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Research article

Mechanistic insights of salicylic acid-mediated salt stress tolerance in *Zea mays* L. seedlings

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ABSTRACT

Elevated sodium level (Na^+) poses significant threat to crop plant physio-biochemical processes, leading to impaired growth followedby decline in productivity. Addressing this challenge, requires an eco-friendly and cost-effective strategy that enhances plant salt stress tolerance capacity. In this context, the exogenous source of plant growth regulators (PGRs) proved to be an efficient approach. Of various PGRs, salicylic acid (SA) is an emerging signaling molecule that boosts plant stress endurance mechanism. This study investigates SA-mediated salt stress tolerance in maize (*Zea mays* L.) seedlings, by examining morpho-physiological and biochemical traits. Maize seedlings were subjected to varying levels of salt stress (0, 25, 50, 75, 100, and 150 mM NaCl) for a period of 10-days. The results revealed that, a substantial decline in germination percentage, shoot and root length, plant biomass, vigour index, and various other physiological parameters under salt stress causing concentrations. Conversely, salt stress increased oxidative stress indicators, including hydrogen peroxide (H_2O_2) and malondialdehyde (MDA), osmolytes and elemental concentrations as well as antioxident enzymes (SOD, CAT, POX, APX, GR, AsA). However, the exogenous supplementation of SA at 0.1 mM significantly restored most morpho-physiological attributes in maize under salt stress conditions. This suggests that SA actively triggers the ascorbate-glutathione (AsA-GSH) pathway and other key enzymes, leading to sodium extrusion and improving antioxidant defense in maize seedlings. This finding provides valuable insights for maize farmers that employing SA could lead to improved maize production in saline soils.

1. Introduction

The excessive salinization of cultivable land led to its degradation that adversely effects crops plant health [1,2]. Approximately 20

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% of agricultural land and 6 % of the total global land are affected by salinity. Elevated levels of sodium salts have a momentous adverse influence on plant growth, water absorption, essential nutrient concentration, and overall agricultural productivity [3]. In their surroundings, plants cope with many environmental problems, including salinity, water shortages, severe temperatures, and excessive amounts of heavy metals. Among these factors, salinity emerges as widely accepted stressor that contribute to environmental degradation and greatly limit the development of plants [2]. Salt stress manifests plants at various levels, leading to reduced yields and triggering a cascade of morpho-physiological and molecular alterations [3].

The detrimental effects of salt stress spread to plant processes like photosynthesis, chlorophyll content, elemental concentrations, osmolyte accumulation, and overall plant biomass [3,4]. Excess sodium (Na^+) builds up in their cytoplasm and imbalance of potassium ion (K^+) uptake taken place in stressed plants [5]. The plants' K^+-dependent enzymatic defence mechanism and K^+ retention are weakened in response to the influx of Na^+ into the root system's cell membrane, which causes membrane depolarization and disruption of K^+ inflow [6]. Interestingly, genotypes that are tolerant to salt have a larger Na^+/K^+ ratio in the cytosol than genotypes that are more vulnerable to salt stress. This modification aids in extenuating the negative effects of high Na^+ buildup [5].

Reactive oxygen species (ROS) including singlet oxygen ($^1O_2$), hydroxyl radicals ($OH^{\bullet-}$), superoxide radicals ($O_2^{\bullet-}$), and hydrogen peroxide (H_2O_2) are produced when Na^+ ion stress occurs. These ROS can cause oxidative damages, lipid peroxidation, and cellular injury [3,7]. Salt stress damages lipids and nucleic acids, denaturates proteins, deactivates enzymes, and compromises the integrity of the cell membrane system [8 9].

To deal with osmotic and oxidative stress, plants have evolved an antioxidant defence system which consist of enzymatic and non-enzymatic components [2,3,7]. Superoxide dismutase (SOD) is a key antioxidant enzyme that considered as the first line of defence against stress [8]. SOD reduces the possibility of producing $OH^{\bullet-}$ radicals by catalysing the dismutation of $O_2^{\bullet-}$ into H_2O_2. Within the complex web of interactions involving metabolites and redox enzyme characteristics, the ascorbate-glutathione (AsA-GSH) pathway is essential [3]. In order to detoxify ROS and avert oxidative damage in plants, this route is necessary. The two main antioxidant enzymes of the AsA-GSH pathway are ascorbate peroxidase (APX) and glutathione reductase (GR) [3,8,9]. Salt-induced osmotic stress decreases water potential, stomatal aperture frequency and size, and chlorophyll pigment degradation, all of which cause plants to perform less during photosynthesis [2,3]. This detrimental effect upsets the osmotic and ionic balance in cells, which hinders plant growth and may even cause plant death. Plants under salinity stress grow more slowly because it reduces their ability to absorb water and damages leaf cells at transpiration sites [10].

Plants respond to oxidative stress by producing antioxidant enzymes catalase (CAT), peroxidase (POX), APX, GR as well as storing osmolytes [11, 3]. Numerous osmolytes support the stability of cell membranes by lowering ROS and lipid peroxidation levels [3]. Sequestering Na^+ and Cl^- ions within cell vacuoles and preventing further Na^+ entry into cells through chemiosmotic or SOS pathway channels are the methods used by plants to adapt to salt stress. These defence mechanisms help the plant withstand against salinity [12]. Even with these defences, plants may find it difficult to combat the impacts of extreme stress with just their internal defences. Therefore, numerous initiatives have been made to lessen the negative effects of stressors and improve crop productivity. Of these methods, using SA externally has shown to be a crucial tactic for enhancing the plant physio-biochemical characteristics in a variety of ecological settings, including salt stress [3,13]. Consequently, there is a great need for affordable and environmentally friendly methods of reducing salt stress in plants.

Plant stress can be effectively mitigated by using plant growth regulator (PGR) or biostimulant [14–16]. According to Barwal et al. [3], SA is an emerging signaling molecule, act as PGR, controls a variety of plant developmental processes and successfully lessens the effects of a number of abiotic stresses. Researches have shown that SA can reduce lipid peroxidation, reduce the build-up of Na^+ and Cl^-, increase proline concentrations, increase the activity of antioxidant enzymes, and help plants recover from a variety of abiotic stressors while preserving their physiological characteristics [13,17–20]. SA-mediating salt stress tolerance at developmental stages in *Zea mays* L. (maize) seedlings has not yet been investigated.

Around the world, maize a cereal grain, is extensively grown as a staple food. According to Walia et al. [21], maize is the third most valuable crop in India. On the other hand, the production of maize has a detrimental impact on world food security. In this situation, a cost- and environmentally-friendly salt mitigation method is quite beneficial for sustainable agricultural production. Thus, investigating the SA-mediating salt tress tolerance in maize seedlings was the aim of the current work. Through exogenous supplementation of SA on maize seedlings, the morpho-physiological and biochemical characteristics were assessed.

2. Materials and methods

2.1. Experimental plan

Seeds of African Tall-09 maize (*Zea mays* L.) were procured from Kucchal Seed Bhandar (officially recognized by the National Seed Corporation) situated in Mangal Panday Nagar, Meerut. African Tall-09 maize is an inbred line. It was developed specifically for maize breeding programs. Tall-09 maize is sensitive to salt stress. The healthy and equal size seeds were selected for experiment. To ensure sterility, the maize seeds underwent a 2-min surface sterilization using a 0.1 % $HgCl_2$ solution, followed by thorough rinsing with distilled water (DW). The seeds were then soaked in DW for 24 h to facilitate moisture absorption, subsequently dried on filter paper, and placed in sterilized Petri plates. These plates, lined with Whatman no. 1 filter paper, were moistened with 10 ml of five distinct NaCl levels (0, 25, 50, 75, 100, and 150 mM), both with and without the addition of 0.1 mM SA. Each treatment was replicated three times, and each Petri plate contained ten seeds, adhering to the guidelines of the International Seed Testing Association [ISTA, 22]. The control group consisted of seedlings grown fast with distilled water. The germination process unfolded over 3–4 days in a dark growth chamber, maintained at a temperature range of 25–30 °C.

After ten days of growth, the treatments were concluded. The 10-days-old maize seedlings were taken and their roots were meticulously washed to eliminate surface salt, and samples were collected for subsequent analysis of morphological, physiological, and biochemical attributes.

2.2. Determination of growth traits

Before seed germination, ten seeds were taken to assess their viability through tetrazolium reaction test. Imbibe 10 seeds placed in a Petri plate containing DW, for viability test. When seed emergence is 2 mm in length then make a small cut with the surgical blade into the seed emerging point and then dipped into 0.1 % of 2, 3, 5 triphenyl tetrazolium chloride solution. Keep the Petri plate in the dark for 4–5 h; the results show that all the seeds get a dark pink color. It is confirmed that all the seeds were 100 % viable.

The seeds germination was recorded from the day of sowing upto the seedling emergence to compare seed germination percentage of seedlings of stress and non-stressed plants. Germination % = (No. of germinated seeds/ Total no. of seeds × 100)

The required number of seedlings (three for each treatment) were gently removed from Petri plates and then measurements were recorded separately for shoot and root length. The shoot and root length (separately) of both treated and control plants were measured using a centimetre (cm) scale. Plant fresh and dry weights were determined using an electronic balance. After estimating the fresh weight, seedlings were placed in paper envelopes and dried in an 80 °C hot oven for 1 h. Subsequently, they were maintained at 65 °C until a consistent weight was achieved.

The percentage of moisture content, plant vigour, and tolerance indices were calculated using the following formulas recommended by Barwal et al. [3]:

Moisture percentage (%) was calculated according to standard formula:

$$\text{Moisture } \% = \frac{\text{Fresh wt.} - \text{Dry wt.}}{\text{Fresh wt.}} \times 100$$

The vigour index (VI) was calculated based on the formula:

$$\text{VI} = \text{Shoot length} + \text{Root length} \times \text{Germination } \%$$

The salt tolerance index (STI) of seedlings was calculated according to formula:

$$\text{STI} = \frac{\text{Mean of longest root in treated seedlings}}{\text{Mean of longest root in control seedlings}} \times 100$$

2.3. Estimation of photosynthetic pigments

The determination of chlorophyll *a*, *b*, and carotenoids were carried out according to the method outlined by Arnon [23]. 50 mg of leaf samples were homogenized and extracted with 5.0 ml of 80 % (v/v) chilled acetone. The solution was then centrifuged at 5000 rpm until the supernatant became colourless. The resulting supernatant was collected, and its volume was adjusted to 10 ml using 80 % chilled acetone. Subsequently, the supernatant was read at 663 nm, 645 nm, and 436 nm for chlorophyll *a*, *b*, and carotenoids, respectively. Finally, the contents of chlorophyll *a*, *b*, and total chlorophyll content and carotenoids were calculated using formulas adopted by Bartucca et al. [24].

$$\textit{Chl. a } \left(mg\, g^{-1}\ F.Wt.\right) = \frac{12.7 * (OD\ 663) - 2.69 * (OD\ 645) \times V}{1000 \times W}$$

$$\textit{Chl. b } \left(mg\, g^{-1}\ F.Wt.\right) = \frac{22.9 * (OD\ 645) - 4.68 * (OD\ 663) \times V}{1000 \times W}$$

$$\text{Total chlorophyll content } \left(\text{mg g}^{-1}\ \text{F.Wt.}\right) = \frac{20.2 \times (A_{645}) + 8.02 \times (A_{663}) \times V}{1000 \times W}$$

$$\text{Carotenoids} \left(\frac{\mu g}{mg}\right) = \frac{[1000\ (A436) - \ 1.28(\text{Chl a}) - \ 56.7(\text{Chl b})]}{256 * 0.906}$$

2.4. Determination of enzymatic antioxidants

The antioxidants activity was assessed by following protocols outlined in our previous study [3]. Initially, plant samples were pulverized into a fine powder using liquid nitrogen. Subsequently, 500 mg of this powder underwent homogenization in 5 ml of 100 mM ice-cold K-phosphate buffer at pH 7.0, supplemented with 1 % polyvinylpyrrolidone and 1 mM EDTA. The resulting solution mixture was then centrifuged at 10,000 rpm for 15 min at 4 °C, and the enzymes assay was conducted using the supernatant.

Superoxide dismutase (SOD, EC 1.15.1.1) activity determination, nitro-blue tetrazolium (NBT) photochemical reduction served as an indicator of activity in a 3 ml reaction mixture containing 2.45×10^{-3} M NBT, 2.8×10^{-5} M riboflavin, 0.1 mM EDTA, and enzyme extract. After exposure to light for 30 min, the absorbance change was observed at 560 nm. The reduction of color by 50 % was quantified as EU mg^{-1} protein per hour by following the method of Beauchamp and Fridovich [25].

Catalase activity (CAT, EC 1.11.1.6) was determined according to the protocol established by Aebi [26]. In this assay, 200 μl of the crude extract was mixed with 2.5 ml of 50 mM Na_2HPO_4 buffer (pH 7.0), and CAT activity was evaluated at 240 nm after the addition of 0.3 ml of 3 % H_2O_2. CAT activity was expressed as A240 EU mg^{-1} protein.

Peroxidase activity (POX, EC 1.11.1.7) was determined using methods of Maehly and Chance [27] and Vimala [28]. In test tubes, 2.25 ml of the crude enzyme extract was combined with 0.25 ml of a solution containing 1 % benzidine, followed by the addition of 0.25 ml of H_2O_2 (7.5 %). Absorbance at 475 nm was recorded, and overall peroxidase activity was expressed as ΔA475 EU mg^{-1} protein.

Ascorbate peroxidase (APX, EC 1.11.1.11) activity was determined according to Nakano and Asada [29] method. The ascorbate oxidation rate at 290 nm was measured to determine APX activity. The reaction mixture contains enzyme extract, 50 mM phosphate buffer (pH 7.0), 0.1 mM EDTA, 0.1 mM H_2O_2, and 0.5 mM ascorbate in a total volume of 1 ml. The reaction was initiated by adding 0.2 ml of 1 mM H_2O_2, and absorbance measurements were taken at 290 nm. APX activity was quantified as EU mg^{-1} protein, employing an extinction coefficient of 2.8 mM cm^{-1} in the calculations.

Glutathione reductase (GR, EC 1.6.4.2) activity was determined by following the method of Jablonski and Anderson [30]. In a 3 ml reaction mixture with 100 mM phosphate buffer at pH 7.0, 1 mM GSSG, 1 mM EDTA, 0.1 mM NADPH, and 25–50 μl of the enzyme extract, the GR test was conducted. NADPH oxidation was monitored by read the absorbance per minute at 340 nm. The extinction coefficient of 6.22 mM^{-1} cm^{-1} was utilized to determine the amount of NADPH oxidized, and GR activity was expressed as EU mg^{-1} protein.

2.5. Determination of non-enzymatic antioxidants

The estimation of ascorbate content (AsA) was carried out in accordance with the method described by Shukla et al. [31]. For the extraction process, 0.2 g of fresh seedlings was homogenized in 5 % (w/v) *m*-phosphoric acid. The extract was then subjected to centrifugation at 5000 rpm for 20 min. A freshly prepared, double-diluted folin reagent (2.5 ml) was combined with a 1 ml aliquot of the supernatant. The resulting test tube was left at room temperature for duration of 40 min. Subsequently, the absorbance was read at 730 nm. The content of AsA was determined and expressed as mg g^{-1} fresh weight.

2.6. Quantification of soluble protein

A fresh sample weighing 0.2 g was homogenized in 5.0 ml of Tris-HCl buffer with a pH of 7.0, and the homogenate underwent centrifugation at 10,000 rpm for 10 min. The supernatant was taken and used to make protein extract. 5.0 ml of Coomassie brilliant blue dye was added to 1.0 ml of this sample extract (plant protein extract). At 595 nm, the absorbance was measured against blank. The standard protein, casein (1.0 mg/ml), was used to create a reference curve, which was reported as mg casein g^{-1} fresh weight. The total protein content was determined by referencing a standard curve of casein protein and calculated as mg g^{-1} fresh weight. The complete protocol of Bradford [32] was adopted.

2.7. Measurement of proline content

The fresh 200 mg leaves were homogenized by crushing in 10 ml 3.0 % sulfosalicylic acid, and centrifuged for 10 min at 5000 rpm. Supernatant was collected, and 2 ml of supernatant was mixed with 2.0 ml of glacial acetic acid and 2.0 ml of freshly made acid ninhydrin solution. For 1 h at 100 °C, the mixture was put in a water bath. The test tubes were placed in an ice bath for terminate the reaction. Toluene (4.0 ml) was added and properly mixed. Spectrophotometrically (Shimadzu UV-2600) at 520 nm, the absorbance of a chromophore-containing toluene layer was determined using a method established by Bates et al. [33]. The curve was created by calculating free proline μg g^{-1} fresh weight using pure proline. The following formula was used to determine the quantity of proline in the sample.

$$\text{Amount of proline } \left(\mu g\ g^{-1}\right) = \frac{\text{A at } 520}{2} \times \frac{\text{vol. of toluene}}{115.5} \times \frac{\text{weight of sample}}{5}$$

2.8. Estimation of total phenolic content or phenolics

A total of 50 mg of the plant sample was homogenized with 5 ml of 80 % ethanol. The resulting mixture was then subjected to centrifugation at 5000 rpm for 10 min, and the supernatant was collected using the method outlined by Bray and Thorpe [34]. The phenolic content expressed in mg g^{-1} of fresh weight, was determined by referencing a calibration curve generated using gallic acid.

2.9. Determination of H_2O_2 and MDA accumulation

In the assessment of oxidative stress indicators in maize seedlings samples, 200 mg of sample was crushed in 3.0 ml of 50 mM KH_2PO_4 buffer with a pH of 6.5, followed by centrifugation at 11,500 rpm for 15 min at 4 °C. A 3.0 ml portion of the resulting supernatant was collected, and a mixture comprising 1.0 ml of titanium tetrachloride (0.1 %) with 20 % H_2SO_4 (v/v) was added. This mixture underwent centrifugation for 15 min at 11,500 rpm at room temperature to quantify hydrogen peroxide by using the method of Yu et al. [35], with results expressed as μg g^{-1} fresh weight. Simultaneously, for the analysis of malondialdehyde (MDA), 200 mg of

shoot tissue was standardized in 3 ml of 5 % trichloroacetic acid (TCA, w/v) and subjected to centrifugation for 10 min at room temperature at 11,500 rpm, following the procedure outlined by Heath and Packer [36]. MDA content was then measured and expressed as μg g^{-1} fresh weight. These assessments provide valuable insights into the oxidative stress status in maize seedlings samples.

2.10. Estimation of sodium (Na^{+}), potassium (K^{+}), and calcium (Ca^{2+}) concentrations

Elemental concentration in maize seedlings samples was determined through a process involving the following steps: 100 mg of plant materials were standardized in 10 ml of 1 N/M HCl (Specific gravity-1.18, 8.823 ml of HCl made to a final volume of 100 ml with distilled water). The mixture was then centrifuged, and the supernatant was collected. The collected supernatant was adjusted to a total volume of 10 ml with 1 N/M HCl and subsequently analyzed for sodium, potassium, and calcium against NaCl, KCl, and $CaCl_2$ (20 ppm) solutions, respectively. This analysis was conducted using a Systronic Flame Photometer #125 according to the method outlined by Prat and Fathi-Ettai [37]. The results of these elemental concentration analysis provide valuable information about the levels of sodium, potassium, and calcium in the maize seedlings sample. Elemental concentration measured and expressed as nmol g^{-1} 1.0 $\times 10^{-9}$ dry weight.

2.11. Statistical analysis

The experimental data was statistically checked through One-Way Analysis of Variance (ANOVA) using SPSS 16.0. To compare the means, Duncan's Multiple Range Test (DMRT) was performed at a significant level of $p \leq 0.05$. The principal component analysis (PCA biplot) and Pearson correlation matrix were conducted by using Origin Pro software (version 2023).

3. Results

3.1. Seed germination and seedling growth

The application of SA possibly mitigates the adverse effects of salt stress on seed germination and seedling growth (Figs. 1 and 2).

Salt concentrations at 100 and 150 mM were shown to reduce seed germination by 42.9 % and 66.7 %, respectively, as compared to the control. The application of SA at 0.1 mM resulted in an increase in seed germination percentage by 41.7 % over without SA treatment under 150 mM NaCl level (Table 1).

The shoot and root length of 10-days-old maize seedlings that were subjected to 25 mM NaCl level showed less effected. However, under high salt levels i.e., 75 and 150 mM NaCl, the shoot length significantly decreased by 45.28 and 80.88 %, and root length decreased by 48.98 and 60.44 % as compared to control. Their maximum reduction was recorded at a concentration of 150 mM NaCl.

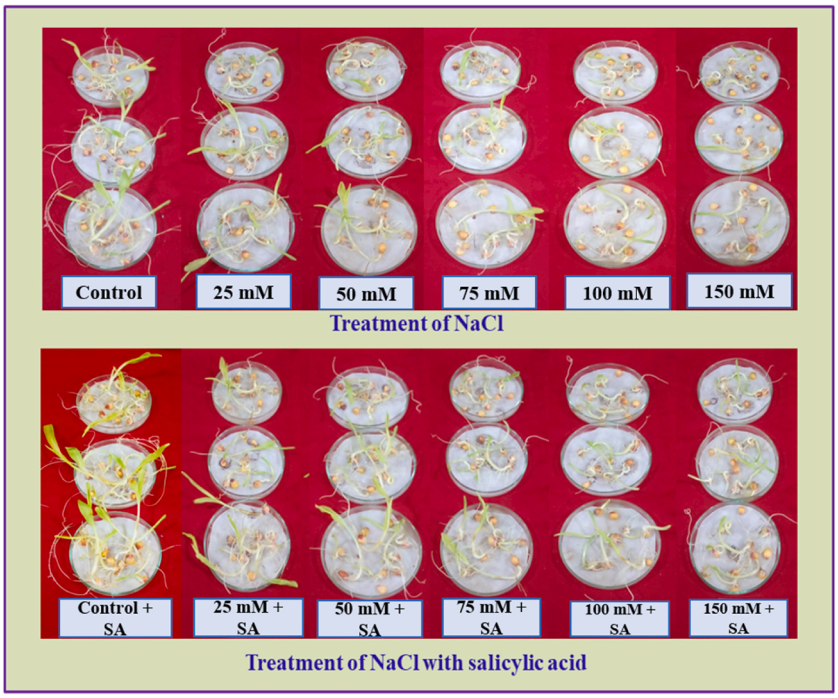

Fig. 1. Seed germination and progress of maize seedlings without and with SA application under various concentrations of NaCl

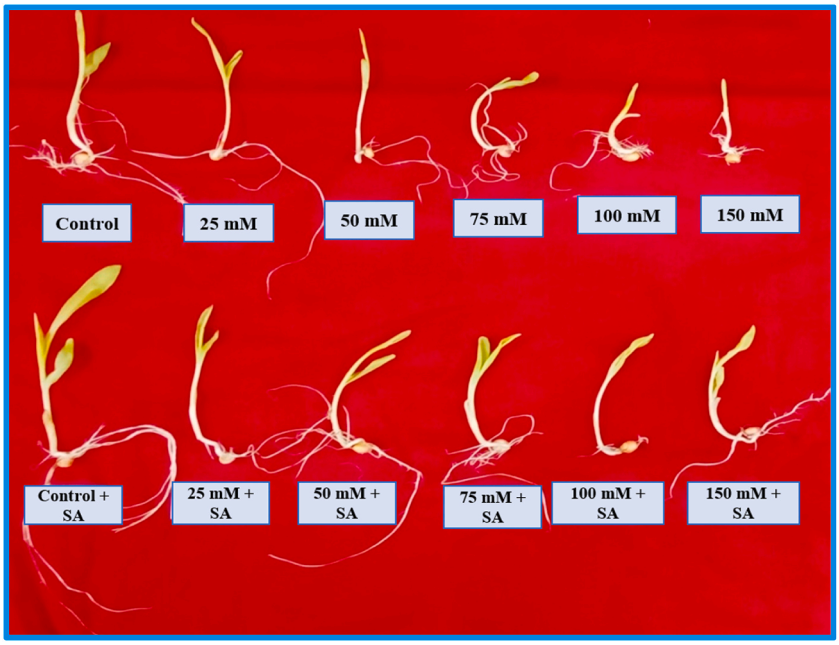

Fig. 2. Progress of maize seedlings without and with SA application under various concentrations of NaCl

Table 1
Effect of different levels of NaCl stress (0, 25, 50, 75, 100 and 150 mM) with or without salicylic acid application on Seed germination (%), Shoot length, Root length, Fresh weight Dry weight and Moisture content (%) of 10-days-old maize seedlings. Data are treatments mean and standard error of three replicates. The alphabet letters show significant differences among treatments confirmed through DMRT at $P \leq 0.05$.

Treatments	Germination %		Shoot length (cm)		Root length (cm)		Fresh weight (mg)		Dry weight (mg)		Moisture %	
NaCl	0 mM SA	0.1 mM SA	0 mM SA	0.1 mM SA	0 mM SA	0.1 mM SA	0 mM SA	0.1 mM SA	0 mM SA	0.1 mM SA	0 mM SA	0.1 mM SA
Control	100 ± 00a	100 ± 00a	12.3 ± 0.6a	14.4 ± 1.2a	4.8 ± 0.96a	7.0 ± 0.50a	1.50 ± 0.02a	1.79 ± 0.19b	0.77 ± 0.03 ab	0.94 ± 0.10b	47.96 ± 1.63a	55.06 ± 1.55a
25 mM	100 ± 00a	100 ± 00a	11.8 ± 0.7a	15.1 ± 0.6a	4.4 ± 0.50 ab	6.4 ± 0.51a	1.66 ± 0.23a	2.18 ± 0.17a	0.85 ± 0.07a	1.15 ± 0.09a	48.73 ± 0.96a	50.22 ± 1.12b
50 mM	80 ± 1.8 ab	100 ± 00a	10.3 ± 1.2 ab	11.2 ± 1.5b	3.9 ± 0.55 ab	5.9 ± 0.41 ab	1.29 ± 0.06b	1.62 ± 0.09bc	0.76 ± 0.00 ab	0.89 ± 0.01b	40.70 ± 1.47bc	49.54 ± 1.98b
75 mM	70 ± 1.9b	90 ± 2.5 ab	8.5 ± 2.0bc	10.7 ± 1.8bc	3.2 ± 0.20b	5.0 ± 0.23bc	1.03 ± 0.07cd	1.28 ± 0.11d	0.65 ± 0.05cd	0.75 ± 0.03cd	42.38 ± 1.44b	49.28 ± 1.06b
100 mM	70 ± 2.9b	90 ± 4.3 ab	9.0 ± 0.4b	9.2 ± 0.82bc	4.0 ± 80 ab	5.9 ± 0.50 ab	1.19 ± 0.02bc	1.52 ± 0.17cd	0.69 ± 0.06bc	0.83 ± 0.04bc	39.80 ± 1.50c	45.19 ± 0.91c
150 mM	60 ± 3.2b	80 ± 3.8b	6.8 ± 1.3c	8.4 ± 0.73c	3.0 ± 50b	4.4 ± 1.13c	0.94 ± 0.10d	1.26 ± 0.08d	0.57 ± 0.04d	0.69 ± 0.05d	38.51 ± 0.75c	45.01 ± 0.84c

However, the significant recovery of shoot length by 20 and 10.47 % happened after the treatment of SA under 100 and 150 mM NaCl, respectively, and of root length by 9.24 % under 75 mM NaCl over the no SA application (Table 1).

The fresh weight of the seedlings increased by 9.39 % under the treatment of 25 mM NaCl and dropped by 58.87 % under the treatment of 150 mM NaCl over the control. The dry weight reduced by 34.04 % under 150 mM of NaCl level. However, the application of SA (0.1 mM) caused the biomass of salt-treated seedlings to rise by 16.54 % of fresh weight under 150 mM NaCl and 17.93 % of dry weight under 25 mM NaCl when compared to seedlings that were not treated with SA (Table 1).

The seedlings that were treated with 25 mM NaCl had a higher moisture% than the seedlings that were treated with 150 mM NaCl, which showed a significant decrease in moisture content by 24.56 % under high salt concentration. The application of SA resulted in an increase in this trait under all salt levels as compared to without SA application (Table 1).

3.2. Vigour and tolerance indices

The vigour index decreased as the levels of NaCl increased as compared to the control. However, with the addition of SA, the vigour index demonstrated a significant increase by 40.12 % over the without SA application (Fig. 3A).

Conversely, the tolerance index decreased with rising concentrations of salt compared to the water-treated control. Notably, with the application of SA, the tolerance index of seedlings increased by 78.14 % under the 150 mM concentration of NaCl over the no SA-treated seedlings (Fig. 3B). These findings underscore the potential positive impact of SA in enhancing vigor and tolerance indices under salt stress conditions, particularly at higher concentrations.

3.3. Photosynthetic pigments content

There was a significant decrease in chlorophyll levels i.e., 186.5 %, 199.6 %, and 212.9 % of chlorophyll *a*, *b* and total under 75, 100, and 150 mM NaCl levels, respectively, compared to the control. However, the addition of SA led to a significant restoration in chlorophyll *a*, particularly under the 75 and 100 mM NaCl treatments, with the increase of 119.2 % and 110.7 %, respectively over the no supply of SA (Fig. 3C). Chlorophyll *b* experienced a noteworthy drop of 121 % under the 150 mM NaCl treatment, which was partially recovered by 30.78 % and 53.24 % in the presence of SA under the 150 mM and 100 mM NaCl concentrations, respectively, than the without SA application (Fig. 3D).

Total chlorophyll content exhibited a decline with the increasing NaCl concentrations compared to the control. Total chlorophyll content decreased by 177.7 % and 147.2 %, respectively, were observed under the 100 and 150 mM concentrations of NaCl. However, the application of SA resulted in an increase in total chlorophyll content under all NaCl treatments with the significant restoration of 89.94 % under the 100 mM NaCl level compared to their respective non-SA treatment (Fig. 3E).

Carotenoids, crucial for preventing photo-oxidation of chlorophyll molecules, demonstrated a significant increase by 46.50 % under 150 mM NaCl treatment compared to the control. Maize seedlings treated with SA under 100 mM NaCl further enhanced carotenoid content by 8.49 % as compared to non-SA treatment (Fig. 3F). These results highlight the positive impact of SA in mitigating the adverse effects of salt stress on chlorophyll and carotenoid contents in maize seedlings.

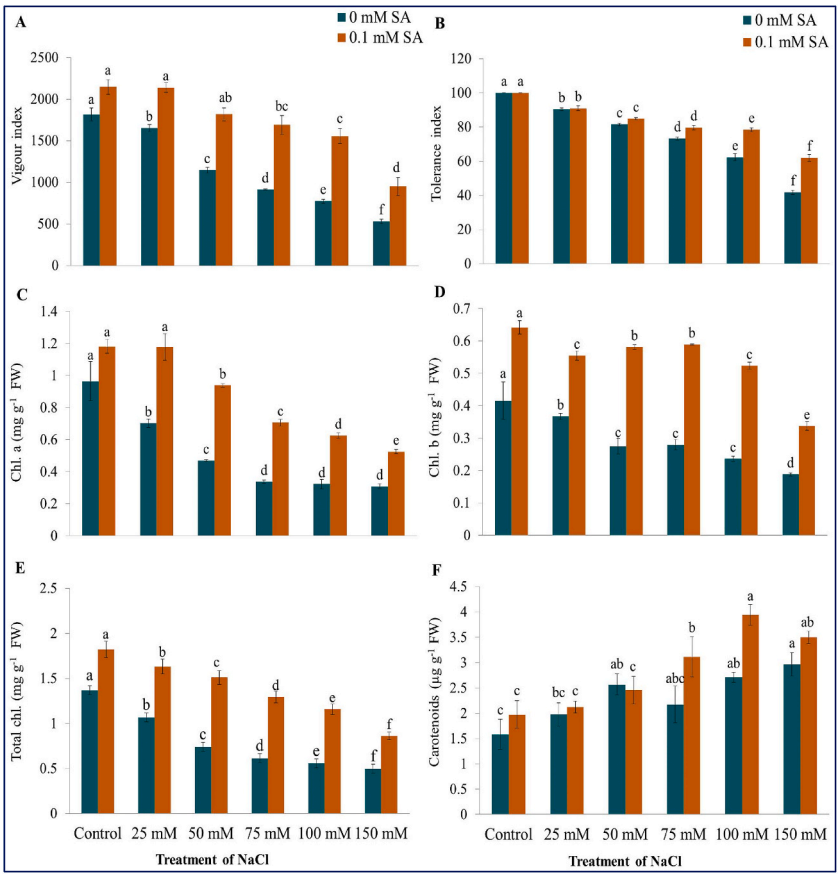

Fig. 3. Effect of different levels of NaCl stress (0, 25, 50, 75, 100 and 150 mM) with or without salicylic acid application on (A) Vigour index (B) Tolerance index (C) Chlorophyll *a* (D) Chlorophyll *b* (E) Total chlorophyll and (F) Carotenoids of 10-days-old maize seedlings. Data are treatments mean and standard error of three replicates. The alphabet letters show significant differences among treatments confirmed through DMRT at $P \leq 0.05$.

3.4. Antioxidant enzymes activity

In maize seedlings, the superoxide dismutase (SOD) activity increase with increasing salt levels and with a significant increase of 47.81 % under the 150 mM NaCl treatment compared to the control. The usage of SA further enhances the SOD activity by 7.16 % under the 100 mM NaCl than the no SA treatment (Fig. 4A).

Both catalase (CAT) and peroxidase (POX) activities significantly increased by 43.70 % and 45.07 %, respectively with the increasing concentration of NaCl upto the 100 mM NaCl treatment compared to the control. The application of SA further elevated CAT activity by 12.27 % and POX activity by 5 % as compared to without SA application (Fig. 4B and C).

Ascorbate Peroxidase (APX) activity significantly increased by 26.34 % under 150 mM NaCl treatment compared to the control. However, the application of SA further augmented APX activity by 25.21 % over no SA treatment (Fig. 4D).

Similarly, glutathione reductase (GR) activity also significantly increased by 25.27 % with the rising concentration of NaCl as compared to control. The application of SA further increased GR activity, with the highest enhancement of 9.15 % compared to without SA application (Fig. 4E).

Ascorbate (AsA) content significantly increased with the increasing concentration of NaCl, with the maximum increase of AsA content by 30.97 % observed under the 100 mM NaCl treatment compared to all other treatments. Interestingly, the application of SA caused no significant improvement in AsA content and led to decreased AsA content in all salt levels, with a maximum reduction of 36.40 % recorded under 50 mM NaCl + SA treatment compared to NaCl alone (Fig. 4F).

3.5. Protein, proline and phenolic contents

A notable decrease in the protein content by 54.86 % and 63.78 %, respectively, was observed under 100 and 150 mM salt concentrations compared to the control. The application of SA resulted in an increase in protein content across all salt concentrations, with

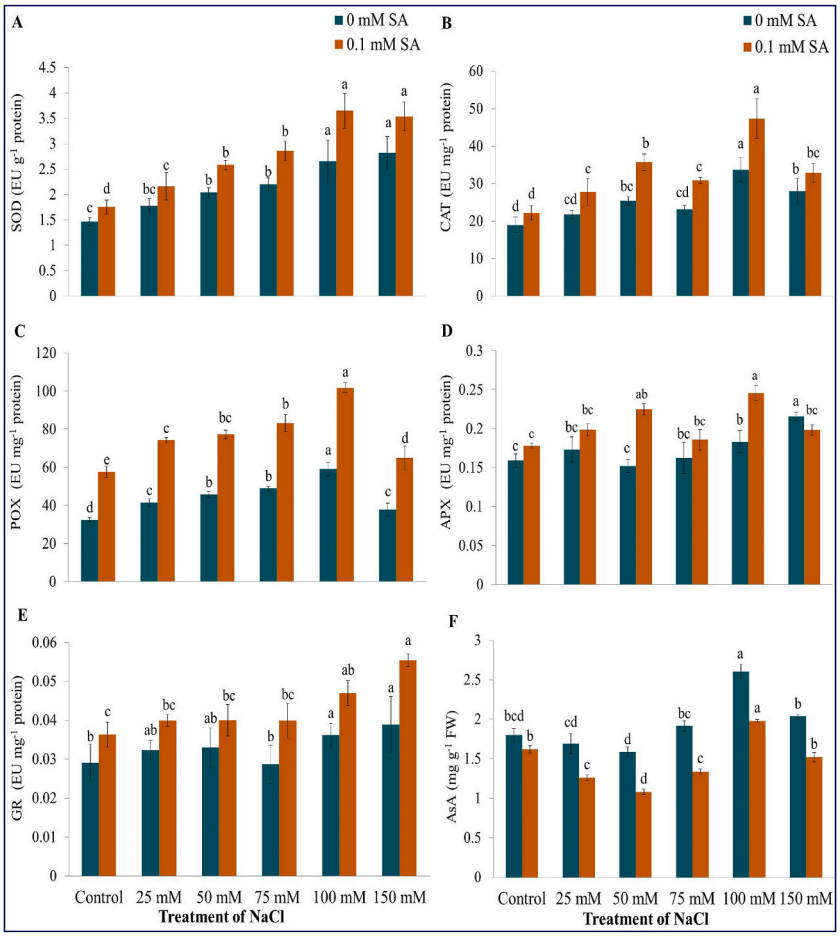

Fig. 4. Effect of different levels of NaCl stress (0, 25, 50, 75, 100 and 150 mM) with or without salicylic acid application on (A) SOD, (B) CAT, (C) POX, (D) APX, (E) GR and (F) AsA contents of 10-days-old maize seedlings. Data are treatments mean and standard error of three replicates. The alphabet letters show significant differences among treatments confirmed through DMRT at $P \leq 0.05$.

a significant enhancement of 16.82 % recorded under 100 mM NaCl treatment as compared to without SA treatment (Fig. 5A).

The accumulation of proline, an important osmoprotectant, reached its highest levels of 80.81 % and 79.86 %, respectively, under 100 and 150 mM NaCl treatments as compared to the control. However, the application of SA further influences proline content progressively over no SA treatment (Fig. 5B).

Phenolic content exhibited a significant increase with the increasing concentration of NaCl upto 75 mM NaCl level as compared to the control. Interestingly, the application of SA further increased phenolic content up to the 75 mM NaCl level over no SA application. However, phenolic content decreased by 23.33 % under high concentration of NaCl (150 mM) + SA treatment (Fig. 6A). These results highlight the dynamic responses of protein, proline, and phenolic content in maize seedlings under salt stress, which were influenced by the application of SA.

3.6. Oxidative stress indicators

The concentration of H_2O_2 increased by 42.81 % under 100 mM NaCl level compared to the control. However, the application of SA resulted in a decrease in H_2O_2 content as the NaCl concentration rose, with the maximum reduction of 11.09 % in H_2O_2 content compared to without SA application (Fig. 6B).

Malondialdehyde (MDA) content increased with the rising NaCl concentrations and with the maximum increase of 54.01 % under 100 mM NaCl level compared to the control. Exogenous application of SA reduced lipid peroxidation by 11.85 % under 150 mM NaCl + SA treatment as compared to without SA treatment (Fig. 6C). These findings highlight the role of SA in mitigating oxidative stress by reducing H_2O_2 concentration and lipid peroxidation in maize seedlings under salt stress conditions.

3.7. Elemental concentrations

The concentration of Na^+ ions increased by 75.05 % with the increasing concentration of NaCl upto 150 mM NaCl treatment compared to the control. The addition of SA led to a significant decrease in Na^+ content, with the most significant reduction of 25.55 % observed under the 50 mM NaCl + SA treatment in maize seedlings over no SA application (Fig. 6D).

Similarly, the highest K^+ content (30.96 %) was recorded under 150 mM NaCl treatment compared to the control. However, the application of SA increased K^+ content, with the maximum increase of 13.82 % observed under 150 mM NaCl + SA treatment in maize seedlings (Fig. 6E).

Ca^{2+} content also significantly increased by 29.27 % with the rising concentration of NaCl as compared to control. The addition of SA further increased Ca^{2+} content, with the maximum enhancement of 8.01 % recorded under the 150 mM NaCl + SA treatment (Fig. 6F). These results illustrate the dynamic regulation of ion homeostasis in maize seedlings under salt stress, influenced by the application of SA.

3.8. Principal component analysis and correlation matrix

The PCA biplot (Fig. 8) consists of two components (PC1 and PC2) designated 70.4 % and 16.8 % of total variation among the characteristics studied. A negative correlation depicted in the left-lower side of the PCA biplot was reported in parameters like K^+, phenolic and Na^+. On the other hand, a strong correlation was observed under SA treatment among various characteristics such as seed germination, shoot and root length, fresh and dry weight, vigour and tolerance index, leaf moisture contents, chlorophyll content, Ca^{2+}, antioxidants (CAT and POX) and non-enzymatic antioxidants (proline and AsA content).

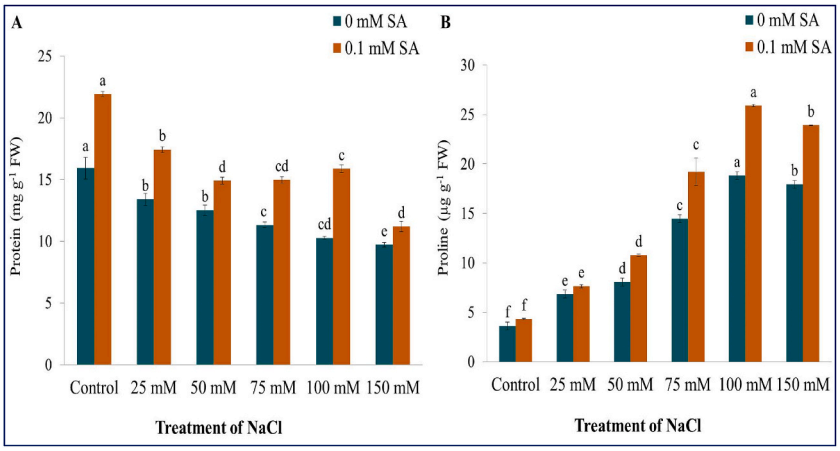

Fig. 5. Effect of different levels of NaCl stress (0, 25, 50, 75, 100 and 150 mM) with or without salicylic acid application on (A) Protein content and (B) Proline content of 10-days-old maize seedlings. Data are treatments mean and standard error of three replicates. The alphabet letters show significant differences among treatments confirmed through DMRT at $P \leq 0.05$.

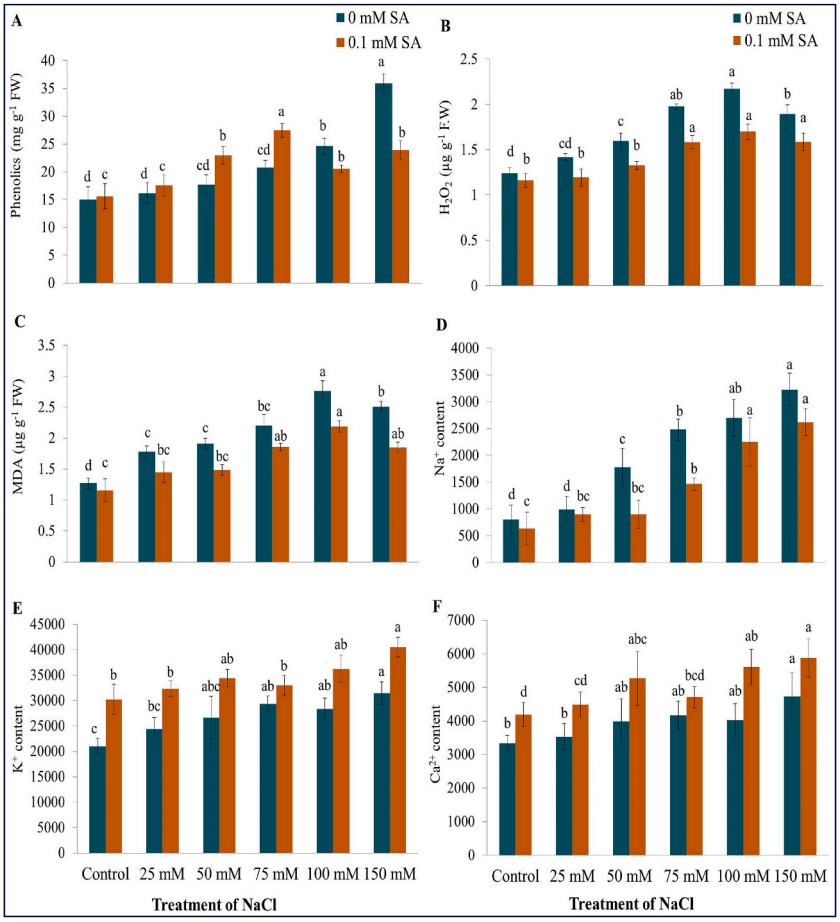

Fig. 6. Effect of different levels of NaCl stress (0, 25, 50, 75, 100 and 150 mM) with or without salicylic acid application on (A) Phenolics, (B) H_2O_2, (C) MDA, (D) Na^+, (E) K^+ and (F) Ca^{2+} contents. Data are treatments mean and standard error of three replicates. The alphabet letters show significant differences among treatments confirmed through DMRT at $P \leq 0.05$.

The correlation matrix (Fig. 9) indicated a substantial positive correlation between various characteristics studied at $p < 0.05$ significant level except between CAT, POX, AsA, MDA, proline, phenolics, Na^+ and K^+ which represent less interactions. The tight ellipses exhibited a significant positive correlation, but large and circular ellipses showed less correlation. The ellipses bending toward the right indicate positive interaction, whereas the ellipses bending toward the left show a negative association.

4. Discussion

The present study provides valuable mechanistic insights into SA-mediated salt stress tolerance in maize seedlings. The observed decline in salt-induced morphological, physiological, and biochemical attributes in maize seedlings correlates with the increasing levels of salt stress. However, the exogenous supplementation of SA significantly restored these impairments and played a protective role in mitigating the adverse effects of salt stress on maize seedlings (Fig. 7). The discussion delves into the involvement of SA in salt stress responses in maize seedlings and exploring the interconnections of the current findings and highlighting areas of agreement or disagreement with other relevant works in the field. This comprehensive analysis contributes to a deeper understanding of the complex interactions between SA and salt stress responses in maize seedlings and it's paving the way for potential applications of this growth regulator to other crop plants to enhance their salt tolerance capacity.

4.1. Growth biomarkers

The current study elucidates that salt stress negatively impacts seed germination, causing delays and decline in seed emergence. The inhibitory effects of salt stress on seed germination might be attributed to the reduction in osmotic potential around the seed due to increased Na^+ accumulation, leading to hindered water uptake and ultimately resulting in diminished germination process [3]. However, the exogenous application of SA at 0.1 mM effectively counteracts the inhibitory effects of salt stress and improved seed germination of maize. This finding aligns with studies in *Arabidopsis thaliana,* where SA was found to enhance seed germination

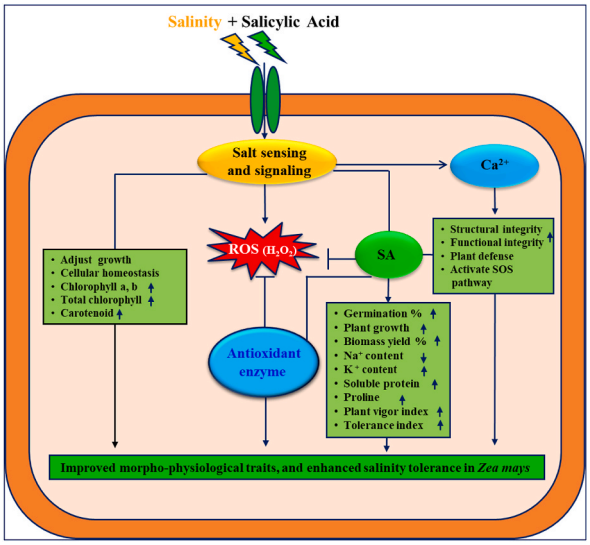

Fig. 7. Mechanistic overview of salinity stress tolerance mediated through salicylic acid and antioxidant system in maize. The blue up and down arrows indicate the increase and decrease level of a particular physiological trait. ROS (reactive oxygen species), SA (salicylic acid) and H_2O_2 (hydrogen peroxide).

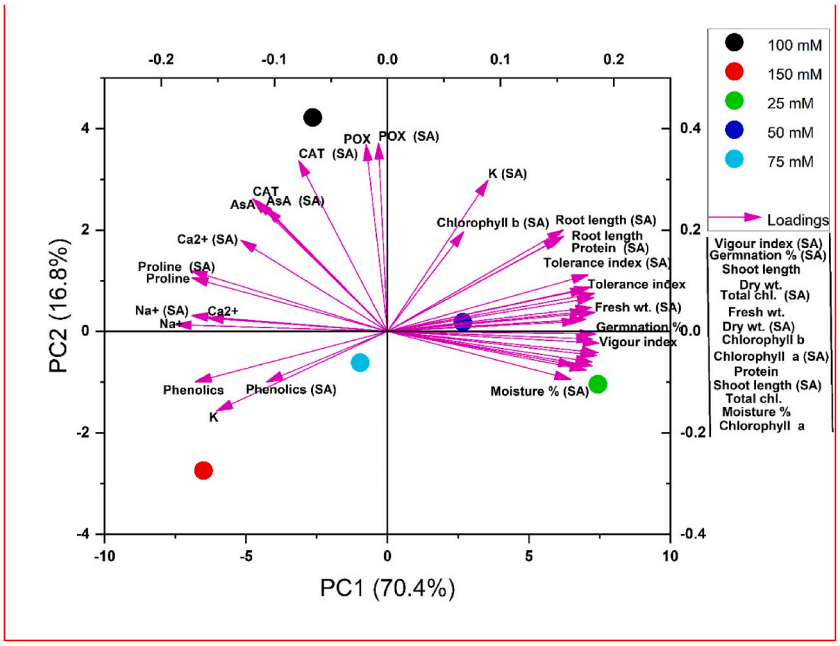

Fig. 8. PCA biplot of graded levels of salt stress (0, 25, 50, 75, 100 and 150 mM NaCl), salicylic acid (SA) treatment and growth and physico-chemical characters studied

percentage by mitigating salt induced oxidative damage [38]. Consistent with the current study, Anaya et al. [39] observed a significant improvement in seed germination and the alleviation of salt stress-induced damage in *Vicia faba* L. through the external application of low-concentration of SA. Additionally, Kaya et al. [9] demonstrated that foliar application of SA alleviated the effects of salinity stress and promoted plant growth. These findings underscore the positive impact of SA in mitigating the inhibitory effects of salt stress on seed germination leading to enhance overall plant performance.

4.2. Photosynthetic pigments, plant vigour and salt tolerance indices

In this study, salt stress has been widely reported to cause decline in photosynthetic pigment content in maize seedlings. The

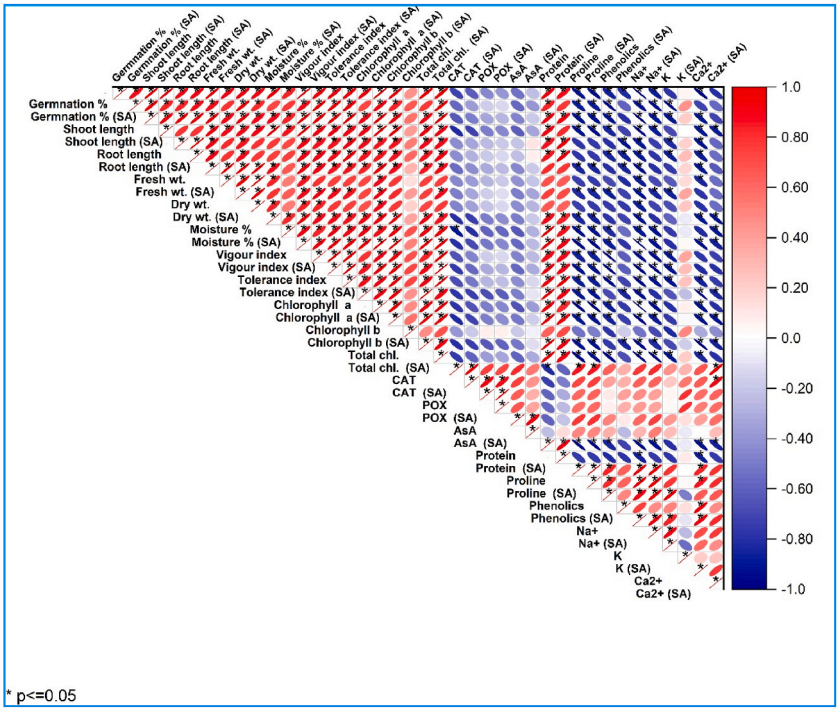

Fig. 9. Pearson correlation matrix between different pairs of studied characters.

reduction in photosynthetic pigments, including chlorophyll *a*, chlorophyll *b*, and carotenoids, is often associated with the oxidation of chlorophyll and other pigment-protein complexes under stressful conditions. The similar results were also highlighted by Barwal et al. [3]. The reduction in photosynthetic pigments is known to impair the photochemical reactions of photosynthesis, ultimately leading to decreased plant performance, as also indicated by Sayyad-Amin et al. [40]. Excessive ROS production induced by salt stress is often implicated in the decline of photosynthetic pigments and the disruption of normal plant physiological processes [41,42,43]. Similar declines in photosynthetic pigments have also been reported under various stressors, such as drought, heat, cold, heavy metals, and mineral deficiencies, leading to plant chlorosis [44–46]. In the current study, salt stress significantly decreased these photosynthetic pigments in maize seedlings, while the exogenous supplementation of SA restored these traits and improved plant performance under salt stress.

Carotenoids, crucial pigments for photosynthetic process, were also influenced by salt stress in maize seedlings. Notably, SA increased carotenoid concentration under salt stress, suggesting its active role in enhancing carotenoids in response to salt stress. Previous studies have also documented the involvement of SA in regulating chlorophyll levels in various plant species [47,48].

This study further observed that the reduction of plant vigour and tolerance indices is associated with severe salt stress in maize seedlings. In contrast, the addition of SA significantly reversed these impairments. These results are in consistent with finding of Lotfi et al. [49]. This finding suggest that SA is actively involved in countering the damaging impacts of NaCl, leading to an enhancement of the photosynthetic rate, as well as improvements in plant vigour and tolerance-related attributes in maize seedlings.

4.3. Antioxidant defense, ROS scavenging, Na^+ extrusion and osmolyte accumulation

Salt stress induce the generation of ROS in many plant species, as documented in the studies of Barwal et al. [3], Rahman et al. [11], and Khan et al. [5]. In response to salt stress, plants have evolved a homeostatic balance in ROS levels by using both non-enzymatic and enzymatic defense [13]. The present study observed that salt stress led to an increase in the levels of $O_2^{\bullet-}$ and H_2O_2, resulting in the accumulation of MDA content. However, exogenous supplementation of SA significantly enhanced the activity of key antioxidants, including SOD, CAT, and POX, in response to salt stress in maize seedlings. This finding indicates that SA is actively involved in enhancing the antioxidant defense system. The increase in the activity of SOD, CAT, and POX plays crucial roles in scavenging toxic ROS and making homeostatic balance against salt stress. SOD activity, involved in $O_2^{\bullet-}$ dismutation, is recognized as the first line of defense in multiple abiotic stress conditions in plants [3,50]. This study also highlights the importance of the ascorbate-glutathione (AsA-GSH) pathway, a fundamental mechanism in plant cells for detoxifying radicals. Ascorbate, serving as a specific electron donor, plays a crucial role in this pathway. Within the AsA-GSH cycle, enzymes such as APX and GR are key players in facilitating the transformation of H_2O_2 into H_2O. These enzymes are vital for regulating plant tolerance to various environmental stresses, including salinity stress [3,18,50]. The current findings align with other studies, where APX and GR activities have been reported to respond differently to salinity stress. The increase in APX and GR activity under salt stress has been documented in various plant species [18,3,

14]. These collective results underscore the crucial role of SA in enhancing the antioxidant defense system, thereby contributing to the plant's tolerance to salt-induced oxidative stress. The salt overly sensitive (SOS) pathway, as elucidated by Almeida et al. [51], stands as a pivotal component in the plant's response to salt stress and its tolerance mechanism. In this pathway, the plasma membrane located Na+/H+ antiporter SOS1 is responsible for regulating the extrusion of Na^+ from the cytoplasm and facilitating the long-distance transportation of Na^+ from roots to shoots [52,53]. This process is initiated through the phosphorylation of the plasma membrane Na^+/H^+ antiporter SOS1 by the serine/threonine protein kinase SOS2, triggering a responsive reaction [51]. The interaction of the SOS2–SOS3 kinase complex induces the calcium sensor protein SOS3, leading to the expulsion of Na^+ from cells and subsequently enhancing the synthesis of SOS1 and thereby regulating cellular ion homeostatic balance [54].

In the current study, escalating NaCl concentrations disrupted the ionic balance, resulting in elevated levels of Na^+, K^+, and Ca^{2+}. However, upon the application of SA, alterations were observed in the concentrations of Na^+, K^+, and Ca^{2+}, thereby affecting the Na^+/K^+ ratio. The enhancement of plant water relations, along with the maintenance of ionic homeostasis and concurrent reduction of oxidative stress in treated seedlings, suggests that SA effectively alleviates the phytotoxic consequences of NaCl. The supplementation of SA not only elevated the Na^+/K^+ ratio but also led to Na^+ extrusion by enhancing K^+ accumulation in maize seedlings. Consequently, the enhanced K^+ and Ca^{2+} concentrations led to a decline in Na^+ concentration in plants. However, the ameliorative effect of SA was more pronounced at lower concentrations of salt. This finding is consistent with observations by others, where the usage of SA led to decreased Na^+ levels and increased K^+ and Ca^{2+} concentrations in plants [55,56].

In this study, it has been observed that the salt stress triggered the accumulation of proline, and this was further increased by the application of SA. Proline, recognized as an osmoprotectant, plays a crucial role in maintaining cellular homeostasis under osmotic stress and preventing the overaccumulation of ROS, such as $OH^{\bullet}$ and $^1O_2$ radicals, thereby inhibiting lipid peroxidation, osmotic damage, and cellular injury [3,13]. Plants employ various other compatible solutes, including sugars, and glycine betaine, to counteract stress-induced oxidative damage by regulating osmotic balance and metabolic equilibrium [13,48,57]. The studies, on *Capsicum annuum*, have shown that proline concentration increases in response to NaCl stress, highlighting the significance of osmolytes in maintaining cellular water balance, protecting against osmotic damages, and mitigating stress in plants [13].

In the current experiment, the supplementation of SA was found to enhance proline synthesis. This improvement contributes to maintaining ionic balance and cellular homeostasis by scavenging ROS generating under salt stress conditions [56,48]. The study observed that exogenous supplementation of SA not only enhanced proline accumulation but also increased the levels of total soluble proteins and phenols. These outcomes collectively contribute to the regulation of osmotic balance and enhance the adaptation of maize seedlings under salt stress.

The present findings revealed the role of SA in orchestrating physiological responses that aid in plant resilience against the detrimental effects of salinity (Fig. 10).

5. Conclusions

In conclusion, this study elucidates the pivotal role of SA in enhancing salt stress tolerance in maize seedlings. Our findings demonstrate that SA treatment significantly mitigates the adverse effects of salt stress by modulating a range of physiological and biochemical parameters. Specifically, SA application leads to a marked improvement in growth parameters, which are typically compromised under saline conditions. This growth enhancement is accompanied by an increase in chlorophyll content, indicating better photosynthetic efficiency and overall plant vigour. At the biochemical level, SA enhances the activity of key antioxidant enzymes, including SOD, CAT, POX, APX, GR and AsA which collectively mitigate oxidative stress by scavenging ROS. The reduction in ROS accumulation is further supported by decreased MDA levels, indicating reduced lipid peroxidation and cellular membrane damage. These findings highlight the role of SA in bolstering the antioxidant defense system, thereby conferring greater resilience to salt-induced oxidative damage. Additionally, SA induced the accumulation of proline, soluble protein, and phenols, enhancing maize adaptability under salt stress. The decrease in AsA content, serving as an indicator of elevated APX activity, signifies an increase in APX activity. This phenomenon aids in the oxidation of AsA, facilitating the neutralization of H_2O_2 and its conversion into H_2O and O_2. Moreover, SA modulates the uptake and distribution of essential ions, such as Na^+ and K^+, maintaining ionic homeostasis under salt stress. The improved K^+, Na^+ and Ca^{2+} ratio observed in SA-treated seedlings underscores its role in preventing ionic toxicity and ensuring proper cellular function. Future research on SA-mediated salt stress tolerance in maize plants, as well as in other crop plants, needs a focus on elucidating the molecular signaling pathways and gene networks involved. Advanced genomic and proteomic techniques can be employed to identify key regulatory genes and proteins.

Data availability statement

The data used in this study cannot be made publicly as statistical data were used.

CRediT authorship contribution statement

Sandeep Kumar Barwal: Writing – original draft, Software, Methodology, Formal analysis, Data curation, Conceptualization. **Sajad Hussain Shah:** Writing – review & editing, Writing – original draft, Data curation. **Anita Pawar:** Writing – review & editing. **Manzer H. Siddiqui:** Writing – review & editing, Visualization, Funding acquisition. **Rajneesh Kumar Agnihotri:** Writing – review & editing, Investigation. **Vimala Yerramilli:** Writing – original draft, Visualization, Validation, Supervision, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Shabir Hussain Wani:** Writing – review & editing, Investigation, Formal

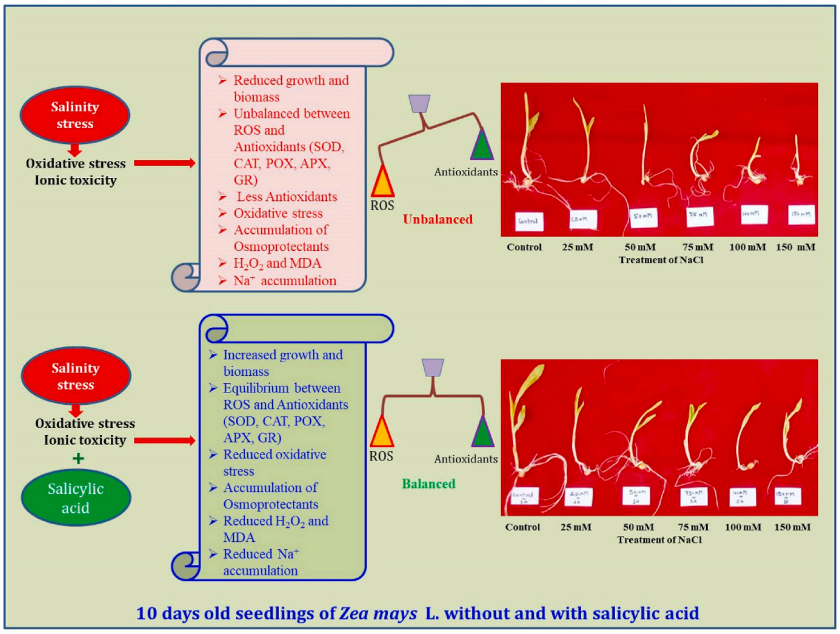

Fig. 10. A summarized representation of effect of salt stress and the mitigating role of salicylic acid

analysis.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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