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Ameliorative effect of ascorbic acid and gibberellic acid on biochemical parameters of wheat (*Triticum aestivum* L.) under salinity stress

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Abstract
The effects of hormonal priming with ascorbic acid and gibberellic acid on wheat (*Triticum aestivum* L.) metabolism during germination phase under saline conditions were studied to determine their effectiveness in increasing relative salt-tolerance. Seeds of wheat var. GW- 496 were pre-soaked in three levels each of ascorbic acid (AsA) *viz*., (50, 100 and 150 mg L^{l}) and gibberellic acid (GA_3) *viz*., (150, 200 and 250 mg L^{-1}) for 2 hrs. Salinity stress was given by germinating pretreated seeds in NaCl @ 50, 75 and 100 mM in Petri plates. The experiment was carried out in four replications. The contents of three osmolytes *viz.,* proline, total soluble sugar and free amino acid were measured. The activity of three antioxidative enzymes *viz.,* polyphenol oxidase, peroxidase and catalase were also estimated. Application of AsA @ 100 mg L^{-1} increased proline and free amino acid content and activity of polyphenol oxidase and peroxidase enzymes. However, total soluble sugar content and catalase enzyme activity were enhanced by 150 mg L^{-1} AsA. GA_3 @ 150 mg L^{-1} enhanced concentration all osmolytes *viz.,* proline and total soluble sugar and free amino acid content, whereas 250 mg L^{-1} GA_3 effectively increased peroxidase and catalase enzyme activity. It could be concluded that, pretreatment of wheat cultivar with AsA and GA_3 could partially alleviate the harmful effect of salinity by increasing antioxidative enzymes activity and accumulation of osmolytes

Keywords: Ascorbic acid, gibberellic acid, biochemical parameters, proline, free amino acid, total soluble sugar, peroxidase and catalase

Introduction
Wheat is the second important cereal crop (after rice) in India. With changing diets and growing world populations, rising prices for fertilizers and pesticides, increasing competition between food and non-food uses, and the negative effects of increased soil salinization resulting from climate change, world wheat production has not met demand in 6 of the past 10 years and yet by 2050 demand will increase by at least 70%. Wheat is consumed by nearly 35% of the world population and providing 20% of the total food calories. India accounts for an area of 31.18 million ha and production of 95.9 million tones with a productivity of 3075 kg ha^{-1}. It occupies about 32% of the total acreage under cereals in the world.

The most common plant response to salt stress is a general reduction in growth and yield. As salt concentration increases above a threshold level, both the growth rate and ultimate size of crop plants progressively decrease. Growth suppression seems to be a nonspecific salt effect that is directly related to the total concentration of soluble salts or osmotic potential of the soil water. Seed germination, one of the most critical phases in plant life, is greatly affected by salinity Abo-Kassem (2007) [1], which either induces a state of dormancy at low levels or completely inhibits germination at higher levels, Iqbal *et al* (2006) [18]. Different mechanisms contribute to salt tolerance of plants through compartment of ions in vacuoles, accumulation of osmotic solutes in the cytoplasm and genetic salt resistance. Osmosolutes, such as proline, free amino acids and carbohydrate compounds, could play important roles in plant salt tolerance by osmotic adjustment (Azooz, 2013) [6].

Presoaking of seed with optimal concentration of phytohormones and antioxidative enzymes has been shown to be beneficial for germination and seedling growth of wheat crop under saline conditions as reported by Mohsen *et al* (2014) [27]. Ascorbic acid, an abundant, relatively small molecule in plants, plays multiple roles in plant growth, functioning in cell division, cell wall expansion, and other developmental processes. In addition, ascorbic acid is a key substance in the network of plant antioxidants, including glutathione and enzymatic

antioxidants that detoxify H_2O_2 to counteract oxygen radicals produced by the Mehler reaction and photorespiration (Noctor and Foyer, 1998) [28]. Ascorbic acid is very sensitive to reversible oxidation (ascorbic acid ↔ dehydroascorbic acid) suggests that it may also be involved in cellular oxidation-reduction reactions, perhaps serving as a hydrogen-transport agent. Attempts have been made to employ active vitamins to overcome the drastic effects of salinity on seed germination and seedling growth as well as on some metabolic mechanisms (Khan and Zaidi, 2006; Ansari and Khan, 1986) [22, 5]. The application of ascorbic through seed soaking enhanced crop growth by increased germination percentage, root and shoot fresh and dry weights, chlorophyll content and higher accumulation osmolytes (proline, free amino acid and total soluble sugar). Experimental studies on different crop have shown that pretreatment with AsA reduced salt induced adverse effects and resulted in a significant increment of growth and yield.

GA_3 is used to revive the plants suffering from salt stress as it overcomes the adverse effects of salt stress on germination Amal and Heba (2014). Hence, the present investigation was carried out in order to investigate the extent of effectiveness of these two growth stimulators in ameliorating the adverse effect of salinity stress. Gibberellic acid (GA_3) is known to induce an increase in phytase and acid phosphatase activities in rye and barley during germination process (Centeno *et al*., 2001) [9, 10]. Kaur *et al*. (1998) [20] stated that the seeds whose germination was inhibited by high levels of salts may be grown in a medium supplied with gibberellic acid under salinity conditions. AsA and GA_3 are reported to enhance the germination and seedling growth of wheat under saline stress.

Materials and Methods

Experimental Materials

Seed of wheat cultivar GW-496, ascorbic acid (AsA), gibberellic acid (GA_3) and sodium chloride (NaCl)

Treatment

A laboratory experiment was carried out with four replications, 100 (25 seeds in each replica) seeds were soaked in distilled water (control), three concentrations of ascorbic acid (AsA) viz., (50, 100 and 150 mg L^{-1}) and three concentrations of gibberellic acid (GA_3) viz., (150, 200 and 250 mg L^{-1}) solutions for 2 hrs and then the same were treated with 2.5 g/l thiram for about 2 minutes. For germination, 25 seeds from each sample were spread in Petri dishes over Whatman No.1 filter paper. The sufficient volume (10 ml from 1st to 5th day and 20 ml from 5th to 11th) of NaCl concentrations (50 mM, 75 mM and 100 mM) were added to induce salinity stress, whereas distilled water was provided as control.

Biochemical Analysis

The final count for all biochemical parameters were taken at 11^{th} day after germination of seedlings.

Osmolyte estimation

Proline content: The proline content in the seed samples was analyzed by the method suggested by Bates *et al*., (1973) [8]. The seeds (0.5 gm) of wheat cultivar were homogenized in a mortar and pestle with 5 ml of 3% sulfosalicylic acid. The contents were centrifuged at 5000 g for 10–15 minutes supernatant was separated and the volume of clear supernatant adjusted to 5 ml with distilled water 1 ml extracted sample adds 2 ml of 3% sulfosalisilic acid and 1 ml of acid ninhydrin was added to it and mixed well. The tubes were placed in boiling water bath for one hour and then cooled. The mixture was then extracted with the addition of 5 ml toluene and allowed to stand for 2-3 minutes. The absorbance of toluene layer was recorded at 520 nm. Blank was also run simultaneously with 5 ml of 1% sulfosalicylic acid without sample. The proline content was calculated from proline standard (10 mg/100 ml in 3% sulfosalicylic acid).

Free amino acid: the estimation of free amino acid determined was done by the method suggested by Lee and Takahashi (1966) [23]. 0.5 g groundnut sample (leaf tissue) was homogenized in mortar and pestle with 10 ml of 80 % rectified spirit for extraction of amino acids. The extract was transferred in 15 ml glass centrifuge tube. The tube was centrifuged for 10 minutes at 2000 rpm. The supernatant was collected in a 25 ml volumetric flask. This was repeated 2 – 3 times and combined supernatant was made 25 ml with 80 % rectified spirit. The extract was used for estimation of free amino acids. Ninhydrin reagent was prepared by mixing following solutions A, B & C in the ratio of 5:12:2.

1% ninhydrin reagent in 0.5 M citrate buffer, pH 5.5, glycerol 0.5 M citrate buffer. Suitable aliquot was taken and made to 1.0 ml with the distilled water. To this, five ml of ninhydrin reagent was added. The content was thoroughly mixed. Tubes were kept in boiling water bath for 10 minutes to develop color. At the end of incubation period, the tubes were removed from water bath. The tubes were cooled and volume was made to 10 ml with distilled water, and observation was taken at 570 nm in spectrophotometer. The reference curve was prepared using glycine (10 mg/100 ml distilled water) as standard amino acid.

Extraction and estimation of total soluble sugars: the sample (seed) 0.5 g was homogenized in 80 % rectified spirit using mortar and pestle and volume was made to 10 ml. The content was refluxed for one hour on boiling water bath at 70˚C. Supernatant was collected and residue was re-extracted twice with 80 % rectified spirit. All supernatant were combined and final volume was made to 25 ml. The extract was used for the assay of total soluble sugars and reducing sugars. Total soluble sugars were estimated by following the method suggested by Dubois *et al*. (1956) [12] with some modifications. Suitable aliquot (0.5 ml) was taken and volume made to 3 ml with the distilled water; followed by 0.5 ml distilled phenol and mixed thoroughly. To this, 5.0 ml concentrated sulphuric acid was carefully added at the side of the tube. After the mixing thoroughly the tubes were kept for 30 minutes at room temperature for color development. The absorbance was measured at 490 nm. The content was calculated with the help of a reference curve prepared from D-glucose as standard and expressed as mg g^{-1}

Antioxidant estimation

Peroxidase (EC 1.11.1.7): Peroxidase activity was determined by the method of Gulibault *et al*., (1976), in which the homogenates by measuring the increase in absorption at 460 nm due to the formation brown color dye Madhaiyan *et al*. (2006). Reaction mixture was prepared as 2.95 ml of 0.01 M phosphate buffer (pH 7.0) containing 3 % H2O2 and 0.1 % orthodianisidine dissolved in methanol. Reaction was initiated

by adding 50 µl enzyme extract. Read the oxidized dye (brown colour) at 460 nm in spectrophotometer every 15 sec interval up to 3 min. Blank was carried out without substrate (H2O2). The specific activity was expressed as U.mg-1protein and Unit activity was defined as Δ OD min -1g -1 fresh weight tissues.

Catalase (EC 1.11.1.6): Catalase activity was determined by Malik and Singh, (1980) [24] Reaction mixture contain 0.01 M phosphate buffer (ph 7.0) containing 16 % H2O2. The reaction was initiated by adding 50 µl enzyme extract and read the bubble formation of H2O2 at 240 nm in spectrophotometer every 15 sec interval up to 3 min. blank was carried out without enzyme. The specific activity was expressed as µmg-1 protein and unit activity was defined as Δ OD min-1g-1 fresh weight tissues.

Statistical Analysis

Data analysis was performed using the software "DSAASTAT" statistical software (Version 1.101). Mean separations were performed by Duncan's Multiple Range Test (DMRT) at 5% level.

Results and Discussion

Osmolytes: A remarkable increase in osmolytes content was observed after pre-treatment of wheat seeds with ascorbic acid and gibberellic acid as compared to control except for total soluble sugar where there was a decrease in the same was found after pre-treatment with GA_3 as compared to control. Salinity treatments sharply increased accumulation of all the three osmolytes under observation. Additional increase in the same was observed after pre-treatment of wheat seeds with AsA. However, application of GA_3 decreased the accumulation of osmolyte.

Proline content: Proline content increased after pretreatment of wheat seeds with ascorbic acid as compared to control (DW). However, gibberellic acid pretreatment exhibited slight irregular increase. Seeds pre-treated with AsA @ 100 mg L^{-1} had highest proline content and treatment with 200 mg L^{-1} GA_3 was at par with it. NaCl, at its mild, moderate and severe stress level increased proline content to the tune of 41%, 50% and 51%. AsA pre-treatment further enhanced proline content under saline condition. The application of AsA @ 150 mg L^{-1} showed maximum proline content. This may be due to increase in synthesis of protein at the expense of proline as a consequence of applied gibberellic acid. Comparing the two biostimulators, seed treatment with AsA was found more effective in ameliorating salinity stress through elevated proline content.

Free amino acid content: Ascorbic acid and gibberellic acid pre-treatment showed a significant increase in free amino acid content as compared to control. However, gibberellic acid pretreatment when compared with control showed slight decrease in free amino acid. The application of AsA @ 100 mg L^{-1} had highest free amino acid content followed by GA_3 @ 250 mg L^{-1}. NaCl, at severe stress levels showed huge induction of (80%) in free amino acid content as compared to moderate and mild concentrations where the increase were to the tune of (66%) and (52%) respectively. This might be due to the fact that salinity induced the biosynthesis of free amino acids. Also, salinity stress might have caused oxidative damage of protein by enzymes like protease, releasing amino acids. A remarkable increase in free amino acid content was observed after pretreatment of wheat seeds with ascorbic acid under saline condition. In contrast, pretreatment with gibberellic acid showed decrease in the amino acid content under saline condition. The highest free amino acid content was found with the application of AsA @ 100 mg L^{-1}. The main reason for such increase might be the activation of amino acid synthetic pathway by ascorbic acid pre-treatment during salinity stress. In case of seeds treated with GA_3 the greater increase in free amino acid content was found with its concentration of 150 mg L^{-1}. This might be due to utilization of free amino acid for the synthesis of new protein after GA_3 pre-treatment, which provides cellular functionality during stress. Among the two AsA @ 150 mg L^{-1} was most effective in increasing free amino acid content. However, both AsA and GA_3 together were involved in maintaining balance between protein and free amino acid content thus imparting tolerance to the wheat seedling against salinity stress

Total soluble sugar content: A considerable increase in total soluble sugar was found after presoaking treatment of wheat seeds with ascorbic acid as compared to control. However, gibberellic acid treatment slightly lowered total soluble sugar content. The application of AsA @ 150 mg L^{-1} increased free amino acid content maximum and it was at par with the application of GA_3 @ 250 mg L^{-1}. NaCl, at its severe stress level showed more increase (68%) in the given parameter as compared to moderate (49%) and mild (42%) concentrations. A remarkable increase in total soluble sugar was found after pretreatment of wheat seeds with ascorbic acid under salinity stress. The highest concentration of AsA (150 mg L^{-1}) showed maximum increment in the given parameter under salinity condition. Such increase might be due the fact that AsA triggered the synthesis of soluble sugar under salinity stress for maintaining osmotic balance. On the other hand, seeds pre-treated with GA_3 showed a noticeable decrease in total soluble sugar content with increasing salinity levels. Under saline condition, GA_3 @150 mg L^{-1} performed well. This may be due the fact that GA_3 might have induced carbon metabolism and activate the metabolic consumption of soluble sugars to form new cell constituents as a mechanism to stimulate tolerance. Comparing the two, seed treatment with AsA was found more effective. Also, it was revealed from the study that AsA and GA_3 together were successful in ameliorating the adverse effect of salinity by maintaining a balance between total soluble sugar and polysaccharide content of seedling, thus both are important for stress tolerance.

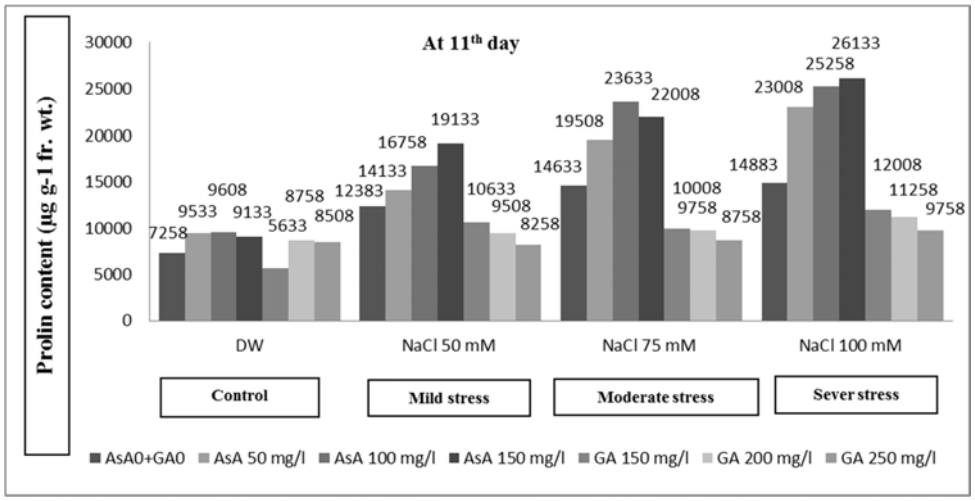

Fig 1: Effect of NaCl induced salinity stress, ascorbic acid (AsA) and gibberellic acid (GA_3) on proline content (µg g^{-1} fr. wt.) at 11th DAG of wheat.

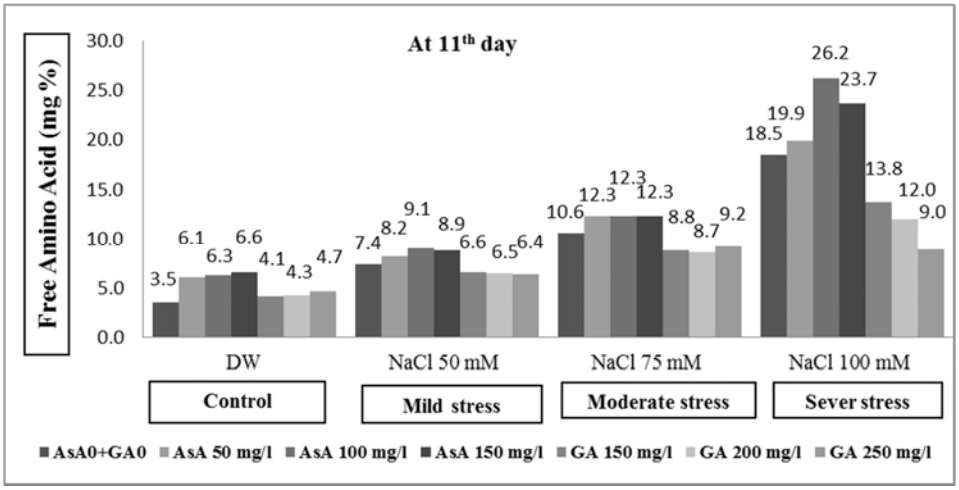

Fig 2: Effect of NaCl induced salinity stress, ascorbic acid (AsA) and gibberellic acid (GA_3) on free amino acid content (mg %) at 11th DAG of wheat.

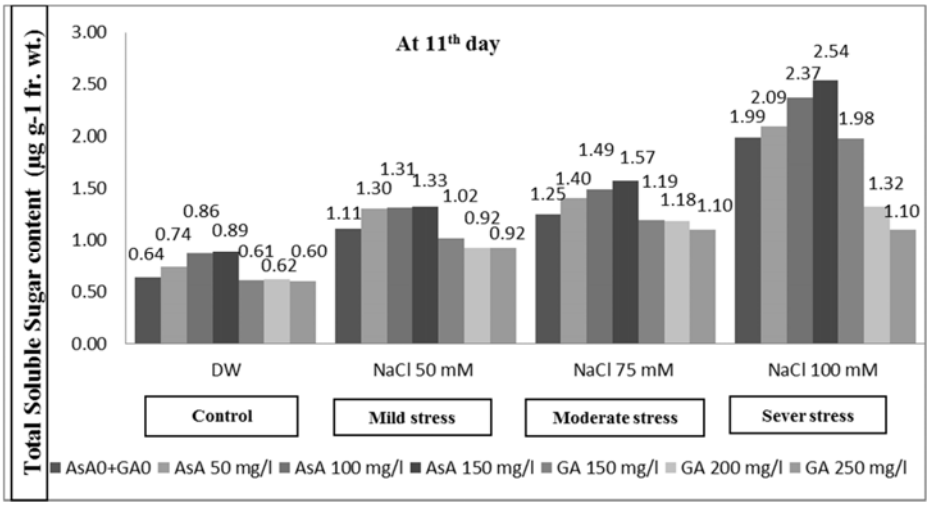

Fig 3: Effect of NaCl induced salinity stress, ascorbic acid (AsA) and gibberellic acid (GA_3) on total soluble sugar content (µg g^{-1} fr. wt.) at 11th DAG of wheat.

Antioxidative enzymes: A remarkable increase in antioxidative enzyme activity was observed after pre-treatment of wheat seeds with ascorbic acid and gibberellic acid as compared to control. Salinity treatments sharply increased accumulation of all the three antioxidative enzymes under observation. AsA and GA_3 pre-treatment of wheat seeds further increased antioxidative enzyme activity under salinity stress condition.

Peroxidase activity: A significant increase in peroxidase activities (POD) was recorded after pretreatment of wheat seeds with ascorbic acid and gibberellic acid as compared to control. The application of AsA @ 100 mg L^{-1} had highest peroxidase oxidase activities and those treated with GA_3 @ 250 mg L^{-1} respectively were at par with it. NaCl, at the severe stress level showed drastic increase of 19% as compared to its moderate (15%) and mild stress (9%) level. This might be due to the toxic effects of the high turnover rate of H_2O_2 or its harmful ROS, which impair enzyme activities. Pretreatment of wheat seeds with AsA and GA_3 caused a remarkable increase in POD activity under saline condition. Application AsA @ 100 mg L^{-1} was the best under all three stress levels. However, GA_3 @ 250 mg L^{-1} was better in nullifying the adverse effect of salt stress. The probable reason for such increase might be due to the role of GA_3 in triggering the synthesis of antioxidative enzyme under saline condition in response to oxidative damage through signal cascade. Considering the two, it was revealed that pre-treatment with AsA was the best in enhancing POD activity.

Catalase activity: A sharp increase in catalase activity (CAT) was recorded after pretreatment of wheat seeds with ascorbic acid and gibberellic acid as compared to control. The application of AsA at its highest concentration (150 mg L^{-1}) showed maximum catalase activity and it was at par with those treated with GA_3 @ 250 mg L^{-1} respectively. NaCl, at the severe stress level showed sharp increase of 56% as compared to its moderate and mild stress level with the increment to the tune of 45% and 35% respectively. To be able to endure oxidative damage under salinity stress, plants triggered efficient antioxidative enzyme system and hence, increased the activity of CAT as an adaptive mechanism to reduce the H_2O_2 and offer protection against oxidative damage (Agarwal & Pandey, 2004) [3]. Pretreatment of wheat seeds with AsA and GA_3 caused considerable increase in CAT activity under saline condition. Seeds pretreated with highest concentration of AsA (150 mg L^{-1}) showed maximum increase in catalase activity under saline conditions. Among the GA_3 pretreated wheat seeds, the maximum catalase activity was exhibited by its application @ 250 mg L^{-1} salt stress conditions. Comparing the two, it was revealed that seed treatment with AsA was found most effective in enhancing catalase activity thus counteracting the harmful effect of salinity stress.

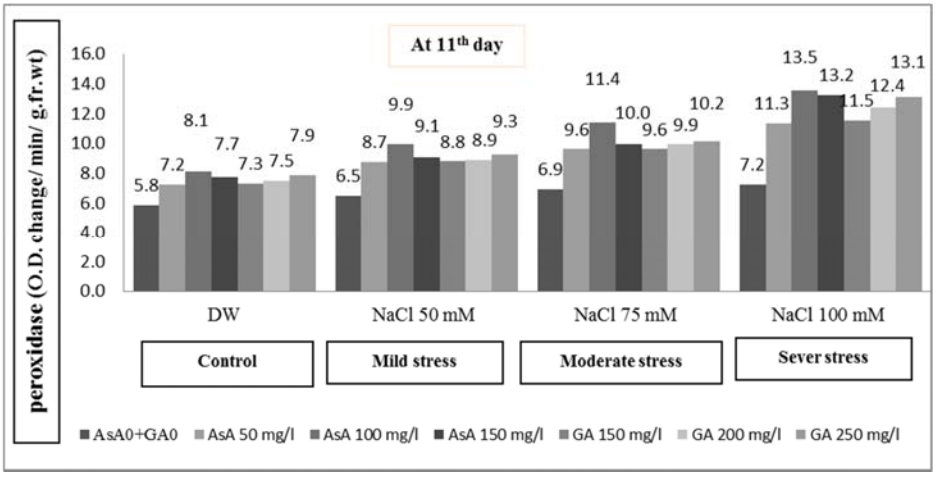

Fig 4: Effect of NaCl induced salinity stress, Ascorbic acid (AsA) and Gibberellic acid (GA_3) on peroxidase activity (O.D. change/min/g.fr.wt) at 11th DAG of wheat.

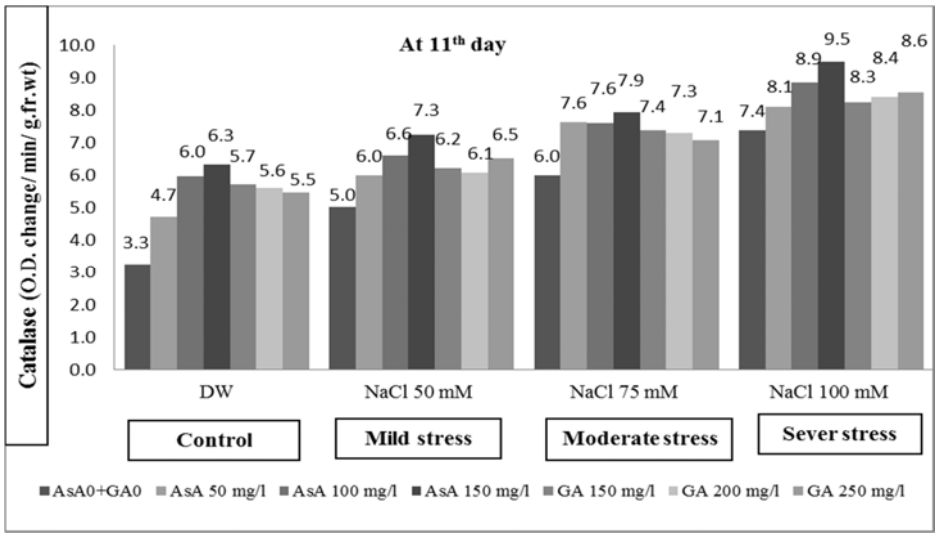

Fig 5: Effect of NaCl induced salinity stress, Ascorbic acid (AsA) and Gibberellic acid (GA_3) on catalase activity (O.D. change/min/g.fr.wt) at 11th DAG of wheat.

Conclusion

Thus, distinct favorable effect of ascorbic acid and gibberellic acid in alleviation of salinity stress could be discerned as evidenced by activated metabolism in terms of increased synthesis of osmolytes and antioxidative enzyme of germinating seedlings of wheat after pretreatment under saline condition. Hence, this study proves the role of these bio-stimulators in ameliorating the deleterious effect of salinity stress.

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