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Mitigating the Effect of Salt Toxicity on Growth, Physiological and Biochemical Processes of Wheat by the Application of Ascorbic Acid

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ABSTRACT

Salt stress is an important factor which reduces plant growth and yield, it reduces nutrient uptake and water balance which adversely effects on plants health. Higher salt concentrations in growth medium hinder nutrient uptake and can lead to dehydration which results in destruction of plant cells ultimately leading to reduced plant growth and yield. Salt toxicity is a major issue to agriculture land globally. After soil erosion it is second most prominent cause of soil degradation. In winter season 2023 a pot experiment according to complete randomized design (CRD) was conducted to examine effects of exogenous application of antioxidant ascorbic acid (ASA) on growth, biochemical and physiological parameters of wheat under salt stress. Two treatments of ASA 0.2 and 0.8 mM were applied to wheat plants grown under normal and stress conditions. Analysis of data revealed that salt stress significantly reduced all growth parameters like root length, shoot length and their fresh and dry weight. When ASA was applied, it significantly reversed the effects of salinity on growth parameters. Application of ASA also significantly improved biochemical and physiological parameters such as carbonic anhydrase (CA) activity, photosynthetic pigments Chl a, Chl b and total chlorophyll. It also increased accumulation of compatible solutes like proline to alleviate toxic effects of salinity. Exogenous application of ASA also reduced the generation and accumulation of reactive oxygen species (ROS) and harmful free radicals such as H_2O_2 and malondialdehyde (MDA). Accumulation of MDA and H_2O_2 can cause deterioration of membrane. So from this study it can be concluded that exogenous application of ASA can enhance wheat plants tolerance to salt toxicity by increasing photosynthetic rate, accumulation of compatible solutes and by reducing the generation and accumulation of ROS.

Keywords: Salt toxicity, wheat, ascorbic acid, morphophysiological, biochemical

Introduction

Salt toxicity is a major issue to agriculture land globally. After soil erosion it is second most prominent cause of soil degradation (Irshad *et al.,* 2023; Ma *et al.,* 2023). 7% of earth area or almost one billion hectares of land is affected by salt toxicity. This causes approximately 2000 hectares loss of agriculture land daily, which ultimately leads to lower productivity of agriculture. Salinity causes 10-25% reduction in crop productivity while in severe cases it can lead to desertification (Ashraf & Chen, 2023). Soil salinity reduces plant growth by causing ionic toxicity, decrease in photosynthetic rate and nutrient deficiencies (Huang *et al.,* 2024). It also produces ROS (Reactive Oxygen Species) which destroy membranes (Kaya *et al.,* 2023; Zahid *et al.,* 2024). Various crops and their genotypes show different levels of tolerance to salt stress (Vantol *et al.,* 2016). Most of the crops can't tolerate stress conditions and are unable to complete life cycles in these conditions (Parida & Das, 2005). Plants have

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developed various complex mechanisms to mitigate this toxicity, one of these mechanisms is osmotic adjustment, in which plants accumulate inorganic ions and solutes such as glycine betaine, proline and some soluble carbohydrates. These accumulated solutes help plants to regulate their enzymes. However in different plant species, inorganic and organic solutes perform different role in osmotic adjustments (Siddiqui *et al.,* 2009). By regulating gene expression, osmoprotectants can help plants to control salt stress (Parida & Das, 2005). So by modulating biochemical and physiological processes, salt tolerance of plants may be enhanced. Some crops which have limited defense mechanisms like wheat are more vulnerable to salt stress (Saeed *et al.,* 2023; Younis *et al.,* 2024).

The most early domesticated cereal crop wheat (*Triticum aestivum* L.) is beneficent for the subsistence of 2.5 billion peoples globally. About 80 million farmers are cultivating wheat (Tayade *et al.,* 2023). About 82% of proteins and 85% calories for the world population are obtained from wheat, which makes it the most valuable cereal crop. Additionally, it is also an important source of minerals for bakery products. Wheat is susceptible to salinity, just like all other cereal crops (Ghane *et al.,* 2011). Salinity effects on almost all biochemical and physiological processes of wheat, which makes salinity a prominent threat for wheat cultivation (El-Sabagh *et al.,* 2021). Accumulation of chloride and sodium ions in soil disturbs plants osmotic balance and leads to dehydration by decreasing water intake (Hasanuzzaman *et al.,* 2013). This toxicity leads to a number of responses in plants like blockage of metabolic paths, varied ion intake and accumulation of reactive oxygen species (ROS) which cause oxidative stress (Hossain *et al.,* 2021).

To mitigate abiotic stresses ascorbic acid (ASA) is considered as the most prominent growth regulator (Conklin, 2001). ASA helps plants to activate various biological defense mechanisms to mitigate abiotic stresses (Conklin & Barth, 2004). ASA also provides electron donors to plants which causes reduction in production of ROS. In addition to ASA also emerged antioxidant properties, which help plants in stress signalling, biochemical, physiological and other growth processes. ASA also helps plants to regulate cell differentiation, cell division and other cellular processes (Venkatesh & Park, 2014). ASA also works as a cofactor for many enzymes and phytohormones. Therefore, this experiment examined the regulation of biochemical and physiological processes by the application of ASA to mitigate salinity in wheat plants. Additionally, exogeneously applied ASA also helps plants to overcome water deficit conditions by improving root growth for nutrient uptake, it also decreases oxidative damages caused by various abiotic stresses (Xu *et al.,* 2015).

As salt toxicity is increasing, so it is important to examine plants growth under salinity. It is also important to understand how biochemical and physiological processes are regulated by ascorbic acid (ASA) to alleviate oxidative damages.

This experiment aims to analyze the potential of ascorbic acid (ASA) and to covers knowledge gap about the ASA optimal application. It is hypothesized that exogenously applied ASA will mitigate the negative effects of salinity and will enhance wheat growth and production.

2. Materials and Methods

2.1 Seeds and Growth Conditions

In chamber growth conditions were set as 25 °C temperature, 60% humidity and 16 hours light and 8 hours dark cycle. Plastic pots and seeds of wheat (Millat variety) were purchased from market. Pots were arranged according to complete randomized design (CRD) and were filled with sand. Then seeds were sterilized with NaClO disinfectant. First, for 24 hours seeds were pregerminated in tap water. Then from these seeds, six seeds were cultivated in each pot. After ten days of sowing or when seedlings grew up to 3 leaves stage, NaCl was applied gradually from 0 mM to 90 mM. Treatments of this experiment are as follows:

1. Controlled
2. 0.2 mM ASA
3. 0.8 mM ASA
4. 90 mM NaCl
5. 0.2 mM ASA + 90 mM NaCl
6. 0.8 mM ASA + 90 mM NaCl.

Three replications of NaCl and ASA were applied on their respective pots during 35 days of this

experiment. After completing all treatments, plants were harvested to measure their growth, biochemical and physiological parameters.

2.2. Growth Parameters

Performance of wheat plants under salt stress by the application of ASA were analyzed by measuring growth parameters such as root length, its fresh and dry weight, shoot length and also its fresh and dry weight. Root length and shoot length were measured by the help of measuring tape. Root fresh weight and shoot fresh weight were measured by weight balance then root and shoot were dried for 48 hours in oven at 70 °C to measure their dry weight.

2.3. Physiological and Biochemical Attributes

From fresh leaves chlorophylls were extracted by using pestle, prechilled mortar and dimethyl sulfoxide. UV spectrophotometer was used to measure pigments absorbance. While Barnes method (Barnes *et al.,* 1992) was used to determine pigment content.

To determine carbonic anhydrase (CA) activity as CO_2 kg^{-1}, fresh leaves were chopped and were placed for 20 minutes in 0.2 M cystine hydrochloride solution at 4 °C in petri dish. Then these leaves were transferred to 0.2 mL of bromothymol blue and 4 mL of 0.2 M sodium bicarbonate solution in test tubes. Then titration process was done with HCL and indicator in this process was methyl red. By this process CA activity was determined as CO_2 kg^{-1} leaf FW s^{-1} (Dwivedi & Randhawa, 1974).

Proline content in leaves was determined by Bates ninhydrin method (Bates *et al.,* 1973). To remove debris from samples, leaves were washed with 3% aqueous sulfosalicylic acid, then these samples were centrifuged at 10.000 × g. For the estimation of proline content supernatant was used. Then supernatant was mixed with glacial acetic acid and acid ninhydrin, after that this mixture was boiled for one hour at 100 °C. Then this mixture was cooled in ice bath and after cooling toluene was added to separate reaction mixture and absorbance was noted at 520 nm.

Heath and Packer method (Heath & Packer, 1968) was used for measuring malondialdehyde (MDA) content to determine lipid peroxidation in leaf samples. While Velikova method (Velikova *et al.,* 2000) was used for measuring H_2O_2. Leaves samples were grounded in 0.1% TCA and grounded samples were centrifuged for 15 minutes at 12.000 rpm. Then these samples were added in mixture containing 1 M potassium iodide and 10 mM potassium phosphate buffer. Supernatant was also added in this mixture and at 390 nm absorbance was noted.

2.4. Statistical Analysis

This experiment was conducted according to complete randomized design (CRD). Three replications of each treatment were applied on their respective pots and each po represents one replicate. ANOVA of recorded data was done using Statistix 8.1 software. To check the significant differences among mean values of each treatment Tuckey's HSD test was used. While R-studio was utilized to measure correlation between all traits and heatmap dendrograms for traits and treatments.

3. Results and Discussion

Analysis of recorded data revealed that salinity caused significant reduction in all growth, biochemical and physiological parameters. However, when ASA was applied exogenously, it reduced oxidative stress and causes significant increase in all these parameters.

3.1. Growth Attributes

In this experiment growth parameters like root length, its fresh and dry weight, shoot length and also its fresh and dry weight were measured to examine the effects of ASA on growth parameters under salt stress. These growth parameters were significantly decreased under salt stress (Tables 1 and 2).

This reduction in growth parameters may be result of disturbances in metabolic processes such as, photosynthesis and nutrients assimilation (Siddiqui *et al.,* 2009). However, exogenous application of ASA helps plants to restore its growth. Under non stress conditions exogenously applied ASA also caused significant increase in growth parameters (Tables 1 and 2). This increase in growth parameters suggests that ASA perform prominent role in regulation of different biochemical and phsiological processes. ASA also helps plant cell division and elongation by providing electrons (Kaviani, 2014). In this study these direct and indirect role of ASA on growth attributes were clearly observed. Application of

ASA showed significant effects on root length (RL) and shoot length (SL) which were supressed under salinity. This increase in RL and SL helps plants in better orientation to capture more solar energy which ultimately lead to higher biomass yield. Application of ASA 0.8 mM provided the most significant results for all growth parameters. So it can be assumed that application of ASA in growth medium helps plants to restore its growth under salinity.

Table 1: Mean values ± SE of shoot length, shoot fresh weight and shoot dry weight

Treatments	Shoot length (cm)	Shoot fresh weight (g)	Shoot dry weight (g)
Control	21.06 ± 0.351	271.82 ± 2.325	26.01 ± 0.337
ASA 0.2	25.82 ± 0.619	380.26 ± 3.129	35.42 ± 0.413
ASA 0.8	32.95 ± 1.043	517.27 ± 2.532	50.26 ± 0.982
NaCl	13.82 ± 0.336	168.14 ± 0.681	18.69 ± 0.228
NaCl + ASA 0.2	18.02 ± 1.043	363.74 ± 1.249	30.45 ± 0.547
NaCl + ASA 0.8	20.29 ± 0.336	418.41 ± 3.689	38.56 ± 0.476

Table 2: Mean values ± SE of root length, root fresh weight and root dry weight

Treatments	Root length (cm)	Root fresh weight (g)	Root dry weight (g)
Control	10.52 ± 0.178	124.2 ± 0.654	8.54 ± 0.243
ASA 0.2	11.74 ± 0.334	138.73 ± 0.584	10.63 ± 0.251
ASA 0.8	17.96 ± 0.419	209.23 ± 0.851	12.273 ± 0.301
NaCl	6.687 ± 0.092	80.56 ± 0.092	7.1533 ± 0.092
NaCl + ASA 0.2	9.87 ± 0.202	147.85 ± 1.157	9.1667 ± 0.179
NaCl + ASA 0.8	12.26 ± 0.367	151.09 ± 0.862	10.603 ± 0.306

3.2 Physiological and Biochemical Attributes

Plants growth is regulated by the process of photosynthesis and photosynthetic pigments are the most important components for the regulation of photosynthesis. Figure 1 shows that when salinity was applied to wheat plants it caused significant reduction in Chl a, Chl b and total chlorophylls. This reduction shows that chloroplasts are filled with Na+ ions under salt stress which leads to chlorophyll degradation (Siddiqui *et al.,* 2018). This accumulation of Na+ ions also cause changes in structure of chloroplasts. In addition to salt stress also generates chlorophyllase enzymes which cause degradation of chlorophylls. When ASA was applied exogenously, its both levels 0.2 and 0.8 mM caused significant increase in chl a, chl b and total chlorophylls under stress conditions as shown in Figure-1. Additionally, ASA also caused increase in chlorophylls under non stress conditions. In this experiment we observed that exogenous application of 0.8 mM ASA caused more increase in chlorophylls production as compared to 0.2 mM ASA. However, both levels of ASA caused significant increase and inhibited chlorophyll degradation. Gul *et al.* (2004) reported that ASA protects the Photosynthetic apparatus from oxidative damages caused by salinity.

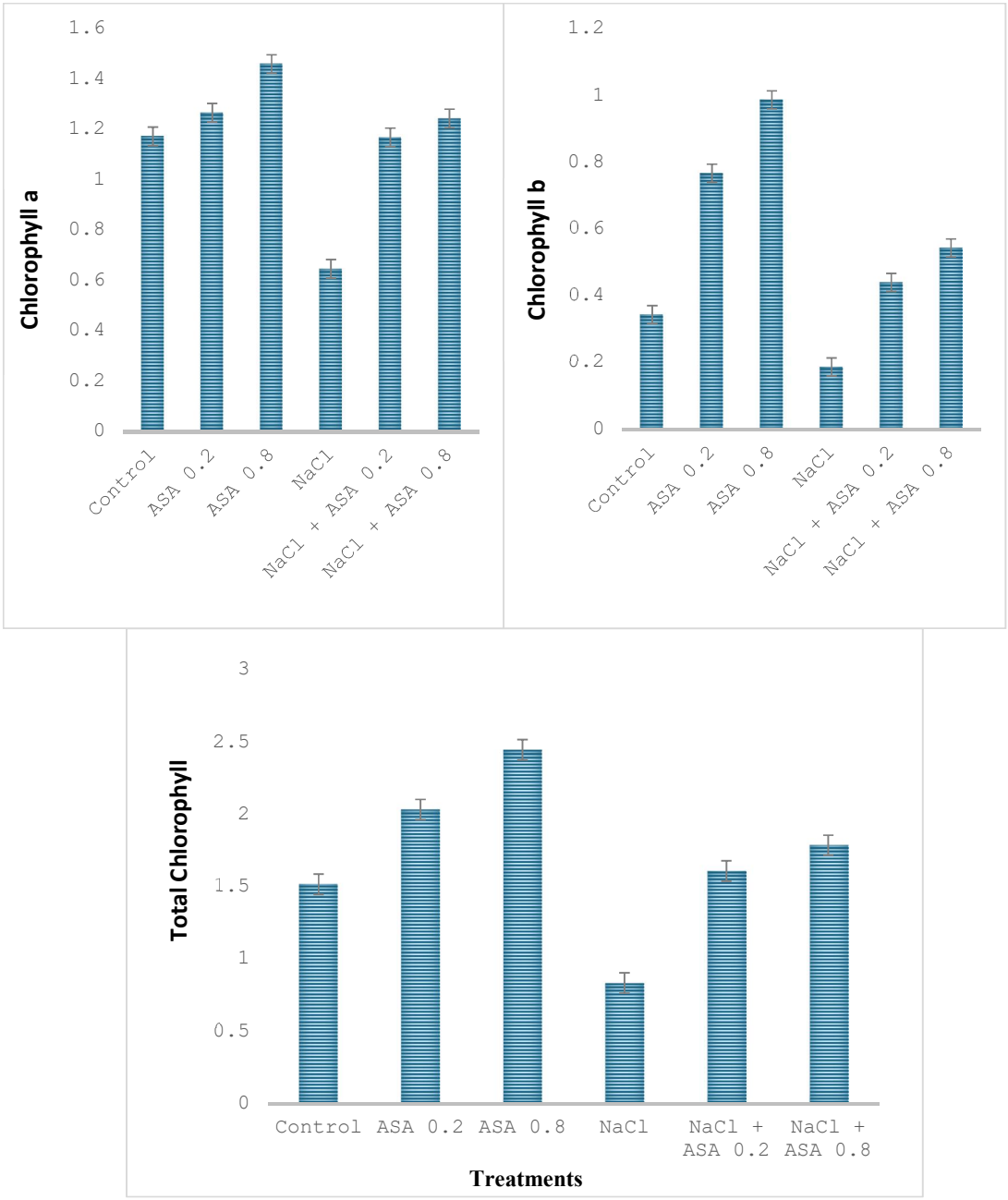

Fig. 1: Graphical Representation of Mean Values of Chl a, Chl b and Total Chlorophyll

CA activity plays a key role in reversible reactions of CO_2 to bicarbonate and bicarbonate to CO_2. So, CA enzyme continuously provide CO_2 to Rubisco. In this experiment activity of CA was monitored and results showed that ASA significantly increased the CA activity under both normal and salt stress conditions as shown in Figure 2. While without application of ASA, CA activity was significantly hindered under salt stress. Like all growth parameters and chlorophylls 0.8 mM provided highest results for CA activity. This enhanced CA activity provided more CO_2 for fixation and enhanced plants growth (Soussi *et al.,* 1998). So, from CA activity perspective it can be assumed that ASA mediate salinity in wheat plants by regulating reversible conversion of CO_2 and by providing more CO_2 for fixation.

When wheat plants are exposed to harsh salinity levels beyond their tolerance, wheat plants synthesize compatible solutes such as proline. These solutes minimize the ROS oxidative damages and stabilize enzymes and proteins. Thus, they maintain the integrity of cell membrane (Pourcel *et al.,*

2007). Figure-2 shows that under stress conditions wheat plants accumulated more proline as compared to controlled or ASA applied plants under normal conditions. While under stress conditions application of ASA significantly enhanced proline accumulation. For the purpose of scavenging ROS, proline plays very important role. As exogenously applied ASA increased proline accumulation, indirectly it increases salt stress tolerance of wheat plants (Zivcak *et al.,* 2016).

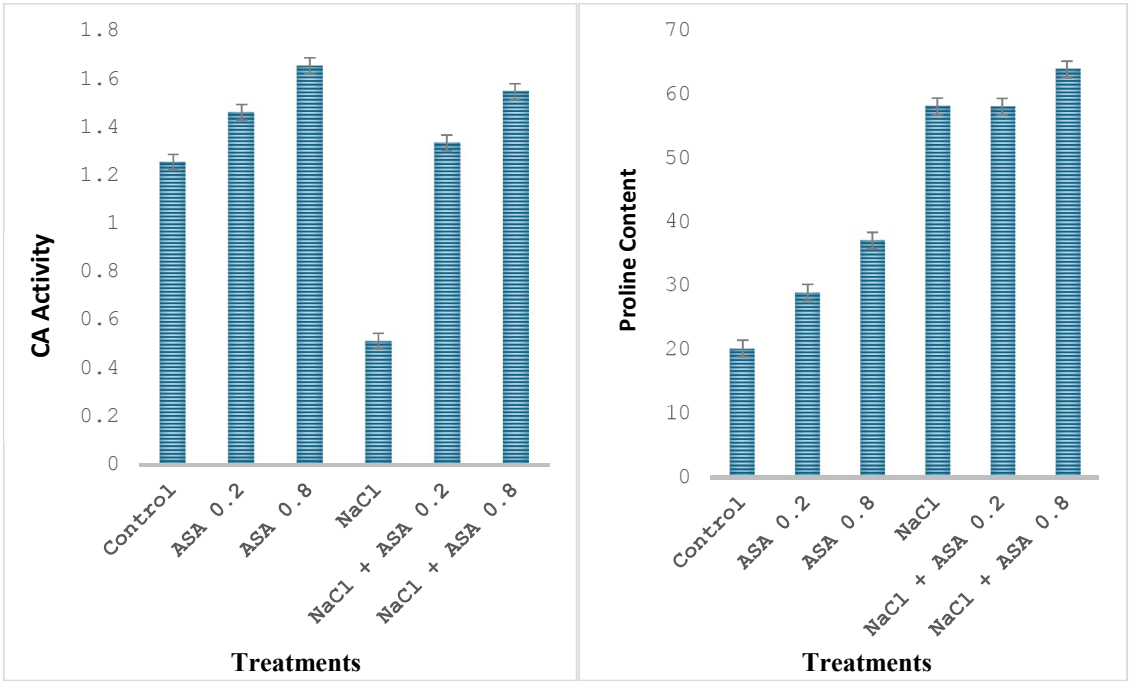

Fig. 2: Graphical Representation of Mean Values of CA Activity and Proline Content

Wheat plants which experience salt stress usually generate higher levels of H_2O_2 and MDA as compared to controlled plants or ASA applied plants under normal conditions (Figure-3). So, plants start various defense processes to limit these radicals. When ASA was applied under normal conditions MDA and H_2O_2 content were significantly reduced. While under saline conditions, both tested levels of ASA significantly mediate harmful impacts of salinity by reducing MDA and H_2O_2 accumulation. Under salt stress ROS species can be detoxified by compatible solutes accumulation or by increasing the activity of photosynthetic enzymes.

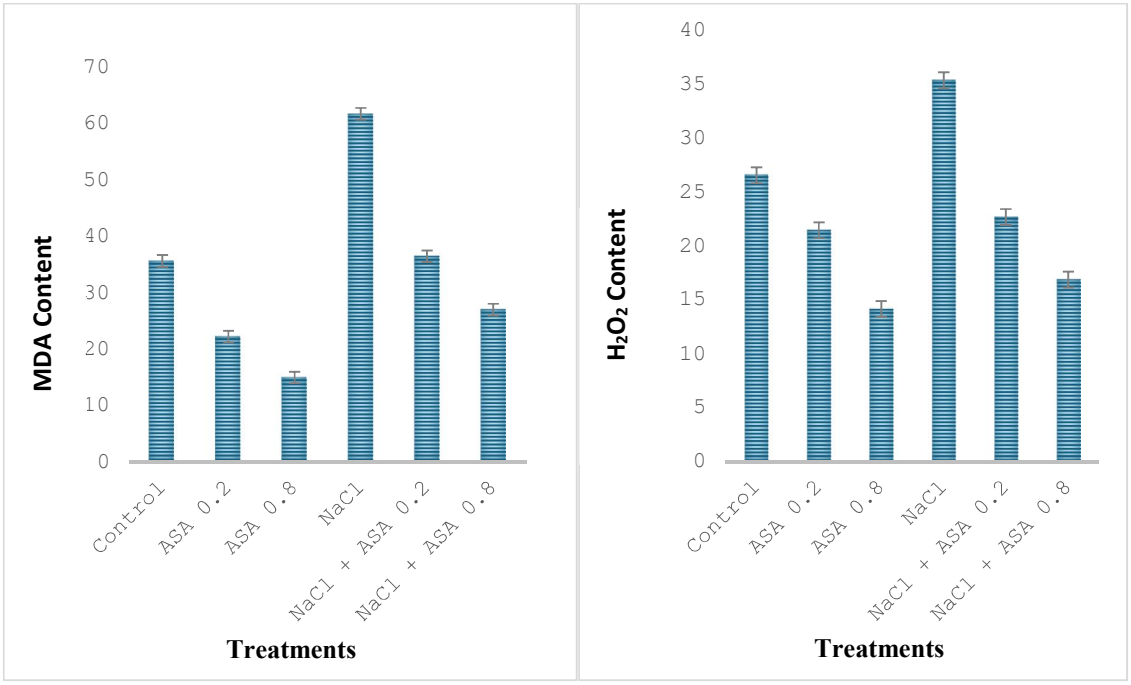

Fig. 3: Graphical Representation of Mean Values of MDA Content and H_2O_2 Content

3.3. Correlation Analysis

Strong positive and negative correlations were found among various growth, biochemical and physiological parameters as shown in Figure-4. Correlation matrix showed that growth parameters like root length, RFW, RDW, shoot length, SFW, SDW, Chl a, Chl b, total chlorophylls and CA activity were positively correlated with each other's. While all these parameters were negatively correlated with MDA and H_2O_2 content.

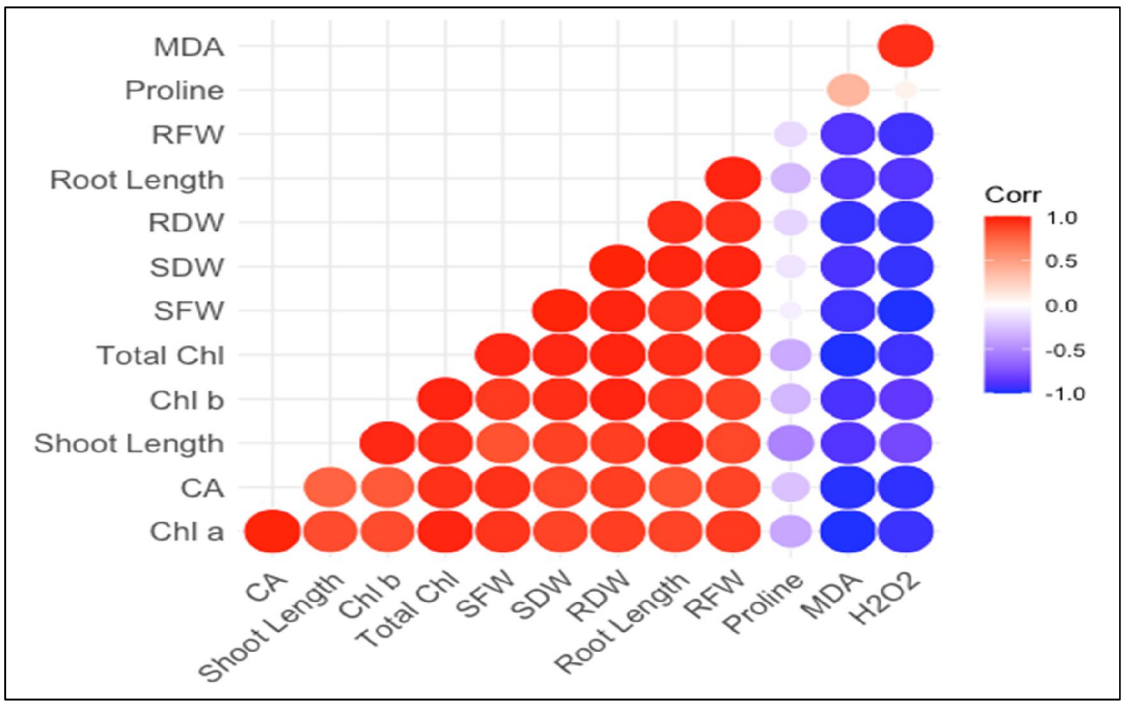

Fig. 4: Correlation matrix between growth, biochemical and physiological parameters of wheat.

3.4. Heatmap Analysis

To examine the effect of ASA on wheat under salinity, a two way heatmap with dendogram were drawn (Figure-5). On the basis of various treatments, the observations were divided into similar groups and relationship among observations were represented in color blocks. The strong redish maroon color represented highly negative and deep blue color represented highly positive correlation among various parameters influenced by ASA under salt stress (Figure-5). Four clusters were drawn in heatmap. In first cluster malondialdehyde and H_2O_2 were clustered. These parameters are strongly correlated with NaCl treatment without ASA and weakly correlated with ASA 0.8 mM treatment. The second cluster includes proline. While third cluster includes root length, root fresh weight, root dry weight, shoot length, shoot fresh weight, shoot dry weight, Chl a and total chlorophylls. These parameters are weakly correlated with NaCl treatment without ASA and strongly correlated with ASA 0.8 mM treatment. These results suggest that ASA 0.8 mM caused significant increase in growth parameters to mitigate salinity.

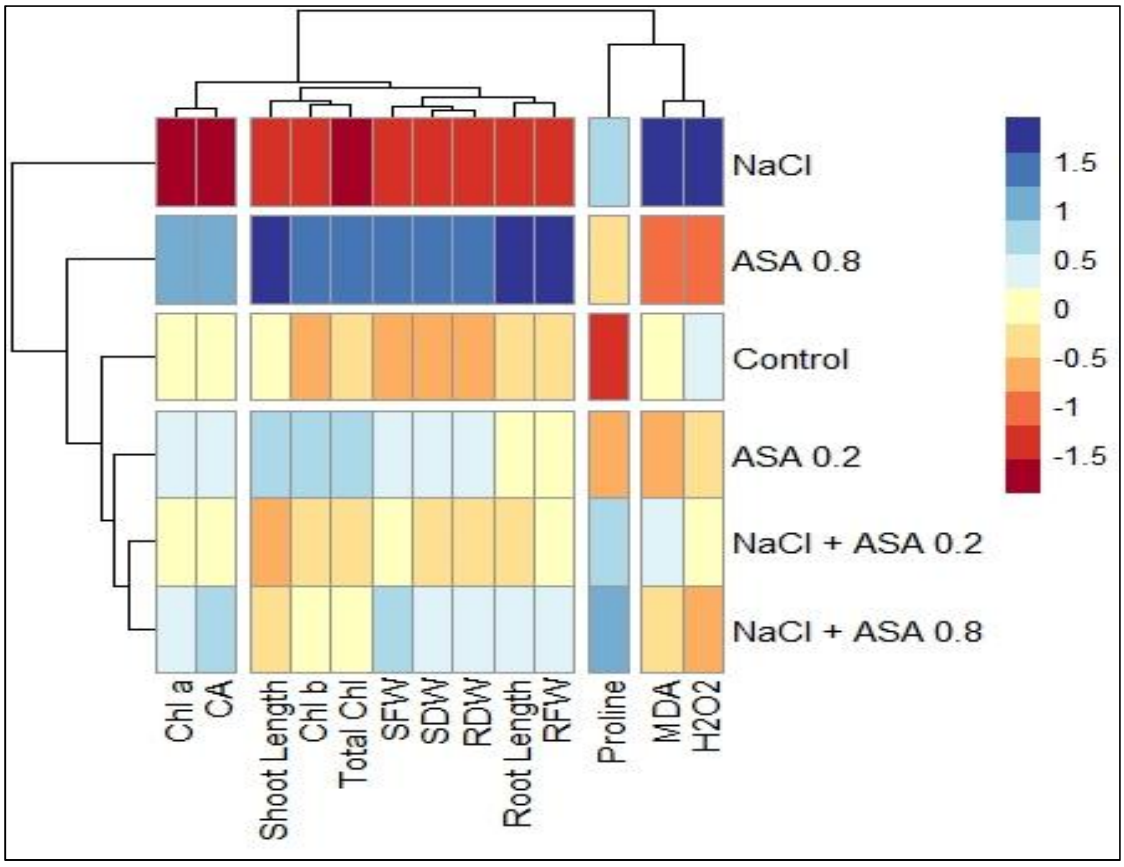

Fig. 5: Heatmap with dendograms between growth, biochemical and physiological parameters of wheat

4. Conclusions

From this experiment it can be concluded that exogenously applied ASA cause significant increase in root, shoot length and their fresh weight by enhancing the activity of CA enzymes which lead to more accumulation of Chl a, Chl b and total chlorophylls under normal conditions. But under saline conditions, application of ASA helped plants to accumulate more compatible solutes, which resulted in enhanced plant growth and tolerance. Application of ASA also helps plants to reduce the accumulation of MDA and H_2O_2. Accumulation of MDA and H2O2 can cause deterioration of membrane. So this study advocates that application of ASA to growth medium can become an effective technique to mitigate salinity.

Abbreviations:
ASA: Ascorbic Acid
CRD: Complete Randomized Design
ROS: Reactive Oxygen Species
CA: Carbonic Anhydrase
MDA: Malondialdehyde
RL: Root Length
RFW: Root Fresh Weight
RDW: Root Dry Weight
SL: Shoot Length
SFW: Shoot Fresh Weight
SDW: Shoot Dry Weight

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