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Research Article
Effect of Nitrogen and Phosphorus on the Growth and Redox Homeostasis of Salt-stressed Mustard Plants

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Abstract

Background and Objective: Salt stress brings about a considerable change in the physiological and biochemical processes which significantly hampers the growth and productivity of the plants to a greater extent. **Materials and Methods:** Indian mustard was chosen as plant material. The seeds were washed thoroughly with water and surface sterilized with 0.01% mercuric chloride and washed again with distilled water prior to sowing in the pots containing a mixture of sand and vermiculite (1:1). Four different treatments were given to 20 days-old plants viz. NaCl (T1), NaCl+N (T2), NaCl+P (T3) and NaCl+N+P (T4). Standard methodology was utilized to carry out the research. **Results:** In the present study four experimental lines were set up simultaneously viz. NaCl (T1), NaCl+N (T2), NaCl+P (T3) and NaCl+N+P (T4). The treatments include 50 mM Nacl, 100 mM Nitrogen and 100 mM Phosphorus. The T4 samples showed significant results with respect to NaCl stressed plants. **Conclusion:** Thus our results suggest that overall growth and productivity of the salt stressed plants can be improved by the combined application of nitrogen and phosphorus fertilizers.

Key words: Salt stress, nitrogen, phosphorous, mustard plants

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

The escalating human population is proposed to cross 9.4 billion by the middle of this century. Meeting the food demands of this expanding population is a burning issue of the hour. The main constrains to the agricultural production are the environmental stresses. Various environmental stresses including heavy metal, salinity, high temperature and drought stress is well-known[1,2]. Salt stress is one of these major environmental predicaments that induce adverse effects both on soil and agriculture, particularly in the areas with high evapotranspiration rates, limiting crop production in arid and semi-arid regions where soil salt content is naturally high and precipitation can be insufficient for leaching. Anthropogenic activities lead to higher accumulation of salts day by day like deforestation, industrialization, overgrazing and improper irrigation practices. Many cellular and physiological processes of plants are being hampered including nutrient uptake and transport, water absorption, photosynthesis, nutrient uptake and metabolism, resulting in decreased growth, development and productivity.

One of the primary effects of salt stress is the alteration of redox homeostasis. The electrons in the transport chains reduce oxygen during salt stress leading to the overproduction of reactive oxygen species including superoxide ions, hydrogen peroxide and hydroxyl radicals. These ROS cause the oxidation of life-supporting biomolecules like lipids, proteins, nucleic acids and carbohydrates disrupting their structure and the metabolic pathways of which they are a part.

Besides oxidative damage, salt stress is known to cause the deficiency of mineral nutrients in the plant by interfering with their availability in the soil, uptake and transport within the plant body. Potassium is one of these essential nutrients which plays indispensable role in the growth and development of plants. It is the most abundant cation in plants found mainly in chloroplast and cytosol and regulates the function of more than 50 enzymes through the stabilization of pH, binding to enzymes and maintaining functional conformation of enzymes. Besides, it helps in osmo-regulation and cell elongation.

Nitrogen, the most important nutrient in plants, plays a key role in different cellular and physiological processes including osmotic adjustment, energy transfer, detoxification of reactive oxygen species, protein synthesis, stomatal regulation, phloem transport, an important indicator of salt tolerance in plants involves the regulation of K^+/Na^+ homeostasis. Plants adapted to higher pH and aerobic soils take nitrogen in the form of nitrate, two nitrate transporter systems coexist in plants and they act in a coordinate manner to take up nitrate from the soil solution and distribute it within the whole plant[3,4]. Another essential element, Phosphorus which helps in maintaining the structural and functional integrity of membranes, stabilization of cell wall and regulation of ion homeostasis. Phosphorus (P) plays an important role in alleviation of the adverse effects of high salinity on whole plant biomass for a variety of crop plants[5]. Nitrogen and phosphorus seem to be readily displaced from its binding sites by other sodium and chloride and the functional aspects associated with these essential nutrients may become critically impaired. Maintaining sufficient concentrations of nitrogen and phosphorus in saline soil is an imperative cause in the domineering of specific ion toxicities, particularly in glycophytes which are more prone to salt damage. For overcoming the negative impact of high salinity, addition of supplemental Ca^{2+} to the growth medium as an ameliorative agent could be necessary.

Indian mustard, an important oilseed crop is grown over a large area in India which ranks second in its production but is still not able to meet the demand. It is cultivated in the north-west climatic zone where the existing soil salinity decreases its production to a large extent. The current study was conducted to evaluate the effect of salt stress and assess the potential of increased levels of the nitrogen and phosphorus and their combination in extenuating salt-induced damage in this important oilseed crop.

MATERIALS AND METHODS

Indian mustard (*Brassica juncea*) was chosen as plant material. Authenticated seeds of *Brassica juncea* L. Czern and coss (genotype CS-54) were procured from the Genetics Division, IARI, New Delhi, India. The seeds were washed thoroughly with water, surface sterilized with 0.01% mercuric chloride and washed again with distilled water prior to sowing in the pots containing a mixture of sand and vermiculite (1:1). After germination, 10 plants were maintained in each pot. The experiment was set in a random design. The plants were grown in Hoagland's growth solution of the one-fourth strength for first 10 days, in half-strength for next 10 days and in full strength for the last 10 days, in a growth chamber under the controlled conditions of light (16 h photoperiods), temperature (27°C) and humidity (60%). Four different treatments were given to 20 days-old plants viz. NaCl (T1), NaCl+N (T2), NaCl+P (T3) and NaCl+N+P (T4). The treatments involve 50 mM Nacl, 100 mM nitrogen and 100 mM Phosphorus. After 30 days, leaves of plants were excised and

used for experimental analysis. Three biological replicates were taken during the experimental procedure.

Biomass accumulation: At the time of harvest, plant weight was recorded before and after oven-drying the samples at 65±2°C for 72 h, when they attained a constant weight for the estimation of biomass accumulation.

Estimation of chlorophyll content: Chlorophyll content in the fresh leaf samples was estimated employing the method of Hiscox and Israelstam[6]. Briefly, 0.1 g leaves taken in moist filter paper in an icebox were washed with cold DDW and chopped. The chopped leaf material was transferred to vials, in triplicates, containing 5 mL of dimethyl sulfoxide (DMSO). The vials were kept in oven at 65°C for 1 h for complete leaching of the pigments. The DMSO was further added to make a final volume of 10 mL and optical density measured immediately. Absorbance of DMSO containing the pigments was noted at 663 and 645 nm using UV-Vis spectrophotometer. Chlorophyll 'a' and chlorophyll 'b' content were estimated employing the formulae given by Arnon[7].

Estimation of soluble protein content: Bradford's method (1976) was used for the quantification of soluble protein content. Briefly, fresh leaf material (0.5 g) was homogenized with the help of pre-cooled mortar and pestle in 0.1 M phosphate buffer with pH 6.8 at 4°C. The homogenate was transferred to 2 mL tubes and centrifuged at 5000 rpm for 10 min at 4°C. The supernatant was added with an equal amount of chilled 10% TCA for protein precipitation and then centrifuged at 3300 rpm for 10 min. The supernatant was discarded and the resultant pellet washed with acetone and then dissolved in 1 mL of 0.1 N NaOH. To 1.0 mL aliquot, 5.0 mL of Bradford's reagent (50 mL of 90% alcohol, 100 mL of o-phosphoric acid, 850 mL of double-distilled water, 0.1 g of Coomassie Brilliant Blue G-250) was added and vortexed. Tubes were kept in the dark for 10 min for optimal colour development and the absorbance noted at 595 nm. Soluble protein content was estimated with the help of standard curve, using bovine serum albumin (Sigma) as standard. The protein content was expressed in mg g^{-1} FW.

Estimation of proline content: Proline content of dried leaves was estimated by employing the method of Bates *et al.*[8]. A 0.5 g of leaf sample was homogenized in 3% sulphosalicylic acid (10 mL) followed by the centrifugation at 10,000 rpm for 10 min. The supernatant (2 mL) was taken in a test tube and 2 mL of acid ninhydrin along with 2 mL of glacial acetic acid were added to it. The mixture was incubated at 100°C in a water bath for 1 h and the reaction was terminated by placing the tubes in an ice bath. Toluene (4 mL) was added to each of it and mixed vigorously on a vortex for 10-15 sec. The toluene layer was taken from the mixture and its absorbance was measured at 520 nm using toluene as a blank. The concentration of proline in the samples was calculated against the standard curve of proline, expressed in µg g^{-1} fresh weight.

Analysis of the enzymatic antioxidants

Superoxide dismutase (SOD) activity: The SOD activity was determined according to the method of Beyer and Fridovich[9] by its ability to catalyze NBT to formazan. The reduced NBT was measured at 560 nm using the absorbance coefficient of 100 mM^{-1} cm^{-1}. The SOD activity was expressed in enzyme units per mg of protein.

Ascorbate peroxidase (APX) activity: The APX activity was determined according to the method of Nakano and Asada[10] in terms of its ability of catalyzing the reduction of hydrogen peroxide to water in the presence of 0.1 M phosphate buffer. The decrease in the absorbance was taken at 240 nm and the APX activity was calculated by using an extinction coefficient of 2.8 mM^{-1} cm^{-1}.

Glutathione reductase (GR) activity: The activity of GR was determined by the method of Foyer and Halliwell[11] estimated by monitoring the glutathione-dependent oxidation of NADPH at its absorption maxima of wavelength 340 nm. The GR activity was calculated using an extinction coefficient of 6.2 mM^{-1} cm^{-1}.

Catalase (CAT) activity: The activity of catalase was determined by the method of Aebi[12]. At 240 nm absorbance was taken to the mixture of 0.1 mL enzyme extract and 0.1 M phosphate buffer both before and after adding 0.1 mL of hydrogen peroxide. The catalase activity was calculated using an extinction coefficient of 0.036 mM^{-1} cm^{-1}.

Statistical analysis: The data obtained were statistically analyzed to check the authenticity of the results.

RESULTS

Biomass accumulation: The biomass accumulation exhibited a range of 35 mg $plant^{-1}$ (control) to 29 mg $plant^{-1}$ (T4 samples). It was found that biomass accumulation

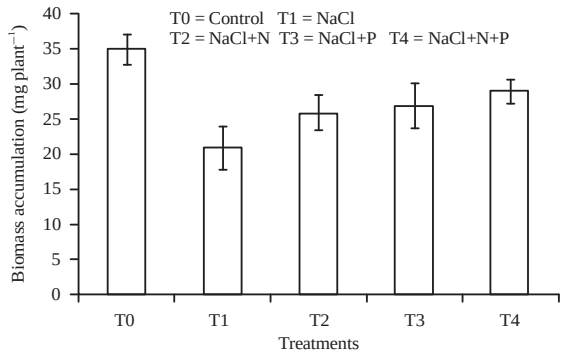

Fig 1: Variation in biomass accumulation in salt-stressed mustard on the application of nitrogen and phosphorus

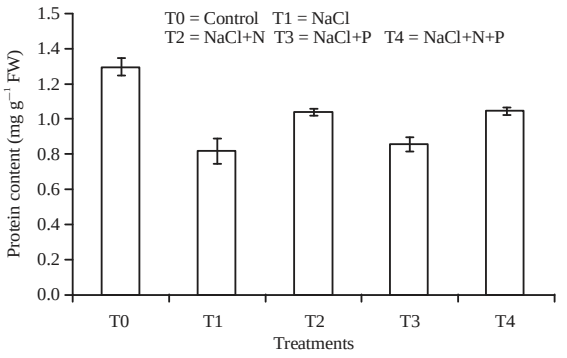

Fig 3: Variation in protein content in salt-stressed mustard on the application of nitrogen and phosphorus

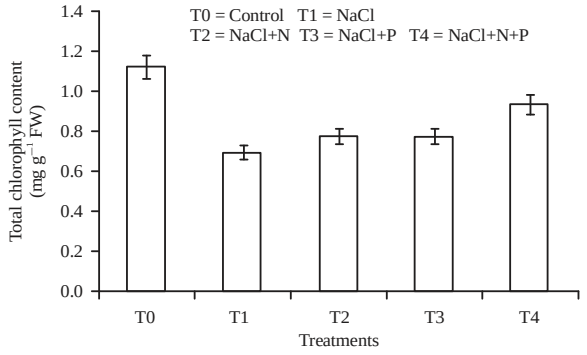

Fig 2: Variation in total chlorophyll content in salt-stressed mustard on the application of nitrogen and phosphorus

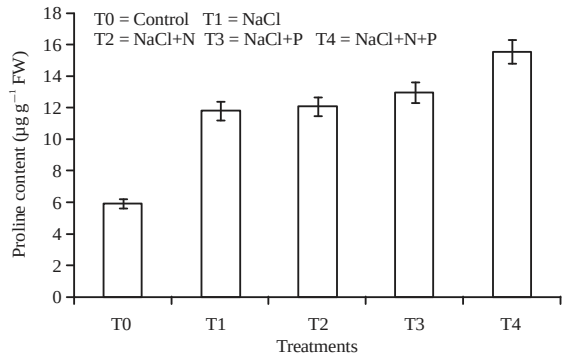

Fig 4: Variation in proline content in salt-stressed mustard induced by application of nitrogen and phosphorus

decreased (21 mg plant^{-1}) during the NaCl salt treatment with respect to the control. In case of nitrogen treatment the accumulated biomass was recorded as 26 mg plant^{-1}. In another trial phosphorous treatment was given and biomass accumulation was recorded as 27 mg plant^{-1}. Furthermore a significant upsurge was observed in T4 samples were 29 mg plant^{-1} biomass was accumulated as compared with the salt stressed plants (21 mg plant^{-1}) (Fig. 1).

Total chlorophyll content: The total chlorophyll content differed between treated samples except between T2 and T3. The chlorophyll content profiles assessed were 1.13, 0.70, 0.78, 0.78 and 0.94 mg g^{-1} FW in T0, T1, T2, T3 and T4 samples, respectively. Here it also found that NaCl salt treatment causes significant decrease in chlorophyll content (Fig. 2).

Protein content of leaves: In this experimental line prominent decrease in the protein content (0.82 mg g^{-1} FW) was observed in plant samples treated with NaCl as compared with control (1.3 mg g^{-1} FW). Moreover the combinatorial application of both nitrogen and phosphorus in T4 plant samples resulted in significant increase in protein content (1.05 mg g^{-1} FW) as compared with the NaCl stressed plants (Fig. 3).

Proline content: Proline is an important amino acid which is involved in osmotic adjustment, protein stability and other stress adaptations. Increased levels of this amino acid were noted during the various treatments in contrast with the control. The highest content were found in T4 samples (15.7 µg g^{-1} FW) followed by T3 (13.1 µg g^{-1} FW), T2 (12.18 µg g^{-1} FW) and T1 11.91 (µg g^{-1} FW), respectively (Fig. 4).

SOD activity: There was a considerable enhancement in the SOD activity within mustard plants in the treated conditions. The SOD activity was found to raise from 80 EU mg^{-1} protein min^{-1} in control to 150 EU mg^{-1} protein min^{-1} in T4 samples (Fig. 5).

APX activity: Striking differences in APX activity were found during the treated conditions. The considerable increase in

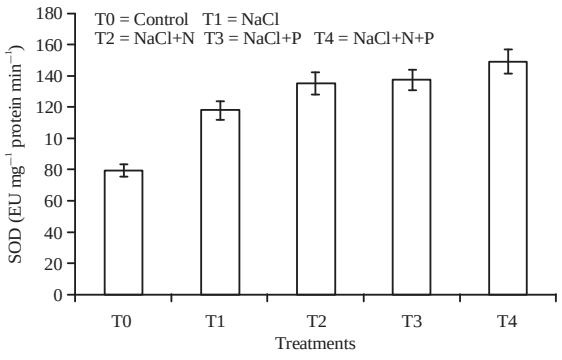

Fig 5: Variation in SOD activity in salt-stressed mustard induced by the application of nitrogen and phosphorus

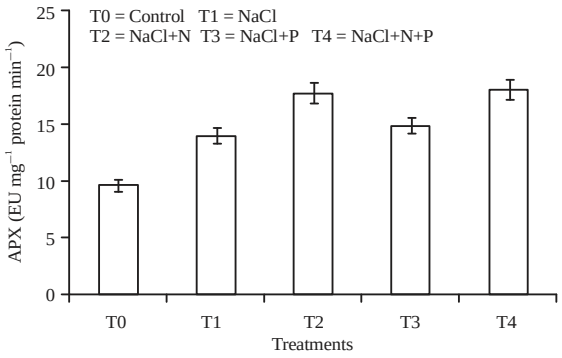

Fig 6: Variation in APX activity in salt-stressed mustard induced by the application of nitrogen and phosphorus

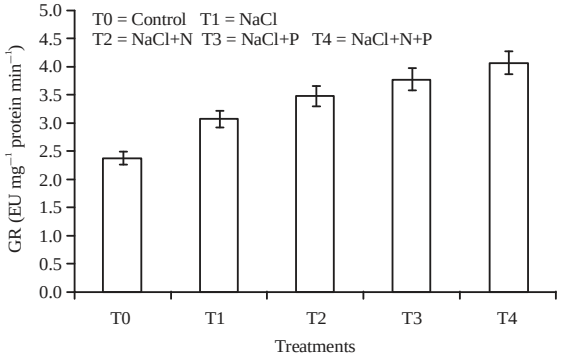

Fig 7: Variation in GR activity in salt-stressed mustard induced by the application of nitrogen and phosphorus

APX activity was analyzed from 9.7 EU mg^{-1} protein min^{-1} in control to 18.2 EU mg^{-1} protein min^{-1} in T4 samples (Fig. 6).

GR activity: The GR activity showed a linear increase during the various treatments. The GR activity profiles assessed were 2.4, 3.1, 3.5, 3.8 and 4.1 EU mg^{-1} protein min^{-1} in T0, T1, T2, T3 and T4 samples, respectively. It was found that NaCl+N+ P treatment resulted in 1.7 fold increment in GR activity as compared to control (Fig. 7).

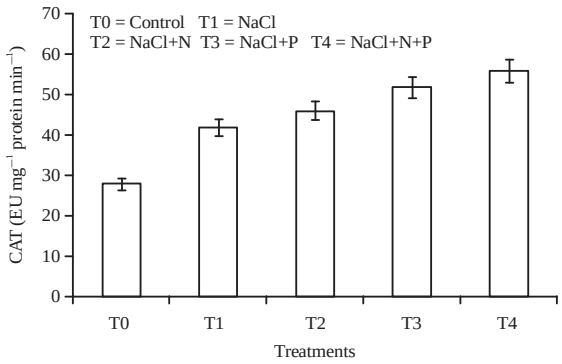

Fig. 8: Variation in CAT activity in salt-stressed mustard induced by application of nitrogen and phosphorus

CAT activity: The CAT activity profiles assessed were 28, 42, 46, 52 and 56 EU mg^{-1} protein min^{-1} in T0, T1, T2, T3 and T4 samples, respectively. The NaCl treatment alone increased the catalase activity by 42 EU mg^{-1} protein min^{-1} with respect to the control (28 EU mg^{-1} protein min^{-1}). The application of nitrogen and phosphorus in the salt-stressed plants ameliorated the catalase activity by 46 EU mg^{-1} protein min^{-1} and 52 EU mg^{-1} protein min^{-1}, respectively and the maximum activity 56 EU mg^{-1} protein min^{-1} was recorded in the NaCl stressed plants treated with nitrogen and phosphorus together (Fig. 8).

DISCUSSION

Biomass accumulation occupies the highest position in the agricultural productivity[13] and forms one of the primary indicators of plant salt tolerance[14]. Mustard biomass accumulation decreased substantially during the application of sodium chloride. The reduction in biomass may possibly be attributed to nutrient deficiency and water stress due to salt. The increase in biomass upon the fertilization by nitrogen and phosphorus nutrients indicates the differential potentials of these nutrients in combating the negative influence of salt stress. Our results were similar with the earlier findings regarding the increase in biomass of salt-stressed plants of sunflower[15], wheat[16] and melon[17] during the application of nitrogen. The highest increase in biomass related to the salt stressed plants was attributed by the combinatorial effect of nitrogen and phosphorus indicate the synergistic effect of these nutrients in minimizing the negative effect of sodium chloride on Indian mustard. Besides biomass the salt stress affected the total chlorophyll content, which determines the

photosynthetic efficiency of the plants. The decrease in the chlorophyll content may be accredited due to salt-induced deficiency of elements including magnesium and iron which play a pivotal role in the synthesis of these photosynthetic pigments and degradation of chlorophyll. Our results were supported by the some earlier studies regarding the decrease in chlorophyll content under salt stress in pumpkin[18,19]. Nitrogen and phosphorus treatments alleviated the levels of chlorophyll content with the highest increase in the application of both these nutrients suggesting their ability in overcoming the salt-induce damage of pigments. Phosphorus act as a secondary messenger in cytokinin mediated chlorophyll-biosynthetic pathway besides directly interacting with light during the pathway. Nitrogen also helps in regulating the amounts of chlorophyll levels by preventing its decomposition. Proteins form the indispensable molecules regarded as the determinants of physiological health decreased in salt-stressed plants. The lower levels of protein content may be due to inhibition of protein synthesis induced by salt stress. Application of nitrogen and phosphorus fertilizers however regained the protein content, the higher potential being exhibited by nitrogen possibly suggesting the increased protein synthesis. Our results were concurrent with the earlier findings of increased protein content in salt-stressed plants during the application of nitrogen[16,20] and phosphorus[21]. Salt stress is likely to interfere with the nitrate uptake in the plants resulting in the alteration in the activities of nitrogen assimilating enzymes. Interestingly, nitrogen and phosphorus treatments help in the reversal of NR activity altered by sodium chloride with the maximum degree during the combined application of these nutrients signifying an enhanced metabolic activity related to N assimilation as a result of the additional supply of N and P.

Proline is one of the main osmoprotectants which besides osmotic adjustment is involved in antioxidant defense and stabilization of proteins and organelles[22]. In our study although the proline levels increased during salinity stress but the application of fertilizers further augmented its levels possibly by accelerating biosynthesis of proline and breakdown of proline-rich proteins. Increased proline levels have earlier been found in the salt-stressed plants including, *Cassia angustifolia*, periwinkle, spinach.

Salt stress induces the divergence of electrons from normal pathways to oxygen reducing ones overproducing reactive oxygen species, which oxidize bio-molecules including lipids, proteins, nucleic acids and carbohydrates. Membranes which are composed of mainly lipids and proteins undergo damage. The lipid per-oxidation by ROS produces aldehydes of which malondialdehyde is the main part. At the individual level phosphorus was more effective than nitrogen which may be because of its specific role in maintaining structural integrity of cellular membranes by linking to lipid bilayer stabilizing phospholipids. In response to the oxidative stress plants possess well-demarcated anti-oxidant defense system, which scavenges the toxic ROS and protect plants from the oxidative damage. These antioxidants including SOD, APX, GR and CAT act through a well-defined glutathione-ascorbate pathway. The SOD forms the first line of defense catalyzing the dismutation of superoxide ion to hydrogen peroxide. Salt treatment increased the SOD activity but the increase was more pronounced by the treatments of P and Ca indicating their potential in conferring superoxide ion-scavenging ability. The SOD activity produces hydrogen peroxide which in itself acts a ROS and can cause membrane damage. Plants own two enzymes, catalase and APX which regulate the levels of H_2O_2 in the cells. Catalase is mainly located in the per-oxisomes where it catalyzes the breakdown of H_2O_2 to H_2O and O_2. Salt treatment induced the up regulation of CAT activities in Indian mustard in our study which was in harmony with the earlier findings in salt-stresses plants including barley[23,24] and tomato[25]. The CAT activity was ameliorated differentially with the addition of N and P indicating the dependence of CAT activity on the nutrient state of the plant. The analogous function of CAT is carried out by APX in the cellular sites including chloroplasts, mitochondria and cytosol. The increased activity of APX in salt-stressed plants indicate the presence of high levels of H_2O_2 and the enhanced levels observed during the phosphorus and nitrogen treatments suggested their roles in maintaining ROS level. GR is an antioxidant which catalyzes the reduction of glutathione disulphide to its sulphydryl form GSH which in provides reducing potential to APX for scavenging H_2O_2. In our study change in GR activity during the treatments was parallel to that of APX. Different workers have find the same results regarding the enhancement in antioxidant activities upon nitrogen fertilization in many plants under abiotic stresses Similarly the increased antioxidant activities were recorded by many workers in plants during water stress cadmium stress and salt stress[26] cadmium stress[27] and salt stress[28].

CONCLUSION

Salt stress hampers the growth and productivity to a greater extent. One of the main causes of salt-induced damage in plants is the nutrient deficiency. The growth and productivity of the salt stressed plants can be improved by the combined application of nitrogen and phosphorus fertilizers.

Nitrogen and phosphorus show a significant potential in combating the salt-induced damage by enhancing the antioxidant defense system with the maximum potential revealed by the combined action of these two nutrients.

SIGNIFICANCE STATEMENT

This research article reports a study on effect of nitrogen and phosphorus on the growth and redox homeostasis of salt-stressed mustard plants. The results reveals growth and productivity of the NaCl stressed plants can be improved by the combined application of nitrogen and phosphorus fertilizers.

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