proline biosynthesis in plants through glutamate pathway and ornithine δ -amino transferase (δ -OAT), the other key enzyme in ornithine pathway of proline biosynthesis are affected under salt stress and N treatment [48]. It was reported that P5CS mRNA level were induced and δ -OAT mRNA level decreased under salt stress and N starvation on other hand OAT mRNA level increased under excess N. The treatments of N and S applied alone or in combination improved proline content in plants under salt stress, and most prominently in Alankar giving more tolerance to this cultivar compared to Chutki. The results of the present study show that there is a positive relationship between proline accumulation and tolerance of mustard plants to salt stress. Proline content increased in both the cultivars subjected to salt stress and also with N and S treatments, but the accumulation was highest in Alankar than Chutki (Figure 5). In fact, it was the difference in potential of both the cultivars to accumulate proline that was influenced by different treatments resulting in difference in salt tolerance capacity of the cultivars. Study of [8] showed that the tolerant genotypes had higher proline accumulation. Adequate N levels were also reported to increase proline content of leaves of salt-treated plants and maintain a higher turgor pressure [50]. Halophytes also exhibit proline accumulation as one of the tolerance mechanisms to NaCl salinity and to other abiotic stresses [51,5]. This extremely water soluble amino acid forms clusters with water molecules which attach to proteins and membranes and prevent their denaturation [5,49,52,53]. Due to its protective function on membranes it can also improve cell water status and ion homeostasis [54,55], and serves as a scavenger for hydroxyl radicals and singlet oxygen and thus reduces oxidative stress [5,51,53].

Conclusively, it may be said that N accumulation, chlorophyll biosynthesis and proline accumulation were more prominently induced in the cultivar Alankar upon treatments of N or S or both. The combined application of N and S was more efficacious in inducing the above mechanisms and salinity stress alleviation in Alankar and promoted plant growth. In contrast, the cultivar Chutki was less efficient in responding to the N and S treatments and therefore, was less tolerant to

salinity stress. Thus, combined N and S fertilization of mustard may be adopted to reduce the negative of salt stress in problem soils.

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