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Responses of photosynthesis, stress markers and antioxidants under aluminium, salt and combined stresses in wheat cultivars

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Abstract: This study was designed to assess the combined effect of both aluminium and salt stress on the processes and molecules pertinent to these stresses and to establish relationship between these two stresses on the basis of photosynthetic attributes and antioxidant enzymes in wheat cultivars. Seven different varieties of wheat grown under environmentally controlled conditions. At 10 days stage of growth, plants were treated with NaCl (100 or 150 mM) and/or Al (05 or 10 mM) through soil. At 30 days stage, treatment of Al and salt stress alone showed the similar deteriorating response in terms of photosynthetic traits and LWP whereas, electrolyte leakage, lipid peroxidation, H_2O_2 content along with enzymes of antioxidant and proline content varied in different varieties of wheat. Moreover, the combination of Al and salt stress further increased the antioxidant enzymes and proline accumulation. However, salt stress restrict the uptake of Al by roots in significant manner under combine treatment of Al and salt stress. The pattern of tolerance was observed on the basis of enhanced antioxidant system and proline accumulation and cultivar LOK-1 and 502 showed maximum and minimum tolerance respectively against the combination of salt and aluminium stress.

Subjects: Bioscience; Environment & Agriculture; Environmental Studies & Management

Keywords: salt stress; aluminium stress; antioxidants; photosynthesis; proline

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Mohammad Yusuf is working as a Young Scientist (Principal Investigator) in the Department of Botany, Aligarh Muslim University, Aligarh, Uttar Pradesh, India. His area of research is to dissect the abiotic stress tolerance mechanism in plants through engineered metabolites and biochemical traits in the presence and absence of phytohormones. Currently he is engaged with the research project sanctioned by Science and Engineering Research Board, Government of India, New Delhi. He has also been awarded with the prestigious Dr D.S. Kothari Postdoctoral fellowship from University Grants Commission, Government of India, New Delhi for outstanding research in abiotic stress tolerance in plants. During the span of four and half years as researcher, he has published more 30 research articles in the Journal of International repute with the total impact factor of more than 45 and 600 citations along with h-index of 13.

PUBLIC INTEREST STATEMENT

In the face of global industrialization and the extensive increase of various anthropogenic activities, plants are frequently exposed to various stresses. These stresses prevent plants from reaching their full genetic potential and limit the crop productivity worldwide. Among these abiotic stresses, metal contamination and salt stress issues are becoming increasingly common in India and elsewhere, with many well documented cases of both salt stress and metal toxicity in agriculture and also the principal cause of crop failure worldwide. While abiotic stress is routinely studied in various crops by applying single stress condition such as salinity, drought, temperature, metal (Cd, Cu, Ni, Al) and these studies do not reflect the conditions that occur in the field where crop plants are subjected to a combinations of different stresses.

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1. Introduction

Unlike animals, plants are frequently exposed to various abiotic stresses either alone or in combinations. These stresses prevent plants from reaching their full genetic potential and limit the crop productivity worldwide (Mahajan & Tuteja, 2005). Among these abiotic stresses, metal contamination and salt stress issues are becoming increasingly common in India and elsewhere, with many well documented cases of both salt stress and metal toxicity in agriculture and also the principal cause of failure of crop productivity worldwide.

Aluminium (Al) toxicity is one of the most important soil constraints for plant growth and development in acid soils. A wide range of toxic effects of Al ions have been demonstrated in plants, although the actual mechanisms of Al toxicity have not been elucidated. When soil pH is less than 5.0, Al ionizes to form phytotoxic ions (Al^{3+}) that are readily absorbed by plant roots (Kinraide, 1990), inhibiting elongation of roots and reducing yields of crops (Matsumoto, 2000). Alterations of root architecture (Doncheva, Amenos, Poschenrieder, & Barcelo, 2005) and inhibition of root elongation (Matsumoto, 2000) are considered primary symptoms of Al-toxicity. Moreover, Al exposure results in alterations to the plasma membrane surface, disruption of cytoskeletal dynamics (Sivaguru, Pike, Grassmann, & Baskin, 2003), changes in Ca^{2+} homeostasis and signaling (Jones, Kochian, & Gilroy, 1998), peroxidative damage to membranes, induction of reactive oxygen species (ROS), and mitochondrial dysfunction leading to Al mediated inhibition of root growth (Yamamoto, Kobayashi, Devi, Rikiishi, & Matsumoto, 2002). On the other hand, the involvement of oxidative stress in Al toxicity has also been suggested, although Al itself is not a transition metal and cannot catalyze redox reactions. Instead, Al ions have a strong affinity for bio-membranes and can cause the rigidification of membranes (Deleers, Servais, & Wülfert, 1986), which seems to facilitate the radical chain reactions mediated by Fe ions enhancing the peroxidation of lipids (Yamamoto et al., 2002).

Most parts of the world facing a serious problem of salt stress that limit agricultural productivity as well as threat to sustainable agricultural practices (Rahdari & Hoseini, 2011). Binzel and Reuveni (1994) revealed that salt affected area is almost three times larger than the arable agricultural land. Moreover, salt stress in the soil largely generates osmotic stress and ion injury to plants (Yasar, Uzal, Tufenkci, & Yildiz, 2006). High salinity level causes ionic imbalance, disturbs the ion homeostasis of plant cells, and affects the distributions and supply of essential mineral nutrients, such as K^{+}, Ca^{2+} and Mn^{2+} and thus affecting the normal physiology of plant cells (Wang, Yan, Jiang, Qu, & Xu, 2012). The outcome of these effects may cause metabolic dysfunctions, like membrane damage, nutrient imbalance, altered levels of growth regulators, enzymatic inhibition and degradation of photosynthetic pigments, including photosynthesis which may ultimately lead to plant death (Chaves, Flexas, & Pinheiro, 2009).

Being sessile in nature, plants are continuously exposed to different abiotic stresses at a single time. It is well documented that traditionally various researchers studied abiotic stresses in plants by considering a single stress condition such as drought, salinity, metal, or heat, and analyzing the different aspects of plant acclimation. However, this type of analysis might not reflect the conditions that occurring in the field, in which plants are subjected to a combination of different abiotic stresses at a single time. Therefore, a considerable gap might exist between the knowledge gained by these studies and the data required to develop crops with enhanced tolerance under field conditions. Taking into the consideration of above reports, the present study was aimed to assess the impact of aluminium and salt stress individually or in combination on the processes and molecules pertinent to these stresses and also establish relationship between these two stresses on the basis of photosynthetic attributes and antioxidant enzymes and also to explore the pattern of stress tolerance in wheat cultivars.

2. Material and methods

The grains of wheat (*Triticum aestivum*) of seven different (DBW-17, LOK-1, 2851, 502, 343, 373, and 550) varieties were procured from National Seed Corporation Ltd., New Delhi, India. The healthy

looking and uniform size grains were surface sterilized with 1% sodium hypochlorite solution for 10 min, followed by repeated washing with double distilled water (DDW).

Aluminium chloride ($AlCl_3$) was used as the source of Al stress. The required concentrations (05 or 10 mM) were added to the nutrient solution for the treatment. The concentrations were selected on the basis of study of Ali et al. (2008).

Sodium chloride (NaCl) was used as the source of salt stress. The required concentrations (100 or 150 mM) were added to nutrient solution for the treatment. The concentrations were selected on the basis of study of Yusuf et al. (2008).

The uniform and healthy-looking grains of seven different (DBW-17, LOK-1, 2851, 502, 343, 373, and 550) varieties of wheat were sown in 189 sand filled plastic pots (6 inch in diameter) under environmentally controlled conditions; 25/20°C (day/night); 70/80% RH (day/night) and 14 h photoperiod and these pots were irrigated with deionized water and nutrient solution (Hewitt, 1966) on alternate days under randomized block design and allowed to grow under these conditions. All the pots were divided into 7 groups with 9 treatments per group and each treatment in the group was replicated three times. These groups represent 7 different varieties (DBW-17, LOK-1, 2851, 502, 343, 373, and 550) of wheat. Grains in sand filled plastic pots were allowed to grow till 10 days stage and 10 days-old seedlings were treated with different levels of Al (5.0, or 10.0 mM) and/or NaCl (100, or 150 mM) for 3 days under pH ≤ 5.5 of sand and allowed to grow till 30 days stage. The plants in all the sets were harvested at 30 days stage of growth to assess various photosynthetic traits, stress biomarkers and activities of antioxidant enzymes. These assays was repeated 3 times with the utilization of 09 plants per treatment (03 plants per pot).

Photosynthetic traits were determined on the fully expanded leaves between 11:00 and 12:00 h by using an infra-red gas analyzer portable photosynthetic system (Li-COR 6400, Li-COR, and Lincoln, NE, USA). To measure net photosynthetic rate (P_N) and its related attributes [stomatal conductance (gs)], the air temperature, relative humidity, CO_2 concentration and photosynthetic photon flux density (PPFD) were maintained at 25°C, 85%, 600 µ mol $mol^{-1}$and 800 µ mol mol^{-2} s^{-1}, respectively.

Maximum quantum yield of photosystem II (Fv/Fm) was measured by using a leaf chamber fluorometer (Li-COR 6400–40, Li-COR, Lincoln, NE, USA). All the measurements carried out at a PPFD of 1,500 µ mol m^{-2} s^{-1} with a constant airflow rate of 500 µ mol s^{-1}. The minimal fluorescence level (Fo) was determined by modulated light, which was sufficiently low (<1 µ mol m^{-2} s^{-1}) not to induce any significant variable fluorescence. The maximal fluorescence (Fm) was determined by a 0.8 s saturation pulse at 4,200 µ mol m^{-2} s^{-1} on dark-adapted leaves (30 min). The sampled leaf was dark-adapted for 30 min prior to measurement of Fv/Fm.

The leaf water potential (LWP) was monitored with the Psyprowater potential system (Wescor, Inc. 370 West 1700 South Logan, Utah 84321, USA) in the third fully expanded leaves of the plant. LWP was measured with a C-52 sample chamber in fully expanded leaves. Leaf discs of about 6 mm in diameter were cut and sealed in the C-52 sample chamber. Samples were equilibrated for 50 s before recording the LWP.

Lipid peroxidation (LPO) rates were estimated by measuring the malondialdehyde equivalents according to Hodges, DeLong, Forney, and Prange (1999). 0.5 g of the leaf was homogenized in a mortar with 80% ethanol. The homogenate was centrifuged at 3,000 × g for 10 min at 4°C. The pellet was extracted twice with the same solvent. The supernatants were pooled and 1 ml of this sample was added to a test tube with an equal volume of the solution comprised of 20% trichloroacetic acid, 0.01% butylated hydroxy toluene and 0.65% thiobarbutyric acid. Samples were heated at 95°C for 25 min and cooled to room temperature. Absorbance of the samples was recorded at 440, 532 and 600 nm. LPO rates equivalent (n mol malondialdehyde ml^{-1}) were calculated by using the formula given by Hodges et al. (1999).

The hydrogen peroxide accumulation was determined by the method proposed by Jana and Choudhari (1981). 500 mg plant sample was homogenized in 3.0 mL of phosphate buffer (50 mM and pH 6.8). The homogenate was centrifuged at 6,000 × g for 25 min. 3.0 mL of extract was mixed with 0.1% titanium chloride in 20% (v/v) sulphuric acid and the mixture was again centrifuged at 6,000 × g for 15 min. The absorbance of the color was read at 410 nm, on a spectrophotometer and was compared with that of the calibration curve. The H_2O_2 content was computed on fresh mass basis using a standard curve of known concentration of H_2O_2.

The total inorganic ions leaked out of the leaves were measured by the method described by Sullivan and Ross (1979). Twenty leaf discs were taken in a boiling test tube, containing 10 ml of deionized water and electron conductivity (EC) was measured (ECa). The contents were heated at 45°C and 55°C for 30 min each in a water bath and EC was measured (ECb). Later the controls were boiled at 100°C for 10 min and EC was again recorded (ECc). The electrolyte leakage was calculated using the formula:

$$\text{Electrolyte leakage}(\%) = [(EC_b - EC_a)/(EC_c) \times 100]$$

For the assay of antioxidant enzymes, the leaf tissue (0.5 g) was homogenized in 50 mM phosphate buffer (pH 7.0) containing 1% polyvinylpyrrolidone. The homogenate was centrifuged at 27,600 × g for 10 min at 4°C and the supernatant was used as source of enzymes catalase, peroxidase and superoxide dismutase and glutathione reductase.

Peroxidase and catalase were assayed following the procedure described by Chance and Maehly (1956). Catalase was estimated by titrating the reaction mixture, consisting of phosphate buffer (pH 6.8), 0.1 M H_2O_2, enzyme extract and 2% H_2SO_4, against 0.1 N potassium permanganate solution. The reaction mixture for peroxidase consisted of pyragallol, phosphate buffer (pH 6.8), 1% H_2O_2 and enzyme extract. Change in absorbance due to catalytic conversion of pyragallol to purpurogallin, was noted at an interval of 20 s for 2 min, at 420 nm on a spectrophotometer. A control set was prepared by using DDW instead of enzyme extract. The activity of superoxide dismutase was assayed by measuring its ability to inhibit the photochemical reduction of nitroblue tetrazolium following the method of Beauchamp and Fridovich (1971). The reaction mixture contained 50 mM phosphate buffer (pH 7.8), 13 mM methionine, 75 mM nitroblue tetrazolium, 2 mM riboflavin, 0.1 mM EDTA and 0.1 mL enzyme extract and was placed under 15 W fluorescent lamp. The reaction was started by switching on the light and was allowed to run for 10 min. The reaction was stopped by switching off the light. 50% inhibition by light was considered as one enzyme unit.

Glutathione reductase was assayed as per the method of Smith, Vierheller, and Thorne (1988). The reaction mixture contained, 66.67 mM potassium phosphate buffer (pH 7.5), 0.33 mM EDTA, 0.5 mM 5,5-dithiobis (2-nitrobenzoic acid) in 0.01 M potassium phosphate buffer (pH 7.5), 66.67 mM NADPH, and 66.67 mM oxidized glutathione and 0.1 mL enzyme extract. The reaction was started by adding oxidized glutathione and the increase in absorbance at 412 nm was recorded spectrophotometrically.

The proline content in fresh leaf samples was determined by adopting the method of Bates, Aldren, and Teare (1973). Sample was extracted in sulphosalicylic acid. To the extract an equal volume of glacial acetic acid and ninhydrin solutions were added. The sample was heated at 100°C to which 5 mL of toluene was added. The absorbance of toluene layer was read at 528 nm on a spectrophotometer.

Soluble sugar content was determined by Yemm and Willis (1954). About 0.05 g ground dry shoots and roots were soaked in 5 mL deionized water. The solution was boiled (100°C) for 30 min to extract soluble sugar and centrifuged at 4,000 × g for 10 min. The extracts were decanted and the residue was re-extracted for two more times, with extracts being completed to 50 mL. In all, 0.1 mL extracts and 3 mL anthrone reagent were mixed and the absorbance of the mixture was recorded at 620 nm.

The content of soluble sugar was calculated from a standard curve of glucose at 620 nm by spectrophotometer.

Al content was determined according to the method described by Zhan, Kou, and He (2008). Fresh treated root tips (10 mm) were rinsed in distilled water, and were weighed and ground to a fine power and extracted for 24 h in 2 M HNO_3. The 0.5 mL of the resulting extraction solution was diluted with 9.5 mL of distilled water. Then 1 mL of diluted solution was added in a 25 mL measuring flasks containing 11 mL reaction mixture composed of 0.1 M HNO_3, 0.05 M cetyltrimethylammonium bromide (CTMAB), 0.05 M EDTA-Zn, 0.05% Chrome Azurol S and mL 40% hexamine, and then incubated for 20 min at 25°C in water bath. The absorbance at 635 nm was monitored against blank. Al content was determined using a standard curve of known concentrations. Al content data was calculated using the following formula:

$$\text{Al content } (\mu g.g^{-1}.\text{ FW}) = \text{Al content/weight}$$

Data were statistically analyzed using SPSS, 17.0 for windows (SPSS, Chicago, IL, USA. Analysis of variance (ANOVA) was performed on the data to determine the least significance difference between treatment means with the level of significance at $P \leq 0.05$.

3. Results

3.1. Net photosynthetic rate and its related attributes

The result obtained showed a significant decrease in net photosynthetic rate (P_N), stomatal conductance (G_S), and maximum quantum yield of PSII (Fv/Fm) of all the varieties tested at 30 days stage of growth. However, out of different levels of stresses (100 or 150 mM of NaCl and/or 5 or 10 mM of Al), NaCl (100 mM) and Al (5 mM) individually showed similar response in comparison to control plants. Moreover, the combination of NaCl (150 mM) and Al (10 mM) proved deleterious and maximum damage to P_N in DBW-17, LOK-1, 2851, 502, 343, 373, and 550 were 36.1, 31.0, 34.1, 47.0, 39.0, 40.0, and 33.0% whereas for G_S the values were 34.1, 31.0, 36.2, 45.0, 38.2, 41.5, and 38.0% less than their respective controls at 30 days stage of growth. The order of photosynthetic damage at 30 days stage of growth for combination of NaCl (150 mM) and Al (10 mM) in the varieties was in the order of 502 > 373 > 343 > 550 > 2851 ≥ 2851 DBW-17 > LOK-1. Similar response was shown by maximum quantum yield of PS II (Fv/Fm) for all the varieties at 30 day stages of growth (Table 1).

3.2. LWP

Out of different level of stresses, the combination of NaCl and Al induced maximum decrease for LWP in all the varieties tested at 30 days stages of growth. Highest level of NaCl (150 mM) and Al (10 mM) in combination proved most deleterious and lowered the LWP in var. DBW-17 (56.9%), LOK-1 (51.2%), 2851 (67.0%), 502 (73.7%), 343 (65.9%), 373 (69.1%) and 550 (61.3%) in comparison to their respective controls at 30 days stage of growth. Moreover, var. LOK-1 and 502 showed maximum and minimum tolerance respectively, against all the level of stresses either in combination or individually at 30 days stage of growth (Table 2).

3.3. LPO and H_2O_2 content

The data generated showed that both the LPO and H_2O_2 accumulation in leaves increased significantly with the increasing level of stresses but NaCl (100 mM) triggered a similar change with Al (5 mM) individually at 30 days stage of growth in all the varieties. The plants exposed to NaCl (150 mM) + Al (5 mM) had increased accumulation of LPO and H_2O_2 in comparison to the non-treated control plants at 30 DAS. However, the LPO content was more than the H_2O_2 accumulation in the presence of Al alone and also in combination with NaCl stress and the maximum increase of LPO was in the order of 502 > 373 > 343 > 550 > 2851 ≥ DBW-17 > LOK-1 at 30 days stage of growth (Table 3).

Table 1. Effect of NaCl (100 or 150 mM) and/or Al (5 or 10 mM) stress on the net photosynthetic rate and stomatal conductance in different varieties of wheat (*T. aestivum*) at 30 days after sowing (DAS)

	Net photosynthetic rate (μ mol CO_2 m^{-2} s^{-1})										Stomatal conductance (mol m^{-2} s^{-1})									
	0	NaCl 100	NaCl 150	Al 05	Al 10	NaCl 100 + Al 5	NaCl 100 + Al 10	NaCl 150 + Al 5	NaCl 150 + Al 10	Mean	0	NaCl 100	NaCl 150	Al 05	Al 10	NaCl 100 + Al 5	NaCl 100 + Al 10	NaCl 150 + Al 5	NaCl 150 + Al 10	Mean
DBW-17	20.41	16.69	15.30	16.53	15.91	15.26	14.28	13.67	13.06	15.67	0.079	0.067	0.061	0.066	0.063	0.061	0.056	0.055	0.052	0.062
LOK-1	22.49	19.00	17.76	19.02	18.44	17.81	16.64	15.96	15.51	18.07	0.087	0.075	0.070	0.076	0.073	0.070	0.066	0.063	0.060	0.071
2851	19.89	16.40	15.55	16.58	15.94	15.41	14.32	13.70	13.10	15.65	0.080	0.066	0.062	0.066	0.063	0.060	0.057	0.055	0.051	0.062
502	14.28	10.92	9.13	10.85	9.99	9.03	8.71	8.28	7.56	19.86	0.060	0.047	0.040	0.047	0.042	0.038	0.038	0.036	0.033	0.042
343	17.58	14.06	12.30	13.97	12.65	12.34	11.95	11.42	10.72	12.99	0.068	0.056	0.049	0.056	0.059	0.049	0.046	0.044	0.042	0.052
373	16.86	13.23	11.26	13.15	11.63	11.12	10.95	10.67	10.11	12.10	0.065	0.052	0.045	0.052	0.046	0.045	0.043	0.040	0.038	0.047
550	18.55	15.02	13.46	14.84	13.91	13.35	11.05	12.79	12.42	13.93	0.071	0.059	0.053	0.059	0.055	0.052	0.049	0.047	0.044	0.054
Mean	18.58	15.04	14.99	14.06	13.47	12.55	12.35	11.78	14.04		0.072	0.060	0.054	0.060	0.057	0.053	0.050	0.048	0.045	
	LSD at 5%	Varieties (V) = 0.70 (Sig)									Varieties (V) = 0.003 (Sig)									
		Treatments (T) = 0.98 (Sig)									Treatments (T) = 0.002 (Sig)									
		V × T = 1.69 (Sig)									V × T = NS									

Notes: V = Varieties; T = Treatments; Sig = Significant; NS = Non-significant

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Table 2. Effect of NaCl (100 or 150 mM) and/or Al (5 or 10 mM) stress on the maximum quantum yield of Photosystem II and leaf water potential indifferent varieties of wheat (*T. aestivum*) at 30 days after sowing (DAS)

	Maximum quantum yield of PS II (Fv/Fm)										Leaf water potential (−Mpa)									
	0	NaCl 100	NaCl 150	Al 05	Al 10	NaCl 100+Al 5	NaCl 100 + Al 10	NaCl 150 + Al 5	NaCl 150 + Al 10	Mean	0	NaCl 100	NaCl 150	Al 05	Al 10	NaCl 100 + Al 5	NaCl 100 + Al 10	NaCl 150 + Al 5	NaCl 150 + Al 10	Mean
DBW-17	0.67	0.54	0.48	0.55	0.51	0.48	0.44	0.42	0.40	0.49	−0.86	−1.04	−1.16	−1.01	−1.06	−1.14	−1.25	−1.30	−1.35	−1.13
LOK-1	0.73	0.60	0.55	0.62	0.58	0.54	0.51	0.48	0.46	0.56	−0.82	−0.96	−1.06	−0.91	−1.01	−1.07	−1.15	−1.20	−1.24	−1.04
2851	0.68	0.55	0.47	0.56	0.51	0.49	0.45	0.42	0.43	0.50	−0.85	−1.02	−1.02	−1.00	−1.10	−1.18	−1.30	−1.36	−1.42	−1.13
502	0.53	0.37	0.32	0.37	0.35	0.32	0.30	0.28	0.27	0.34	−0.99	−1.38	−1.38	−1.32	−1.48	−1.05	−1.62	−1.68	−1.74	−1.40
343	0.60	0.45	0.40	0.45	0.42	0.39	0.37	0.36	0.34	0.42	−0.91	−1.19	−1.19	−1.14	−1.27	−1.27	−1.41	−1.45	−1.51	−1.26
373	0.57	0.42	0.37	0.41	0.39	0.37	0.34	0.33	0.30	0.38	−0.94	−1.27	−1.27	−1.22	−1.27	−1.36	−1.50	−1.56	−1.59	−1.33
550	0.64	0.50	0.44	0.51	0.48	0.43	0.41	0.39	0.37	0.46	−0.88	−1.11	−1.11	−1.01	−1.18	−1.26	−1.32	−1.37	−1.42	−1.18
Mean	0.63	0.49	0.43	0.49	0.46	0.043	0.40	0.38	0.36		−0.89	−1.13	−1.17	−1.08	−1.19	−1.19	−1.36	−1.41	−1.46	
	LSD at 5%	Varieties (V) = 0.02 (Sig)									Varieties (V) = 0.06 (Sig)									
		Treatments (T) = 0.03 (Sig)									Treatments (T) = 0.05 (Sig)									
		V × T = NS									V × T = 0.12 (Sig)									

Notes: V = Varieties; T = Treatments; Sig = Significant; NS = Non-significant

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Table 3. Effect of NaCl (100 or 150 mM) and/or Al (5 or 10 mM) stress on the lipid peroxidation and H2O2 content in different varieties of wheat (*T. aestivum*) at 30 days after sowing (DAS)

	Lipid peroxidation (μ mol g^{-1} fresh mass)										Hydrogen peroxide content (μ mol g^{-1} fresh mass)									
	0	NaCl 100	NaCl 150	Al 05	Al 10	NaCl 100 + Al 5	NaCl 100 + Al 10	NaCl 150 + Al 5	NaCl 150 + Al 10	Mean	0	NaCl 100	NaCl 150	Al 05	Al 10	NaCl 100 + Al 5	NaCl 100 + Al 10	NaCl 150 + Al 5	NaCl 150 + Al 10	Mean
DBW-17	2.40	2.64	2.71	2.60	2.73	3.68	3.26	3.36	3.94	3.03	5.94	6.89	7.48	6.83	7.60	7.90	8.13	8.49	8.79	7.56
LOK-1	2.25	2.54	2.65	2.55	2.70	2.85	2.94	3.06	3.15	2.74	5.40	6.04	6.48	5.94	6.53	6.80	7.12	7.50	7.77	6.62
2851	2.38	2.62	2.74	2.64	2.78	3.11	3.21	3.35	3.90	2.97	5.91	6.90	7.45	6.84	7.50	7.94	8.18	8.52	8.82	7.56
502	2.83	3.76	3.93	3.72	3.96	4.21	4.35	4.49	4.66	3.99	6.80	9.11	9.86	9.04	9.79	9.99	10.40	10.74	11.15	9.65
343	2.58	3.22	3.40	3.20	3.43	3.58	3.71	3.87	4.02	3.44	6.37	8.02	8.72	9.76	8.79	9.04	9.36	9.74	10.06	8.87
373	2.70	3.51	3.69	3.47	3.72	3.88	4.05	4.21	4.32	3.72	6.48	8.42	9.20	8.35	9.33	9.59	9.84	10.17	10.49	9.09
550	2.49	2.93	3.21	2.95	3.23	3.38	3.48	3.63	3.75	3.22	6.15	7.38	7.99	7.44	8.11	8.42	8.67	9.10	9.47	8.08
Mean	2.51	3.03	3.19	3.01	3.22	3.52	3.57	3.71	3.96		6.15	7.53	8.16	7.74	8.23	8.52	8.81	9.18	9.50	
	LSD at 5%	Varieties (V) = 0.12 (Sig)									Varieties (V) = 0.15 (Sig)									
		Treatments (T) = 0.12 (Sig)									Treatments (T) = 0.37 (Sig)									
		V × T = 0.31 (Sig)									V × T = 0.52 (Sig)									

Notes: V = Varieties; T = Treatments; Sig = Significant; NS = Non-significant

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3.4. Leaf electrolyte leakage

Availability of different level of stresses NaCl (100 or 150 mM) and/or Al (5 or 10 mM) in the sand caused a significant increase in the electrolyte leakage from the leaves of all varieties, compared with their respective controls. However, the leakage of electrolyte from the leaves was more prominent in the varieties 502, 373, 343, and 550 under combination of NaCl (150 mM) and Al (10 mM). The variety LOK-1 grown in sand devoid of NaCl and/or Al i.e. control plants showed minimum values for electrolyte leakage in comparison to NaCl and/or Al treated sand at 30 days stage of growth. However, electrolyte leakage increased in proportion to levels of NaCl and/or Al whereas, NaCl (100 mM) or Al (5 mM) individually triggered similar response and maximum values were generated by NaCl (150 mM) in combination with Al (10 mM) in all varieties. Variety LOK-1 showed maximum tolerance against NaCl and/or Al stress (Table 4).

3.5. Activities of CAT, POX, SOD and GR

Activities of catalase (CAT), peroxidase (POX), superoxide dismutase (SOD) and glutathione reductase (GR) in all the varieties increased in proportion to the level of stresses (NaCl and/or Al) at 30 days stage of growth. Out of different varieties tested, the highest activities of CAT (45.0%), POX (84.9%), SOD (67.5%) and GR (73.9%) in comparison to their respective controls was noted in variety LOK-1 with NaCl 150 mM in combination of 10 mM of Al at 30 days stage of growth. However, the variety 502 showed least activities of these enzymes at early stage of growth in comparison to later stage of growth (Tables 5 and 6).

3.6. Leaf proline and soluble sugar content

Leaves of plants grown in the presence of NaCl (100 or 150) and/or Al (5 or 10 mM) treatment showed a significant accumulation of proline and soluble sugar in all the varieties at both the stages of the growth. However, the accumulation of proline was higher at 30 days stage of growth in comparison to sugar. Out of different level of stresses, NaCl (100 mM) or Al (5 mM) individually showed similar response. Furthermore, NaCl (150 mM) alone and NaCl (100 mM) in combination with Al (5 mM) also showed no-significant response. Maximum proline and sugar content in all the varieties were noted under combination of NaCl (150 mM) and Al (10 mM) stress and the pattern of increasing accumulation was in order of LOK-1 > DBW-17 ≥ 2851 > 550 > 343 > 373 > 502 (Table 7).

3.7. Root Al content

All the varieties respond differentially in the presence of NaCl and/or Al stress at 30 days stage of the growth. Availability of NaCl (100 or 150 mM) in sand showed no-significant uptake of Al by roots in comparison to control plants. However, Al (10 mM) alone showed maximum uptake of Al by roots in variety 502 (65.9% in comparison to control plant) at 30 days stage of growth whereas, the combination of NaCl and Al stress showed lower uptake of Al by roots. The accumulation of Al in the root at higher concentration of Al (10 mM) was in the order 502 > 373 > 343 > 550 > 2851 ≥ DVW-17 > LOK-1 whereas, LOK-1 showed least content of Al in roots at all the concentrations of Al (Table 4).

4. Discussion

In our previous study we reported that salt (Yusuf et al., 2008) and aluminium (Ali et al., 2008) stress alone induced inhibition of net photosynthetic rate and its related attributes. Moreover, in the present study, we also found that individually salt (100 and 150 mM) and aluminum (5 or 10 mM) stress showed similar deteriorating responses whereas, the maximum damage for net photosynthetic rate and related attributes were caused by the combination of salt (150 mM) and Al (10 mM) stress and these two combination proved lethal and worked in aggressive manner showed in Table 1. It is believed that excess salt in soil prevents uptake of water from soil lead to the conditions of physiological drought conditions and makes the soil more acidic. This believe authenticated by the present study in which salt stress alone and in combination with Al lowered the LWP in Table 2. In order to conserve water, plants close their stomata which simultaneously restricts the entry of CO_2 into the leaf, reducing photosynthesis efficiency. Moreover, the reduction in the photosynthetic capacity under salinity might be largely due to the stomatal closure, brought about by salt-induced ABA

Table 4. Effect of NaCl (100 or 150 mM) and/or Al (5 or 10 mM) stress on the electrolyte leakage and root Al content in different varieties of wheat (*T. aestivum*) at 30 days after sowing (DAS)

	Electrolyte leakage (%)										Root Al content (μg g^{-1} fresh mass)									
	0	NaCl 100	NaCl 150	Al 05	Al 10	NaCl 100 + Al 5	NaCl 100 + Al 10	NaCl 150 + Al 5	NaCl 150 + Al 10	Mean	0	NaCl 100	NaCl 150	Al 05	Al 10	NaCl 100 + Al 5	NaCl 100 + Al 10	NaCl 150 + Al 5	NaCl 150 + Al 10	Mean
DBW-17	6.49	7.65	8.11	7.72	8.17	8.56	8.89	9.21	9.73	8.28	28.45	28.21	28.72	34.14	42.10	33.00	36.41	38.12	39.83	34.33
LOK-1	6.01	6.91	7.27	6.85	7.33	7.63	7.99	8.35	8.77	7.45	27.10	27.21	27.01	31.97	38.48	30.35	32.52	34.68	36.31	31.73
2851	6.50	7.63	8.14	7.76	8.20	8.59	8.91	9.18	9.75	8.29	28.70	28.07	28.81	34.24	42.30	33.13	36.01	38.80	40.00	34.45
502	7.55	10.19	10.57	10.26	9.81	11.55	11.85	12.30	12.83	10.76	34.14	34.01	34.34	49.50	56.67	47.79	51.21	53.59	54.96	46.24
343	6.99	8.37	9.22	8.94	9.29	10.06	10.34	10.55	11.18	9.43	33.01	33.45	32.94	43.90	51.82	42.25	45.88	47.86	49.84	42.32
373	7.21	9.51	9.87	9.58	9.94	10.81	11.10	11.31	11.26	10.06	31.90	31.81	32.02	44.02	51.35	42.40	45.93	48.16	50.08	41.96
550	6.79	8.35	8.97	8.41	8.82	9.37	9.64	9.91	10.52	8.97	31.57	31.89	31.30	40.09	47.67	38.19	41.98	43.88	46.09	39.18
Mean	6.79	8.37	8.87	8.50	8.79	9.51	9.81	10.11	10.57		30.69	30.66	30.73	39.69	47.19	38.15	41.42	43.58	45.30	
	LSD at 5%	Varieties (V) = 0.23 (Sig)									Varieties (V) = 0.20 (Sig)									
		Treatments (T) = 0.36 (Sig)									Treatments (T) = 0.50 (Sig)									
		V × T = NS									V × T = NS									

Note: V = Varieties; T = Treatments; Sig = Significant; NS = Non-significant

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Table 5. Effect of NaCl (100 or 150 mM) and/or Al (5 or 10 mM) stress on the activities of catalase and peroxidase in different varieties of wheat (*T. aestivum*) at 30 days after sowing (DAS)

	Catalase activity (m mol L^{-1} H2O2 decomposed g^{-1} fresh mass)										Peroxidase activity (unit g^{-1} fresh mass)									
	0	NaCl 100	NaCl 150	Al 05	Al 10	NaCl 100 + Al 5	NaCl 100 + Al 10	NaCl 150 + Al 5	NaCl 150 + Al 10	Mean	0	NaCl 100	NaCl 150	Al 05	Al 10	NaCl 100 + Al 5	NaCl 100 + Al 10	NaCl 150 + Al 5	NaCl 150 + Al 10	Mean
DBW-17	368	415	425	411	441	433	467	478	496	437	18.12	26.09	27.90	25.91	28.81	28.08	30.26	30.80	32.25	27.58
LOK-1	401	461	481	470	497	472	521	533	553	487	19.70	29.55	31.32	29.74	32.30	31.71	33.68	34.47	35.85	30.92
2851	370	420	432	415	450	440	470	483	501	442	18.18	26.16	28.17	26.10	28.72	27.99	29.99	30.72	32.17	27.57
502	324	340	356	350	364	350	369	375	382	356	15.76	20.17	21.27	19.70	22.22	21.00	22.85	23.95	25.05	21.33
343	348	382	417	391	403	422	410	416	423	401	17.01	23.81	24.87	23.98	25.68	25.00	26.19	27.21	28.40	24.68
373	336	359	409	375	379	401	389	395	401	382	16.44	22.02	23.18	21.86	24.00	23.01	24.82	25.81	26.63	23.08
550	356	395	416	404	420	421	427	434	441	412	17.53	25.24	26.29	25.41	27.17	26.47	28.04	28.74	30.15	26.11
Mean	357	396	419	402	422	419	436	444	456		17.53	24.72	26.14	24.67	26.98	26.18	27.95	28.81	30.07	
	LSD at 5%	Varieties (V) = 20.71 (Sig)									Varieties (V) = 1.01 (Sig)									
		Treatments (T) = 22.90 (Sig)									Treatments (T) = 1.25 (Sig)									
		V × T = 43.61 (Sig)									V × T = NS									

Notes: V = Varieties; T = Treatments; Sig = Significant; NS = Non-significant

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Table 6. Effect of NaCl (100 or 150 mM) and/or Al (5 or 10 mM) stress on the activities of superoxide dismutase and glutathione reductase in different varieties of wheat (*T. aestivum*) at 30 days after sowing (DAS)

	Superoxide dismutase activity (unit g^{-1} fresh mass)										Glutathione reductase activity (Unit g^{-1} fresh mass)									
	0	NaCl 100	NaCl 150	Al 05	Al 10	NaCl 100 + Al 5	NaCl 100 + Al 10	NaCl 150 + Al 5	NaCl 150 + Al 10	Mean	0	NaCl 100	NaCl 150	Al 05	Al 10	NaCl 100 + Al 5	NaCl 100 + Al 10	NaCl 150 + Al 5	NaCl 150 + Al 10	Mean
DBW-17	162	199	220	201	228	215	236	243	251	217	12.91	14.84	15.09	14.81	15.75	15.12	16.52	17.68	18.59	15.70
LOK-1	178	231	249	224	258	252	267	274	284	246	14.04	16.56	17.00	16.50	17.83	16.97	18.53	21.06	23.58	18.00
2851	160	201	222	195	232	222	241	251	244	218	12.88	14.90	15.12	14.90	15.80	15.11	16.60	17.71	18.64	15.74
502	137	154	161	148	167	166	176	183	189	164	10.95	11.71	12.03	11.69	12.41	12.00	12.70	13.14	13.68	12.25
343	151	173	191	170	197	188	205	212	221	189	11.79	13.08	13.32	12.98	13.67	13.30	14.14	14.65	15.32	13.58
373	144	158	177	150	182	180	191	198	204	176	11.37	12.39	12.50	12.34	12.62	12.55	12.79	13.18	13.70	12.60
550	156	184	202	180	212	200	219	227	235	201	12.49	13.98	14.36	13.95	14.86	14.30	15.36	16.00	16,79	14.41
Mean	155	185	203	181	210	203	218	226	232		12.34	13.92	14.20	13.88	14.70	14.19	15.23	16.20	17.25	
	LSD at 5%	Varieties (V) = 10.11 (Sig)									Varieties (V) = 0.70 (Sig)									
		Treatments (T) = 12.11 (Sig)									Treatments (T) = 0.90 (Sig)									
		V × T = 22.21 (Sig)									V × T = 1.60 (Sig)									

Notes: V = Varieties; T = Treatments; Sig = Significant; NS = Non-significant

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Table 7. Effect of NaCl (100 or 150 mM) and/or Al (5 or 10 mM) stress on the proline and soluble sugar content in different varieties of wheat (*T. aestivum*) at 30 days after sowing (DAS)

	Proline content (μ mol g^{-1} fresh mass)										Soluble sugar content (mg g^{-1} dry mass)									
	0	NaCl 100	NaCl 150	Al 05	Al 10	NaCl 100 + Al 5	NaCl 100 + Al 10	NaCl 150 + Al 5	NaCl 150 + Al 10	Mean	0	NaCl 100	NaCl 150	Al 05	Al 10	NaCl 100 + Al 5	NaCl 100 + Al 10	NaCl 150 + Al 5	NaCl 150 + Al 10	Mean
DBW-17	10.30	12.77	13.39	12.56	13.39	13.28	14.42	15.86	16.48	13.60	34.15	39.57	40.94	39.95	42.34	41.32	44.05	45.76	47.46	41.72
LOK-1	11.20	14.56	15.68	14.44	16.68	15.79	17.36	17.80	18.48	15.77	37.12	44.52	47.51	44.91	47.88	47.14	49.74	51.96	53.82	47.17
2851	11.35	12.80	13.36	12.50	13.40	13.20	14.50	15.90	16.60	13.73	34.21	39.60	40.40	40.00	42.50	41.00	44.25	46.01	47.99	41.77
502	8.28	8.94	9.27	9.74	9.35	9.35	9.85	10.26	10.76	9.53	28.21	29.05	29.90	29.33	30.74	30.18	31.59	32.72	33.56	30.58
343	9.52	10.56	11.42	11.13	11.61	11.51	12.18	12.85	13.32	11.56	30.80	32.95	34.18	32.64	35.11	33.88	36.65	38.19	40.04	34.93
373	8.84	9.81	10.25	9.90	10.25	10.16	10.87	11.40	12.02	10.38	29.32	30.78	31.37	30.80	32.25	31.40	33.42	34.89	36.65	32.32
550	9.96	11.95	12.35	12.05	12.64	12.25	13.24	13.94	14.74	12.56	33.03	36.33	37.65	36.66	39.90	37.32	40.95	42.60	44.92	38.81
Mean	9.21	11.64	12.45	11.76	11.47	12.22	13.20	14.00	14.62		32.40	36.11	37.42	36.32	38.67	37.46	40.09	41.73	43.49	
	LSD at 5%	Varieties (V) = 0.75 (Sig)									Varieties (V) = 2.35 (Sig)									
		Treatments (T) = 0.65 (Sig)									Treatments (T) = 1.75 (Sig)									
		V × T = 1.30 (Sig)									V × T = 4.01 (Sig)									

Notes: V = Varieties; T = Treatments; Sig = Significant; NS = Non-significant

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accumulation, which will limit automatically the photosynthetic CO_2 assimilation (Yusuf et al., 2008). The poor net photosynthetic rates, under salt stress, were noted to be positively related to the observed decrease in stomatal conductance and internal CO_2 concentration (Ali et al., 2008). In addition to this, presence of excess Al induce damage of photosystem II (Ali et al., 2008), disturbance in chloroplasts ultrastructure (Saleem, Ashraf, & Akram, 2011), decreases in Fv/Fm (maximum quantum efficiency) (Lu, Cao, Feng, He, & Jiang, 2009). Furthermore, Pereira et al. (2006) revealed that Al treatment decreased 5-aminolevulinic acid (ALA) dehydratase activity in cucumber and in maize, the enzyme responsible for chlorophyll synthesis. Similar deteriorating effects have been shown by various workers for both salt and Al stress (Chen, Qi, Smith, & Liu, 2005).

It is a common phenomenon of plants to generate ROS under the exposure of different stressors either alone or in combination (Suzuki, Rivero, Shulaev, Blumwald, & Mittler, 2014). LPO, H_2O_2 accumulation and electrolyte leakage is an excellent biomarker of stress induced tissue damage (Monteiro, Rocha, Mancera, Fontaínhas-Fernandes, & Sousa, 2009) and excess production of ROS (Thirupathi, Jun-Cheol, Changsoo, Kumariah, & Wook, 2011). In the present study, stress biomarkers (LPO, H_2O_2 content and Electrolyte leakage) increased significantly in the presence of salt and/or Al stress with varied intensity in different varieties of plants showed in Tables 3 and 4. Salt stress induces ROS, which cause injury to the cellular proteins, nucleic acids and also affects the membrane integrity by peroxidation of membrane lipids (Attia, Sayed, Ibrahim, Mohammed, & El-Alfy, 2011). The extent of LPO and thus membrane disintegration due to induced oxidative stress by salinity determines the sensitivity of the plant species towards salinity stress. Plant species which show improved integrity of plasma membrane and thus lesser electrolyte leakage under salt stress conditions contributed to improved salt tolerance (Fariduddin, Khalil, Mir, Yusuf, & Ahmad, 2013). Al stress also induces oxidative stress (Parker, Zelazny, & Kinraide, 1988) due to the involvement of Al ions in various processes, including an increase in enzyme activity (SOD and POX) related to ROS and LPO in *Glycine max* (Cakmak & Horst, 1991), as well as changes in the expression of various genes induced by Al in Arabidopsis (Richards, Schott, Sharma, Davies, Gardner, 1998). Recently, Yamamoto, Kobayashi, and Matsumoto (2001) have suggested that peroxidation of lipids stimulated by Al ions in peas is an early symptom and may be the cause of poor growth of plants and development.

To balance excess ROS and maintain coordination, regulation of phosphoproteins, activation of signalling pathways by ROS responsive regulatory genes and buffering of ROS by ROS scavenging enzymes (Dietz, 2011), plants should enhanced the activities of antioxidant enzymes under the exposure of various stressors alone or in combination. Plants with higher levels of antioxidants have been reported to possess greater tolerance against various environmental stresses (Dionisio-Sese & Tobita, 1998). In the present study, the activities of CAT, POX and SOD in plants increased exposed to NaCl stress alone and in combination with Al stress, maximum activities of these enzymes were shown under combination of stressor in Tables 5 and 6. Plants have multiple genes encoding SOD and different iso-enzymes of SOD are specifically targeted to chloroplasts, mitochondria, peroxisomes, and cytosol. Mn-SOD is most often found in the mitochondrial and peroxisomes (Palma et al., 1998), and Fe-SOD is found in chloroplast (Bowler, Montagu, & Inze, 1992). Cu/Zn SODs provide less protection than Fe-SODs when localized in the chloroplast (Sen Raychaudhuri & Deng, 2000). Therefore, our results suggested that mitochondrial and peroxisome compartments might be more important in scavenging $O_2^{\cdot-}$ in the stressed leaves of salt-tolerant wheat plants. The greater tolerance of the wheat cultivar seems to be a result of the joint action of the enzymes CAT, POX, and SOD. Similar to our results, Ma, Gao, Zhang, Cui, and Shen (2012), showed that two maize and rice cultivars with different tolerance capacity to Al, respectively, showed that the improvement in protection against Al toxicity was obtained by an increase in the activity of the antioxidant system.

Accumulation of carbohydrate in the plants are well known for osmotic adjustment under salt stress (Zheng et al., 2008) that is evident from the increased sugar content in Table 7 in tolerant varieties of wheat along with higher LWP showed in Table 2 and these two parameters further increased in the combination of salt and aluminium stress showed in Table 2. On the other hand, soluble sugars are strongly related to stress induced ROS accumulation in plants. Nishizawa, Yabuta, and

Shigeoka (2008) analysed the ROS scavenging ability of soluble sugars *in vitro* and demonstrated similar antioxidant capabilities for sugars similar to GSH. In addition, their concentrations are suitably ranged to protect plant cells from oxidative damage (Nishizawa et al., 2008). Interestingly, raffinose concentrations in chloroplasts of stressed plants are comparable with those of AsA and GSH, suggesting that this soluble sugar can directly scavenge ROS in chloroplasts (Zheng et al., 2008). Proline act as an osmoprotectant, potent non-enzymatic antioxidant and also accumulates under wide range of biotic and abiotic stresses (Szabados & Savoure, 2009). As a singlet oxygen quencher (Alia Mohanty & Matysik, 2001) and scavenger of hydroxyl radicals (Smirnoff & Cumbes, 1989), when it accumulates in plant tissue proline may be important in preventing oxidative damage caused by ROS. Proline may stabilize DNA, membranes and protein complexes, and provide a source of carbon and nitrogen for growth after stress relief. Proline metabolism is involved in the regulation of intracellular redox potential and the storage and transfer of energy and reducing power (Szabados & Savoure, 2009). Changes in proline metabolism may be more beneficial for plant tolerance to environmental stresses than properties of the amino acid itself. These properties of proline accumulation under abiotic stress showed conformity with findings of present study in which different cultivars of wheat showed contrasting accumulation of proline under salt stress alone and also in combination with Al stress revealed in Table 7. Moreover, an additional reason for increased proline level in the present study seems to be the water stress generated by Al (Barceló & Poschenrieder, 2002) as evident from decreased LWP in the leaves shown in Table 2. The synthesis of proline is a gene-regulated process that involves the activation of genes of its biosynthesis and down regulation of those involved in its degradation (Sumithra & Reddy, 2004), under stress conditions.

5. Conclusions

In the present study, salt and aluminium stress alone showed the similar response in terms of photosynthetic attributes, electrolyte leakage and LPO whereas, in combination the deleterious effect was more pronounced in the cultivars of wheat with varied levels. However, the salt stress restrict the uptake of aluminium by root under the combination of salt and aluminum stress in all the cultivars of wheat. On the other hand, the pattern of tolerance was observed on the basis of enhanced antioxidant system and proline accumulation and cultivar LOK-1 and 502 showed maximum and minimum tolerance respectively, against the combination of salt and aluminium stress.

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Competing Interests
The authors declare no competing interest.

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