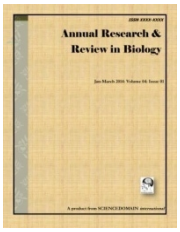

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Exogenously Applied H_2O_2 Promotes Proline Accumulation, Water Relations, Photosynthetic Efficiency and Growth of Wheat (*Triticum aestivum* L.) Under Salt Stress

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Authors' contributions

This work was carried out in collaboration between all authors. Author FA carried out the experimental work and searched literatures for the work on which this article is based. Author MIRK designed the experiment, and carried out the analyses and prepared the manuscript. Author NAK supervised the work and was involved in the design of the experiment, preparation and presentation of the manuscript. All authors read and approved the final manuscript.

Research Article

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ABSTRACT

Aim: To determine the role of hydrogen peroxide (H_2O_2) in the alleviation of salt stress in wheat (*Triticum aestivum* L.).
Design of the Study: Wheat plants were grown with or without 100 mM NaCl and were treated with 0, 50 or 100 nM H_2O_2 treatments.
Place and Duration of Study: The experimental work was carried out in the naturally illuminated green house at the Department of Botany, Aligarh Muslim University, Aligarh, India between November to December, 2012.
Methodology: Plants were sampled at 30 days after seed sowing to determine physiological, biochemical and growth parameters.
Results: Treatment of plants with H_2O_2 significantly influenced the parameters both under non saline and salt stress. The application of both 50 and 100 nM H_2O_2 reduced the severity of salt stress through the reduction in Na^+ and Cl^- content; and the increase in proline content and N assimilation. This resulted in increased water relations,

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photosynthetic pigments and growth under salt stress. However, maximum alleviation of salt stress was noted with 100 nM H_2O_2 and 50 nM H_2O_2 proved less effective. Under non saline condition also application of H_2O_2 increased all the studied parameters.
Conclusion: The treatment of 100 nM H_2O_2 maximally benefitted the wheat plants under non saline condition and alleviated the effects of salt stress. The treatment of H_2O_2 increased proline content which might help increased photosynthetic pigments and growth under salt stress. The mechanism of proline metabolism by which H_2O_2 treatment may protect against salt stress will be investigated further.

Keywords: Hydrogen peroxide; salt stress; proline; photosynthetic pigments; wheat.

1. INTRODUCTION

Salt stress is one of the abiotic stress factors that adversely affect crop production in different regions, particularly in arid and semi-arid regions. It has been estimated that over 800 million hectares of land in the world is affected by salinity [1]. High salt concentration causes ion toxicity and osmotic stress, leading to the excessive generation of reactive oxygen species (ROS), which causes damage to lipids, proteins and DNA [2]. Salt stress caused osmotic stress (drought problem), ion imbalance and the direct toxic effects of ions on the metabolic processes are the most important and widely studied physiological impairments [3]. These effects under salt stress are usually caused by elevated Na^+ and Cl^- concentration in the soil. Salt stress changes the morphological, physiological and biochemical responses of plants [4-5]. Salt stress decreases photosynthetic attributes [6-7] and plant growth and development [8]. Plants develop several mechanisms to induce tolerance to overcome salt stress effects. The osmotic adjustment by proline is considered as of great significance. The role of proline in cell osmotic adjustment, membrane stabilization and detoxification of toxic ions in plants exposed to stress has been documented [9].

Research works have shown that H_2O_2 is a signalling molecule in plants [10-12] and acts as a second messenger in response to wounding, heat, chilling, drought stress, salt stress in plants [13-17]. H_2O_2 has been shown to act as a key regulator in a broad range of physiological processes including photosynthesis [18], growth and development [19]. It plays dual role in plants under optimal and stressful environments [10]. At high concentration, it initiates programmed cell death [10], whereas at low concentration, it acts as a signal molecule and involved in acclamatory signalling molecule triggering tolerance against salt stress [17,20]. The present study was carried out to evaluate the potential of exogenously applied H_2O_2 in alleviation of salt stress in wheat (*Triticum aestivum* L.) and study proline accumulation, photosynthetic pigments, nitrogen metabolism, and growth in the presence of salt and H_2O_2 treatments.

2. MATERIALS AND METHODS

2.1 Plant Materials and Growth Condition

Seeds of wheat (*Triticum aestivum* L.) cultivar "711" were sown in 23-cm diameter earthen pots filled with 5 kg of reconstituted soil (sand: clay: peat; 70:20:10; EC: 8.8 dS m^{-1}; pH: 6.8) in the net house of Botany Department, Aligarh Muslim University, Aligarh, India under natural day/night conditions with a photosynthetically active radiation (PAR), 960 µmol/m^2/s.

Day and night temperatures were 24/18 ± 3°C, and relative humidity 68 ± 5%. After seedling establishment, three plants per pot were maintained. Treatments of 0, 50 or 100 nM H_2O_2 in the presence or absence of 100 mM NaCl were given to pots after one week of the seed germination. The sampling was done at 30 days after sowing. The experiment followed a factorial randomized complete block design and the number of replicates for each treatment was three. Among three replicates, first plant from each treatment was used for determination of ions and proline content, while another plant was used for estimation of water relations, N assimilation and photosynthetic parameters. The third plant was used for determination of growth parameters. Sampling of plants was done at 30 d after sowing (DAS) to record physiological and growth parameters.

2.2 Determination of Na^+ and Cl^- Content

The content of Na^+, Cl^- and K^+ was determined in the digested plant samples using Tri acid mixture (TAM), which is a mixture of nitric acid, sulphuric acid and perchloric acid in the ratio of 10:5:4. The content of Na^+ and K^+ was estimated using flame photometer (Khera-391: Khera Instruments, New Delhi), whereas Cl^- content was determined by titration against 0.02 N silver nitrate solution using 5% K_2CrO_4 as indicator.

2.3 Estimation of Proline Content

Proline content was determined spectrophotometrically by adopting the ninhydrin method of Bates et al. [21]. Fresh leaves (300 mg) were homogenized in 3 ml of 3% sulphosalicylic acid. The homogenate filtrate was reacted with 1 ml each of acid ninhydrin and glacial acetic acid for 1h in a test tube placed in a water bath at 100ºC. The mixture was extracted with toluene and the absorbance was measured at 520 nm using L-proline as a standard.

2.4 Water Potential and Osmotic Potential

Leaf water potential was measured on the second leaf from top (fully expanded youngest leaf) of the plant by using water potential system (Psypro, WESCOR, USA). The leaf used for water potential measurement was frozen in liquid nitrogen in sealed polythene bags which was thawed and cell sap was extracted with the help of a disposable syringe. The extracted sap was used for the determination of osmotic potential using a vapour pressure osmometer (5520, WESCOR, USA).

2.5 Nitrate Reductase Activity and Leaf Nitrogen Content

Nitrate reductase (NR) activity in leaves was measured by preparing an enzyme extract using the method of Kuo et al. [22]. Leaf tissue (1.0 g) was frozen in liquid N_2, ground to powder with a chilled mortar and pestle, and then stored at −80ºC. The powder was thawed for 10 min at 4ºC and was homogenized in a blender in 250 mM Tris–HCl buffer, pH 8.5, containing 10 mM cysteine, 1 mM EDTA, 20 M FAD, 1 mM DTT, and 10% (v/v) glycerol. The homogenate was centrifuged at 10,000 × g for 30 min at 4ºC. NR activity was assayed as the rate of nitrite production at 28ºC adopting the procedure of Nakagawa et al. [23]. The assay mixture contained 10 mM KNO_3, 0.065 M HEPES (pH 7.0), 0.5 mM NADH in 0.04 mM 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-napthylethylene-di-amine-dihydrochloride (NED). The absorbance was read at 540 nm after

10 min. Leaf N content was determined in acid–peroxide digested material using the method of Lindner [24].

2.6 Net Photosynthesis and Photosynthetic Pigments

Net photosynthesis was measured in fully expanded uppermost leaves of plants in each treatment using infra red gas analyzer (CI-340, Photosynthesis System, CID Bio-Science, USA). The measurements were done between 11.00 and 12.00 h at light saturating intensity and at 370 ± 5 µmol mol^{-1} atmospheric CO_2 concentrations. Total chlorophyll and carotenoid content was extracted by using the method of Hiscox and Israelstam [25] by using dimethyl sulphoxide as an extraction medium and estimated and calculated by the method of Arnon [26].

Fresh leaves (100 mg) were cut into small pieces and collected in the test tube containing 7 ml of DMSO. The test tubes were covered with black paper and incubated at 45ºC for 40 min. for the extraction. The content was transferred to a graduated tube and the final volume was made to 10 ml with DMSO. Extract measuring 3 ml was transferred to cuvette and absorbance was read at 645 and 663 nm for chlorophyll content and at 480 and 510 nm for carotenoid content on Spectrophotometer (SL 164, Elico, Hyderabad, India).

2.7 Determination of Growth Parameters

Shoot length and root length were measured by a meter scale. After recording fresh weight, plants were dried in an oven at 80ºC for 48 h till constant weight dry weight of plants was determined. Leaf area was measured by a leaf area meter (LA 211 Systronics, New Delhi, India).

2.8 Statistical Analysis

Data were analyzed statistically using analysis of variance (ANOVA) by SPSS 17.0 for Windows, and presented as treatment mean ± SE (n=3). Least significant difference (LSD) was calculated for the significant data at $P < 0.05$. Bars showing the same letter are not significantly different by LSD test at $P < 0.05$.

3. RESULTS AND DISSCUSSION

Salt stress affects processes from seed germination to growth and flowering that ultimately cause decrease of plant productivity [8]. Saline soil possesses high concentration of Na^+ and Cl^- content and thus exerts adverse effects on physiological and metabolic processes, finally diminishing growth and yield.

Plants adopt several strategies to avoid salt stress effects at all levels of organisation, such as ion homeostasis, osmotic adjustment, and enhancement of antioxidant defense system and increase of the photosynthetic ability [27]. Both the concentrations of H_2O_2 promote defense system in plants under salt stress and improve growth and photosynthetic ability of plants. The evidences have shown dual role for H_2O_2 during biotic and abiotic stresses. As an element of oxidative stress, it deleteriously influences cell components on the excess accumulation; and simultaneously it induces protective mechanisms, especially at the early stage of plant stress response. H_2O_2 can act as a signalling molecule for stress adaptation and programme cell death and regulates plant development [28].

3.1 H_2O_2 Retarded Ions Accumulation under Salt Stress

Salt treatment resulted in higher leaf Na^+, Cl^- content and Na^+/K^+ ratio in comparison to control. Plants treated with 50 or 100 nM H_2O_2 exhibited leaf Na^+, Cl^- content and Na^+/K^+ ratio lesser than the control. Maximum reduction in the content of leaf Na^+ and Cl^- was recorded with 100 nM H_2O_2. Application of 100 nM H_2O_2 resulted in reduction of leaf Na^+, Cl^- content and Na^+/K^+ ratio by 23.4%, 31.2%, and 20.1% and 13.3%,19.5% and 9.4% with 50 nM H_2O_2 in comparison to control (Fig. 1). The reduction in photosynthesis, NR activity, N content and growth of salt treated mungbean plant has been associated with the disturbance in homeostasis of Na^+ and Cl^- ions [29], but the reduction in accumulation of ions with H_2O_2 may be related to the regulation of the expression and activity of Na^+ transporters and H^+ pumps that generate the driving force for transport [30]. It has been shown that salinity-induced ROS formation can lead to programmed cell death, and a high cytosolic K^+/Na^+ ratio is essential for triggering salinity-induced PCD [31]. Enhanced uptake of K^+ at the cost of reduced uptake Na^+ in the cells of salt stressed plants is considered vital for maintaining high cellular K^+/Na^+ ratio [32]. Exogenous application of H_2O_2 showed ameliorating effects in terms of lowering the levels of Na^+ and thereby maintaining lower Na^+/ K^+ ratio as compared to that in salt stressed plants. The most distinctive effect on ion homeostasis could be observed when 100 nM H_2O_2 was applied under saline condition.

3.2 Application of H_2O_2 Improves Proline content and Water Relations

Proline content increased with the H_2O_2 application under non saline and salt stress compared to control. Maximum increase of about 2-times in proline content was noted with the 100 nM H_2O_2 under salt stressed plants compared to control (Fig. 2). Proline can protect plants from stress through different mechanisms, including osmotic adjustment, detoxification of ROS, protection of membrane integrity, and stabilization of proteins/enzymes [9]. Exogenous H_2O_2 may increase the proline content in *Nitraria tangutorum*, which could be alleviated by H_2O_2 scavengers [33]. Exogenous H_2O_2 treatment has been shown to prevent the increase of oxidative stress and endogenous H_2O_2 concentration in plants and enhance tolerance of plants to salt stress by enhancing the production of enzymatic and non-enzymatic antioxidants which can quench the ROS and decrease lipid peroxidation [17,34]. In the present study, accumulation of proline content under salt stress was increased by the application of H_2O_2 and reversed the deleterious effects of salt stress. The reversal of adverse effects of salt stress on photosynthesis by H_2O_2 may be attributed to the accumulation of proline which maintained the cellular osmotic adjustments by increasing osmotic potential and water potential and protected photosynthetic machinery from salt stress by acting as an oxygen radical scavenger. It has been documented that proline helps in detoxification of toxic ions and protects cell from ROS damages [9]. Yang et al. [35] suggested that H_2O_2 might be involved in signal transduction events, leading to proline accumulation in maize seedlings, and that the H_2O_2-induced proline accumulation. In contrast, Lv et al. [36] have shown higher ion leakage and higher ROS levels with the increased activity of proline metabolizing enzymes and proline accumulation under heat treatment in Arabidopsis seedlings.

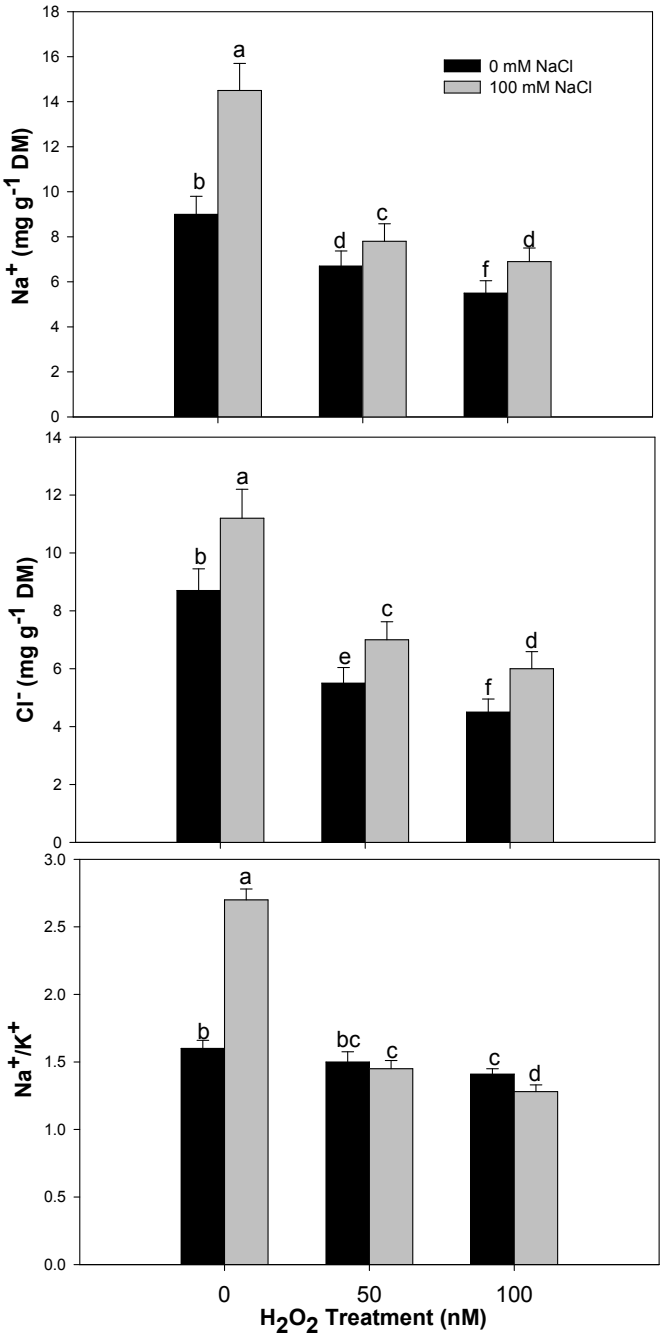

Fig. 1. Leaf Na^+, Cl^- content and Na^+/K^+ ratio in wheat (*Triticum aestivum* L.) cv. 711 plants grown with or without 100 mM NaCl and treated with 0, 50 or 100 nM H_2O_2. Data are presented as treatments mean ± SE (n=3). Data followed by the same latter are not significantly different by LSD test at p<0.05

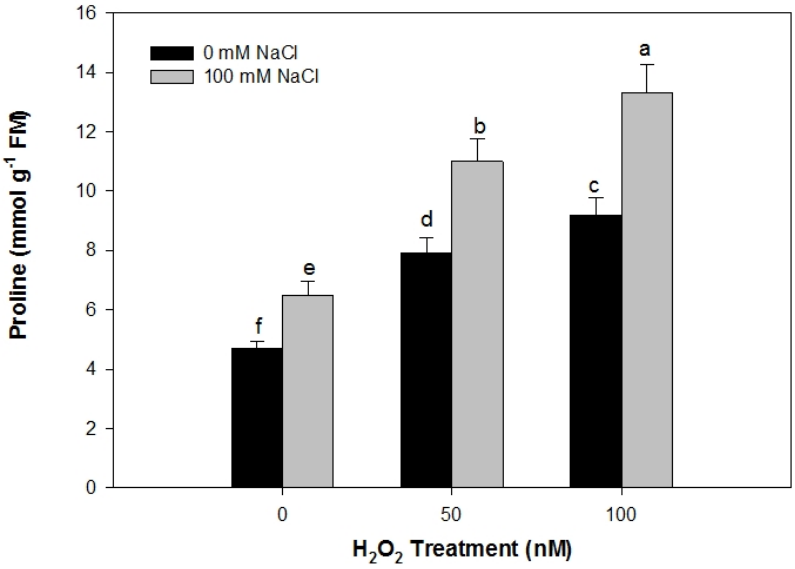

Fig. 2. Proline content in wheat (*Triticum aestivum* L.) cv. 711 plants grown with or without 100 mM NaCl and treated with 0, 50 or 100 nM H_2O_2. Data are presented as treatments mean ± SE (n=3). Data followed by the same latter are not significantly different by LSD test at p<0.05

Reduction of water potential by 40.1% and osmotic potential by 64.1% was observed with NaCl treatment compared to control plants. Application of 100 nM H_2O_2 improved water potential and osmotic potential by 33.3% and 14.1%, respectively of salt stressed plants compared to control. The treatment of 50 nM H_2O_2 to NaCl treated plants could increase water potential by 15.8% and osmotic potential by 8.4% compared to control (Fig. 3).

Salt stress decreases water potential and osmotic potential of crop plants [35,37-38]. The decrease in water potential in salt stressed plant results from decreased water use efficiency [39]. Effect of salinity on water content has been reported by many crops including alfalfa, cotton, pea, tomato, mentha, balm etc. [40]. Under salt stress, osmotic stress is triggered by an excess of salt in the soil, and ionic stress is caused by the over-accumulation of salt in the cells. These stresses individually affect the physiological status of plant [41]. Low concentration of H_2O_2 (50 nM) reduced the effects of salt stress, but 100 nM H_2O_2 was most beneficial in alleviating salt stress. It may be said that increased proline accumulation by 100 nM H_2O_2 in salt stressed plants increased osmotic potential, thereby increasing water potential of plants and resulted in the reversal of water deficiency. This increase in water potential and osmotic potential might helped stabilization of protein and increase in photosynthesis. H_2O_2 alleviates water uptake-reductive effect under salinity stress [42].

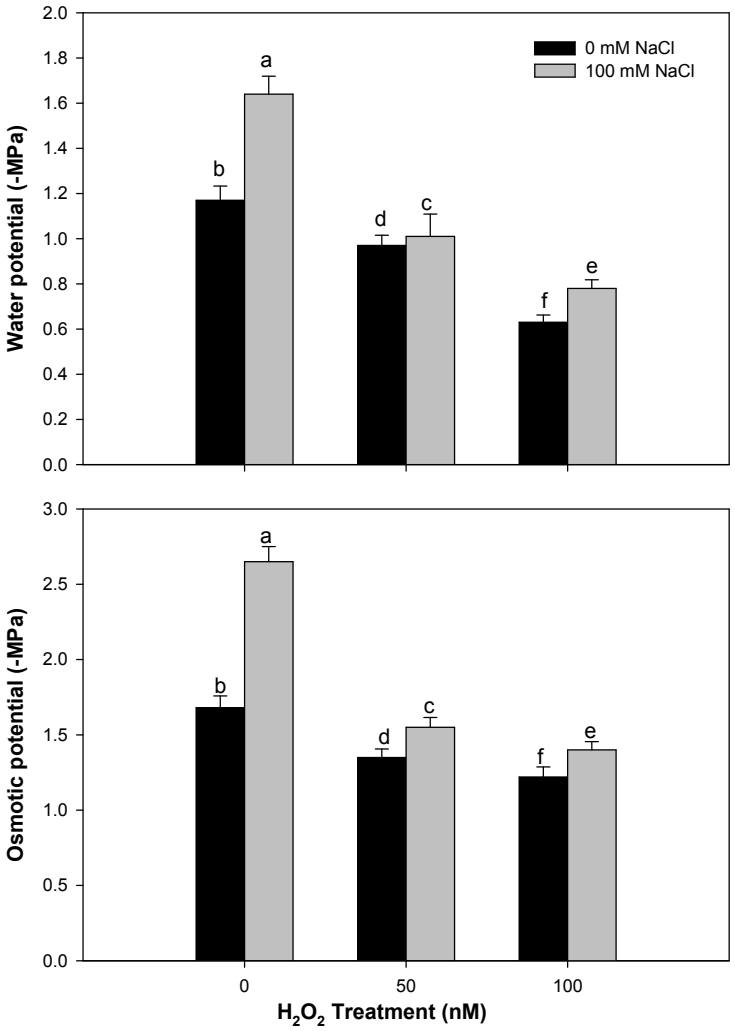

Fig. 3. Leaf water potential and leaf osmotic potential in wheat (*Triticum aestivum* L.) cv. 711 plants grown with or without 100 mM NaCl and treated with 0, 50 or 100 nM H_2O_2. Data are presented as treatments mean ± SE (n=3). Data followed by the same latter are not significantly different by LSD test at p<0.05

3.3 Effect of H_2O_2 on Nitrate Reductase Activity and Leaf Nitrogen Content under Salt Stress

In the present study, NR activity and N content were decreased under salt stress. However, H_2O_2 application significantly enhanced these characteristics under non saline and salt stress. Application of 100 nM H_2O_2 under salt stress maximally increased NR activity by 16.5% and N content by 11.3% compared to control (Fig. 4). Nitrate reductase is a primary enzyme in the nitrate assimilation pathway and a limiting factor in plant growth and development. Its activity is negatively influenced by salt stress [35], but H_2O_2 may alleviate

the adverse effects of salt stress on N assimilation. Fontaine et al. [43] proposed that H_2O_2 would activate the oxidative Pentose Phosphate Pathway resulting in promotion of germination. The alleviation of salt-induced effects on net photosynthesis by H_2O_2 may be attributed to the allocation of nitrogen to leaves. Higher allocation of N to leaf through the increase in the activity of NR with 100 nM applications under salt stress increases photosynthesis to a greater extent in wheat plants and this nitrogen may influence the structure and composition of photosynthetic apparatus.

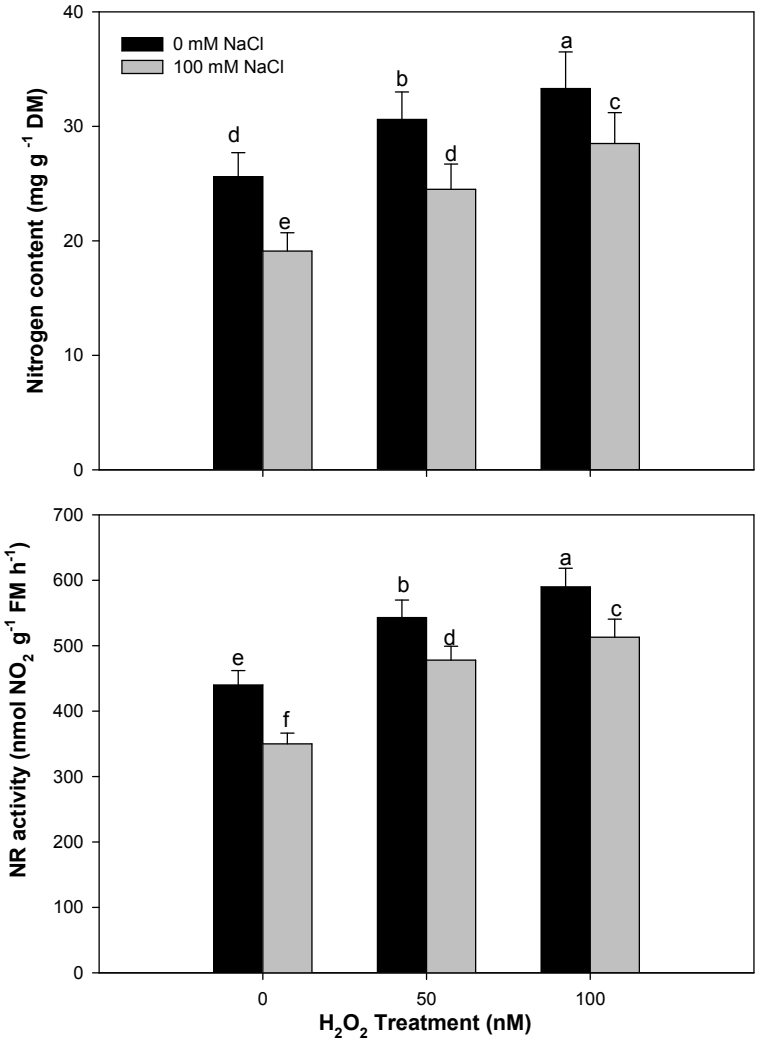

Fig. 4. Nitrogen content and nitrate reductase activity in wheat (*Triticum aestivum* L.) cv. 711 plants grown with or without 100 mM NaCl and treated with 0, 50 or 100 nM H_2O_2. Data are presented as treatments mean ± SE (n=3). Data followed by the same latter are not significantly different by LSD test at p<0.05

3.4 H_2O_2 Treatment Increases Net Photosynthesis and Photosynthetic Pigments

Under salt stress, net photosynthesis, content of chlorophyll and carotenoid decreased by 24.1%, 40.5% and 44.2%, respectively compared to control. Application of 50 and 100 nM H_2O_2 increased net photosynthesis by 36.6%, and 50.1%, total chlorophyll content by 20.5% and 28.7%, carotenoid content by 24.3% and 35.7%, respectively compared to control under non saline condition. Under salt stress, supplementation of 100 nM H_2O_2 proved better than 50 nM H_2O_2 in alleviation of salt stress. Treatment of 100 nM H_2O_2 increased net photosynthesis by 30.3%, chlorophyll content by 17.4% and carotenoid content by 21.4%, whereas 50 nM H_2O_2 could increase net photosynthesis by 11.7% chlorophyll content by 9.7% and carotenoid content by 11.4% compared to control plants under salt stress (Fig. 5). Despite the recognition of H_2O_2 as the central signalling molecule in stress responses little is known about how it affect photosynthetic machinery [44]. The alleviation of salt stress-inhibited photosynthesis by H_2O_2 application may be associated with the H_2O_2-mediated increase in proline content which acted as an antioxidant and protected photosynthetic machinery from salt-induced ROS. Moreover, higher water potential was responsible for maintaining enzyme integrity under salt stress and increased N allocation to the photosynthetic machinery helped increase in photosynthetic potential of plants. It has been shown that pre-treatment of seeds with H_2O_2 increase the net photosynthetic rate in wheat seedlings [45]. In the present study, chlorophyll content decreased under salt stress through the decrease in intermediates of chlorophyll biosynthesis [46], leading to a decreased absorption of light by the chloroplast and thus indirectly impairing photosynthesis. The decrease in chlorophyll content might have been due to salt induced increase in the activity of chlorophyll degrading enzyme chlorophyllase [47].

3.5 H_2O_2 Improves Growth under Salt Stress

Plant growth monitored as shoot length, root length, leaf area, fresh mass and dry mass reduced under salt stress, but the decrease in growth was ameliorated by H_2O_2 application. Root length, shoot length, fresh mass, dry mass and leaf area were increased with the application of both concentration of H_2O_2 under non saline and saline conditions compared to control, but maximum increase was noted with the application of 100 nM H_2O_2. Salt stress decreased plant root length, shoot length, leaf area, fresh mass and dry mass as compared to control. Exogenously sourced 100 nM H_2O_2 was better than 50 nM H_2O_2 in alleviation of salinity stress. Application of 100 nM H_2O_2 increased shoot length, root length, leaf area, fresh mass and dry mass by 30.1%, 42.2%, 37.1%, 27.3% and 36.3 % respectively, compared to control (Figs. 6-7). Li et al. [17] observed that exogenous H_2O_2 treatment decreased the deleterious effect of salt stress on growth of wheat. Present study suggested that application of 100 nM H_2O_2 was involved in greater salt tolerance than 50 nM H_2O_2, and increased growth characteristics and photosynthetic pigments. Amjad et al. [48] suggested the multiple effects of H_2O_2 on root system, leaf and coleoptiles growth of wheat seedlings.

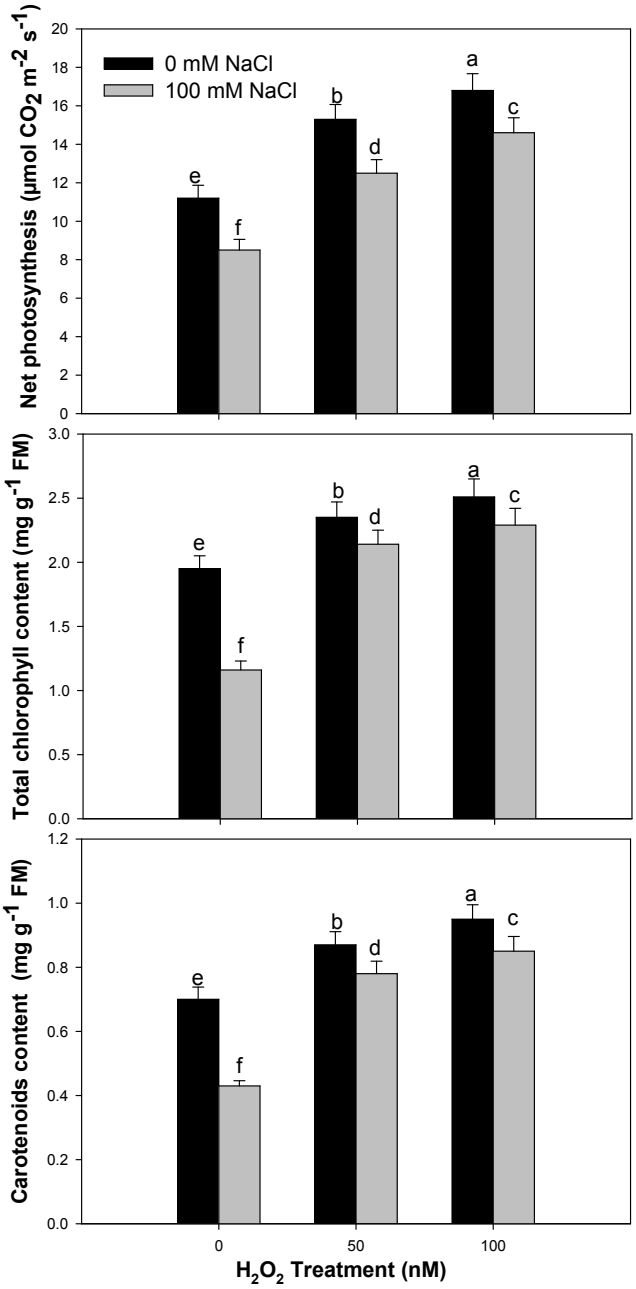

Fig. 5. Net photosynthesis, total chlorophyll content and carotenoids content in wheat (*Triticum aestivum* L.) cv. 711 plants grown with or without 100 mM NaCl and treated with 0, 50 or 100 nM H_2O_2. Data are presented as treatments mean ± SE (n=3). Data followed by the same latter are not significantly different by LSD test at p<0.05.

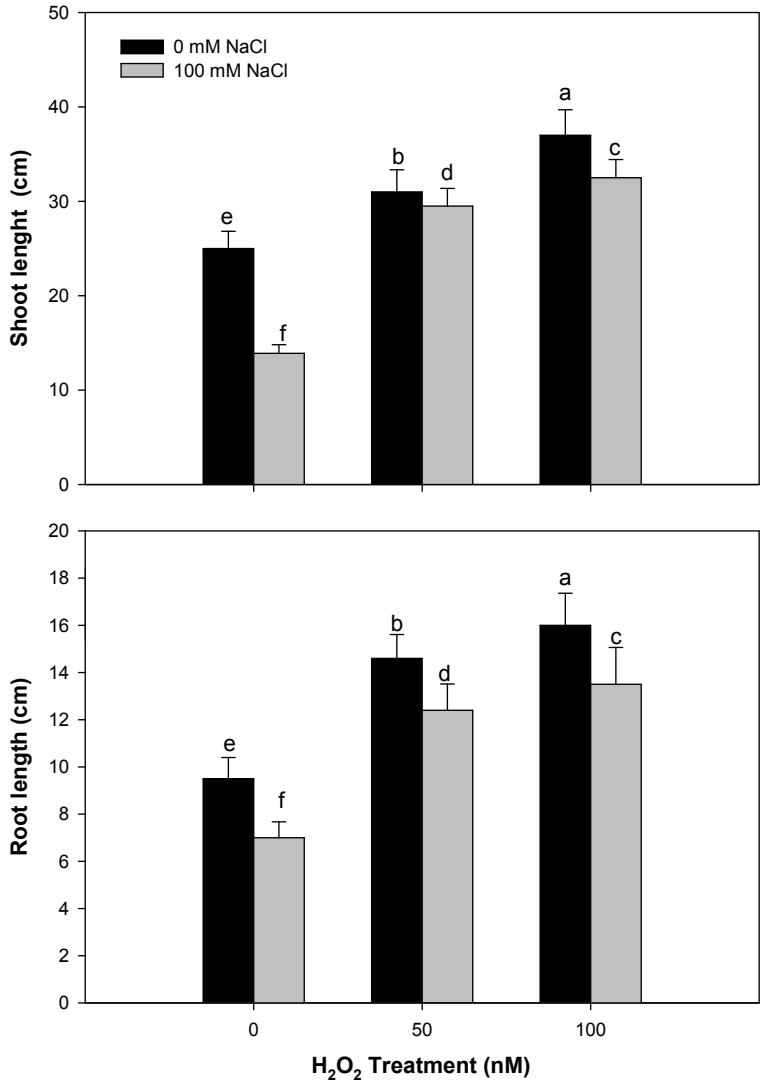

Fig. 6. Plant shoot length and plant root length in wheat (*Triticum aestivum* L.) cv. 711 plants grown with or without 100 mM NaCl and treated with 0, 50 or 100 nM H_2O_2. Data are presented as treatments mean ± SE (n=3). Data followed by the same latter are not significantly different by LSD test at p<0.05.

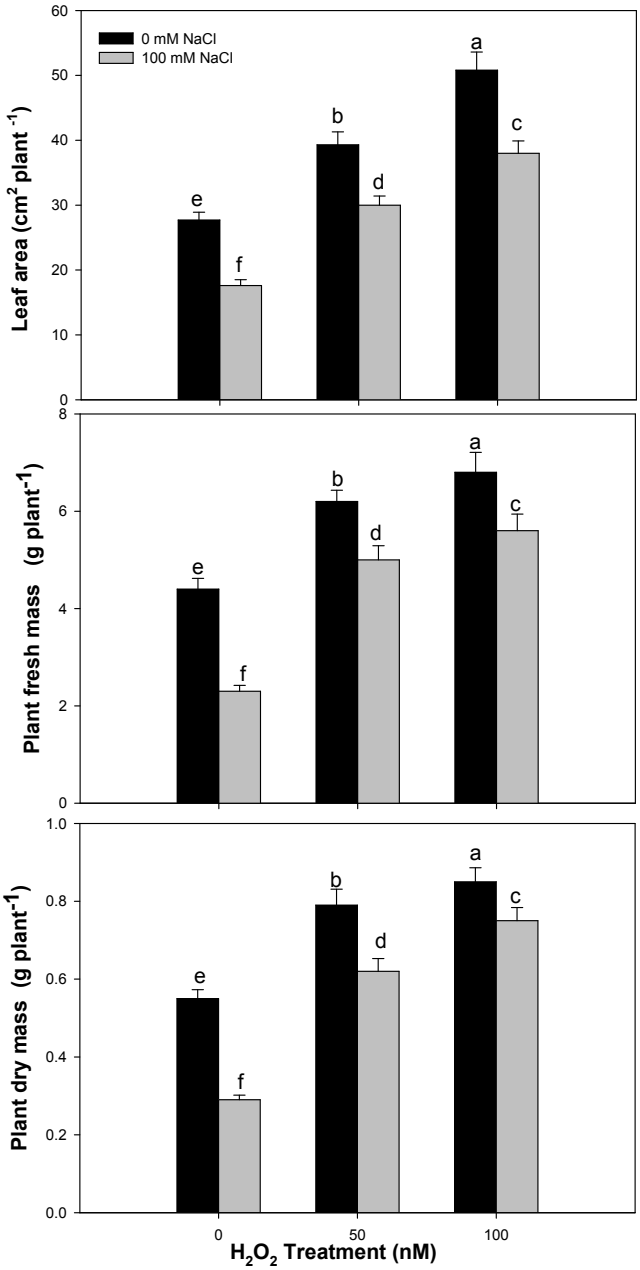

Fig. 7. Leaf area, plant fresh mass and plant dry mass in wheat (*Triticum aestivum* L.) cv. 711 plants grown with or without 100 mM NaCl and treated with 0, 50 or 100 nM H_2O_2. Data are presented as treatments mean ± SE (n=3). Data followed by the same latter are not significantly different by LSD test at $p<0.05$.

4. CONCLUSION

It may be said that H_2O_2 induces salt tolerance by avoiding the risk of ion toxicity by improving proline accumulation to maintain water relations of plants which helped in maintaining enzyme integrity and improving nitrate assimilation and photosynthetic ability of plants. It may be employed in inducing defense against salt stress and reducing stress-induced damage to crops.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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