International Journal of Chemical Studies

P-ISSN: 2349–8528
E-ISSN: 2321–4902
IJCS 2018; 6(4): 2107-2116

Received: 20-05-2018
Accepted: 26-06-2018

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Effect of exogenous application of salicylic acid on antioxidative enzymes in black gram (*Vigna mungo* (L.) Hepper) irrigated with saline water

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Abstract
Black gram [*Vigna mungo* (L.) Hepper] is one of the most important pulse crops grown in India. India is the largest producer and consumer of black gram in the world. Salt stress is one of the most serious limiting factors for growth and production in most of the crops and salicylic acid an important phenolics known to alleviates its adverse effect. Thus, the green house experiment was conducted to investigate the effect of exogenous application of salicylic acid (SA) under salt stress on physiological, biochemical parameters and enzymatic activities of black gram. The experimental design was factorial completely randomized design with four salicylic acid concentrations (0.0, 0.5, 1.0 and 1.5 mM), four level of irrigation to induce salinity stress (< 2 EC-tap water, 4.0 EC, 6.0 EC and 8.0 EC). The pots were irrigated with different concentration of saline water and plants were sprayed with salicylic acid after 20 and 40 DAS (Days after Sowing), samples were withdrawn after 21, 41 and 61 DAS for the analysis. The observations were recorded for the antioxidant enzymes *viz*, polyphenol oxidase, peroxidase and catalase. Antioxidant enzymes activities like peroxidase and polyphenol oxidase in leaf was increased with higher concentration of salt stress however, the catalase activity was decreased. Antioxidant enzymes activity showed similar trends. Higher dose of salicylic acid decreased the catalase and polyphenol oxidase enzyme activity.

Keywords: antioxidative enzymes, Black gram, Salicylic acid, Salinity stress, *Vigna mungo* L.

Introduction
Black gram or uradbean [*Vigna mungo* (L.) Hepper] belongs to family *Fabaceae* sub family *papilionaceae*, is one of the most highly prized pulses in tropical countries especially in India. Black gram has a surprising number of health benefits, including its ability to boost energy, protect cardiovascular health, reduce pain and inflammation, improve immunity, aid in maintaining skin health, prevent diabetes, build strong bones, strengthen the nervous system, and optimize digestion. Seed of black gram is perfect combination of all nutrients, which includes proteins (25-26%), carbohydrates (60%), fat (1.5%), minerals, amino acids and vitamins. Black gram crop is sensitive to saline and alkaline soils (Sharma *et al.*, 2011) [13]. Soil salinity is one of the major factors responsible for losses in crop production and quality. Numerous factors such as salt composition (mainly Na^+ and Cl^- ions), land topography, human activities etc. are the causes of salinization. Under high salinity stress, reactive oxygen species (ROS) formed and accumulated in plant cells causes severe damage to plants, including growth inhibition, impaired metabolism, change in biochemical parameters like lipid peroxidation, changes in antioxidant enzyme activities, necrosis and finally death of cells. However, plants equipped with a variety of defense mechanism scavenging ROS formed due to biotic as well as abiotic stresses like accumulation of phenolics or induction of antioxidant and its related enzymatic system (Kandoliya and Vakharia, 2013; Patel *et al.* 2015; Kandoliya and Vakharia, 2015; Joshi *et al* 2018) [5, 6, 9, 4]. Salicylic acid commonly called "Aspirin" in acetylated form. Salicylic acid belongs to an extraordinary diverse group of plant phenolic defined as substance that possess an aeromatic ring bearing hydroxyl group (Raskin, 1992). Thus, the green house experiment was conducted to investigate the effect of exogenous application of salicylic acid (SA) under salt stress on antioxidative enzymatic activities in black gram.

Materials and Methods

The green house experiment was conducted during *Rabi* 2016-17 at Food testing Laboratory, [*Vigna mungo* (L.) Hepper] seeds of varieties Black gram Gujarat-1 were obtained from Main Pulse Research Station, Junagadh Agricultural University, Junagadh.

Treatments

a. Salinity Level (4): S_1-≤2 ds.m^{-1} (Control Tap Water), S_2-4 ds.m^{-1}, S_3-6 ds.m^{-1}and S_4-8 ds.m^{-1}
b. Salicylic acid (4) : T_1-0.0 mM, T_2-0.5 mM, T_3-1.0 mM and T_4-1.5 mM
c. Growth stage (3): D_1-21DAS, D_2-41DAS and D_3-61DAS
d. Salicylic acid spray of appropriate concentration (T_1 to T_4) was done and at 20 days after of sowing and days after sowing of crop growth. The sample was collected at 21, 41 and 61 (D_1 to D_3) days after sowing respectively. Pot irrigated with saline water having a different concentration (S_1 to S_4) and packed in plastic bag and brought to the laboratory under ice cold condition

Enzyme Assay

1. Polyphenol oxidase (PPO) activity (EC 1.14.18.1): Leaf tissue weighed 0.1 gm and grind in 5 ml of 100 mM sodium phosphate buffer, pH 6.5. The homogenate was centrifuged at 10,000 rpm for 15 min at 4°C and the supernatant was used for enzyme assay. The reaction mixture contained 2.9 ml of catechol (10 mM catechol in 10 mM phosphate buffer, pH 6.5) and reaction was initiated by the addition of 100 µl of enzyme extract. The changes in the colour due to the oxidized catechol were read at 490 nm for one minute at an interval of 15 second. Blank was carried out without substrate. The enzyme activity was expressed as Δ OD.min.$^{-1}$g.$^{-1}$fr.wt. tissues (Esterbaner *et al*. 1977) [2].
2. Peroxidase (POX) activity (EC 1.11.1.7): Leaf tissue (100 mg) was homogenized in a pre-chilled mortar and pestle with 2 ml of extraction buffer, containing 50 mM sodium phosphate buffer pH 7.0. The homogenates were centrifuged at 10,000 rpm for 15 minutes and the supernatant was used for the assay of antioxidant enzymes *viz*. peroxidase and catalase. The reaction mixture contained 2.99 ml of 0.03% H_2O_2 in 0.1M phosphate buffer (pH 6.0) containing 0.01 % orthodianisidine dye (freshly prepared, dissolved in methanol). The reaction was initiated by the addition of 10 µl of enzyme extract. The change in color of oxidized dye was read at 460 nm up to 1 minute at the interval of 15 seconds. Blank was run without the addition of enzyme (Malik and Singh (1980) [7]|. The enzyme activity was expressed as Δ OD.min.$^{-1}$g.$^{-1}$fr.wt.
3. Catalase activity (EC 1.11.1.6): Catalase activity was measured immediately in fresh extract and was assayed as described by Aebi (1984). Three ml reaction mixture contained 50 mM sodium phosphate buffer (pH 7.0), 18 mM H_2O_2 and 50 µl enzyme extract. The hydrogen peroxide dependent oxidation was estimated by measuring the decrease in the absorbance at 240 nm. The enzyme activity was expressed as Δ OD.min.$^{-1}$ g.$^{-1}$fr. wt.

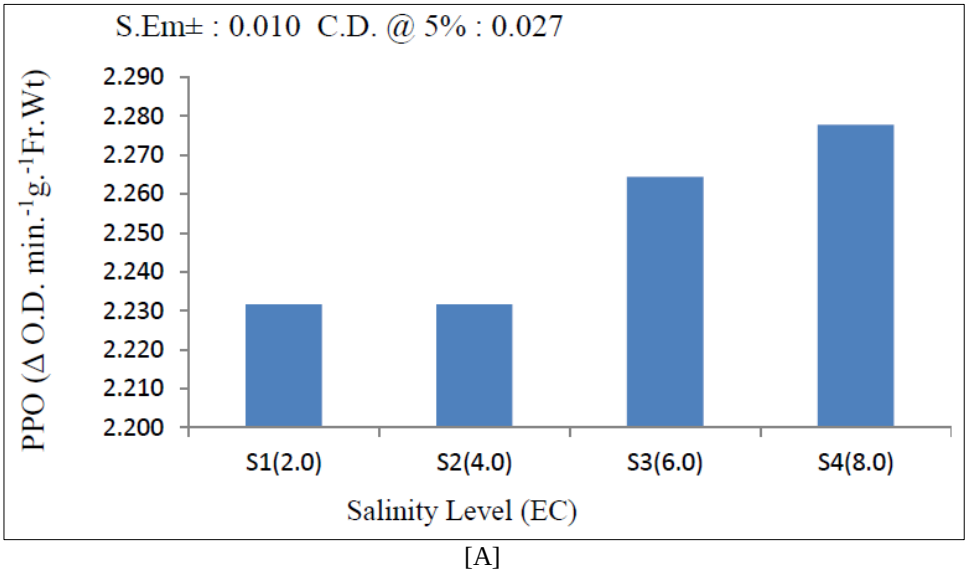
Results and Discussion

Polyphenol Oxidase (EC 1.14.18.1)

The data on enzyme activity of polyphenol oxidase activity (Δ O.D. min.$^{-1}$g.$^{-1}$fr.wt) analyzed from leaf tissue of black gram collected from plants treated with different concentration of salicylic acid (T_1 to T_4) grown in a pot irrigated with different concentration of saline water concentration (S_1 to S_4) at different stages (D_1 to D_3) are depicted in Fig.1 and 2.

Mean effect of salinity level irrespective of salicylic acid and growth stage were found to be significant for polyphenol oxidase activity (Fig. 1 A). Among the salinity level, treatment S_4 (irrigated with saline water 8 EC) showed highest amount of polyphenol oxidase activity (2.278 Δ O.D. min.$^{-1}$g.$^{-1}$Fr.Wt) while the S_1 (pot irrigated with saline water 2 EC) showed lowest amount of polyphenol oxidase activity (2.232 Δ O.D. min.$^{-1}$g.$^{-1}$Fr.Wt). In general, activity increased with the increase in salinity in pot.

Imposition of different treatments of salicylic acid resulted significant difference for the polyphenol oxidase activity (Fig. 1 B). The tissues obtain from black gram pots treated with salicylic acid 0.0 mM (T_1) revealed higher amount of mean polyphenol oxidase activity (3.098 Δ O.D. min.$^{-1}$g.$^{-1}$Fr.Wt.) and which was followed by T_2-0.5 mM Salicylic acid (2.370 Δ O.D. min.$^{-1}$g.$^{-1}$Fr.Wt.) and T_3-1.5 mM salicylic acid (1.976 Δ O.D. min.$^{-1}$g.$^{-1}$Fr.Wt) irrespective of salinity level and growth stage. The mean lowest content was noted for the tissues received from T_4- pots treated with salicylic acid 1.5 mM (T_4) (1.578 Δ O.D. min.$^{-1}$g.$^{-1}$Fr.Wt.) irrespective of salinity level and growth stage. In general the exogenous application of salicylic acid decreased the enzyme activity.



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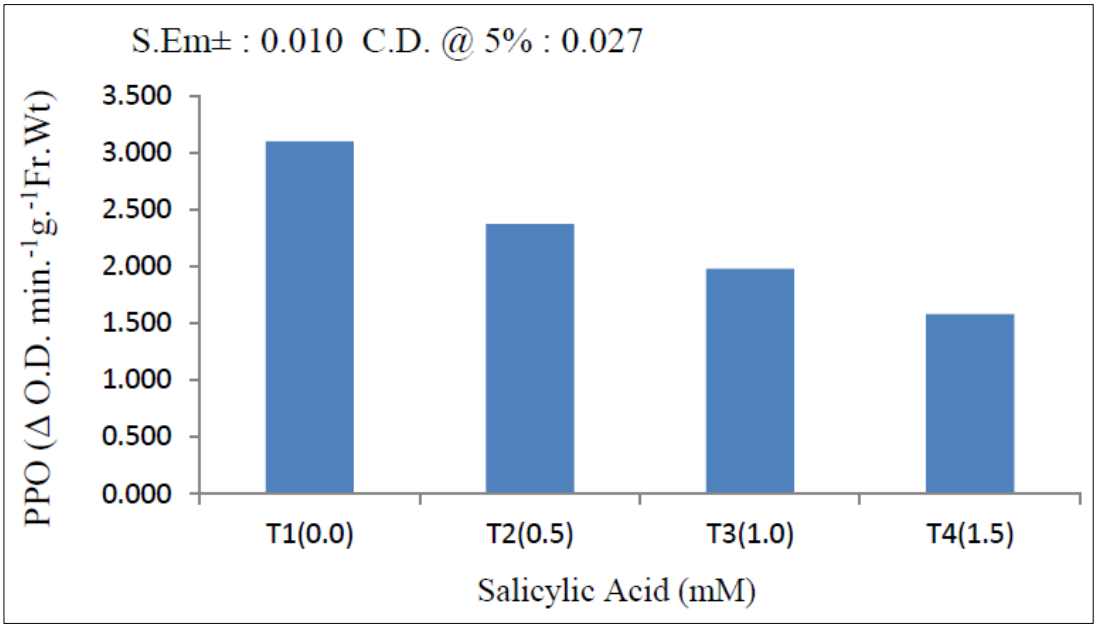

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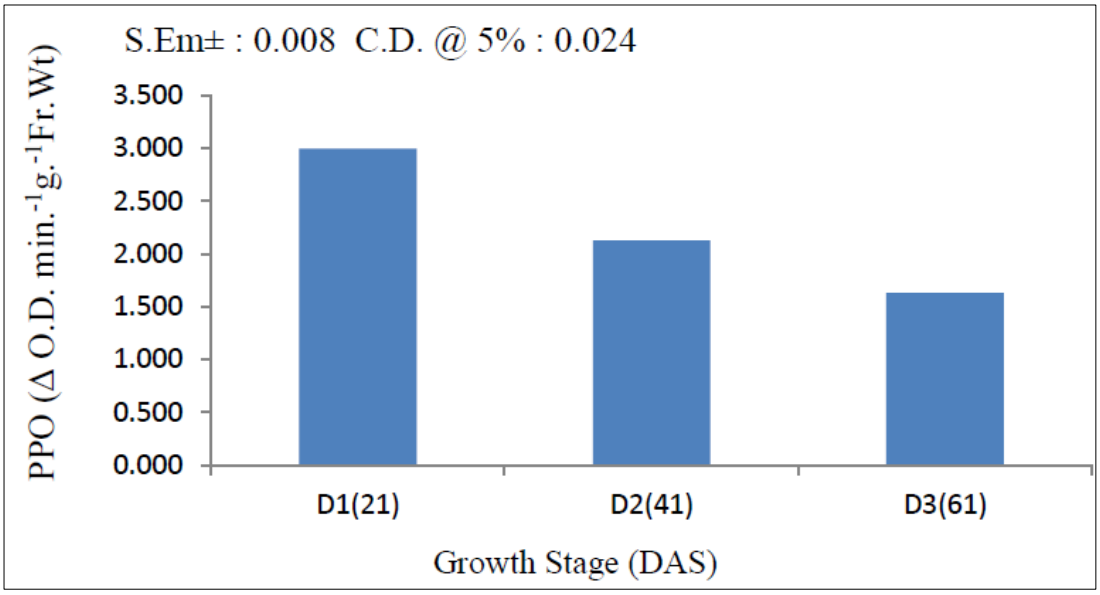

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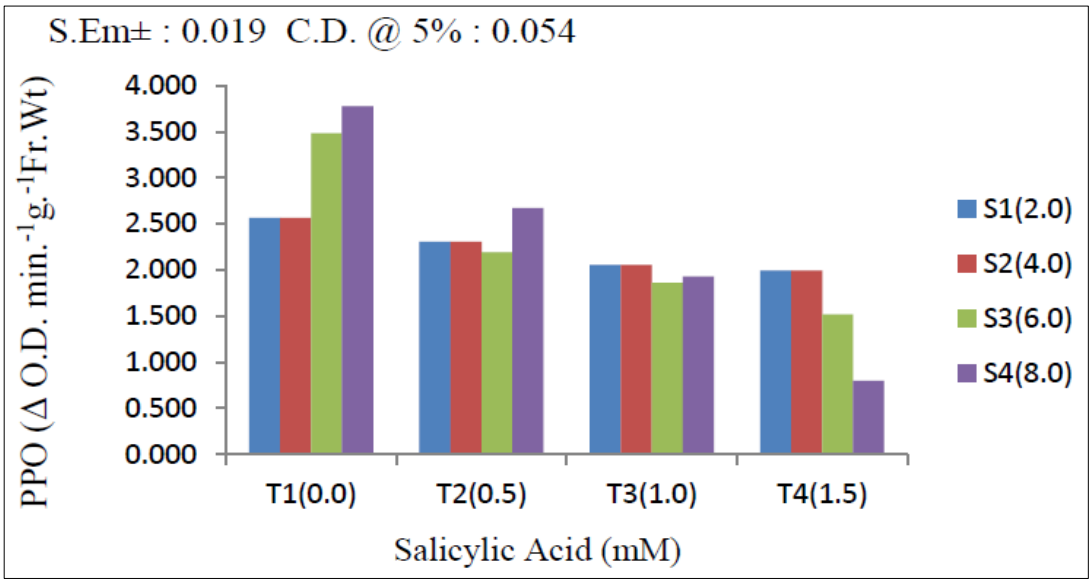
Fig 1: Mean effect of (A) Salinity, (B) Salicylic acid and (C) Growth Stage on Polyphenol Oxidase (Δ O.D. min.$^{-1}$g.$^{-1}$Fr.Wt) in seedling of Black gram.



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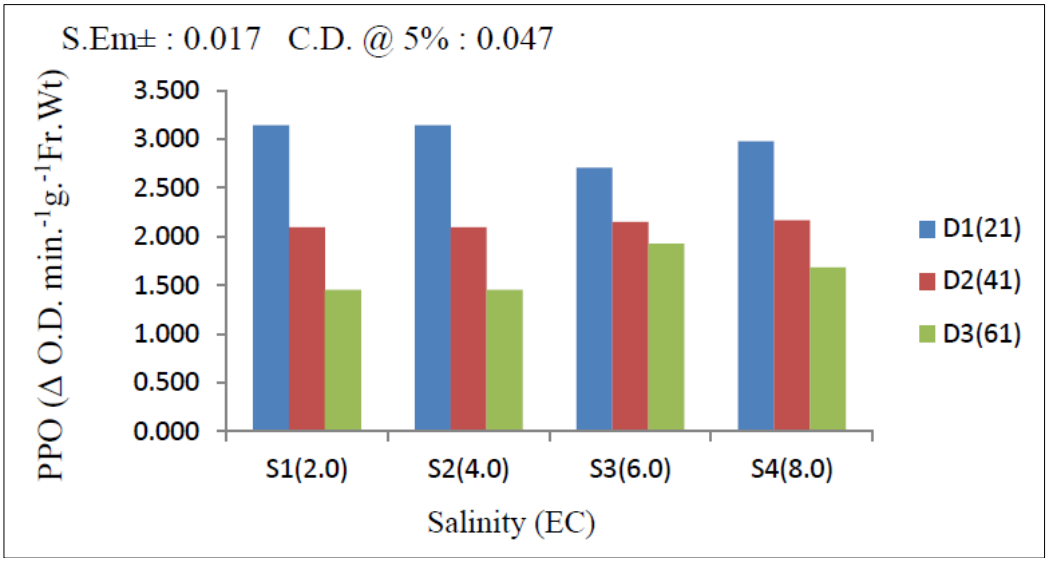

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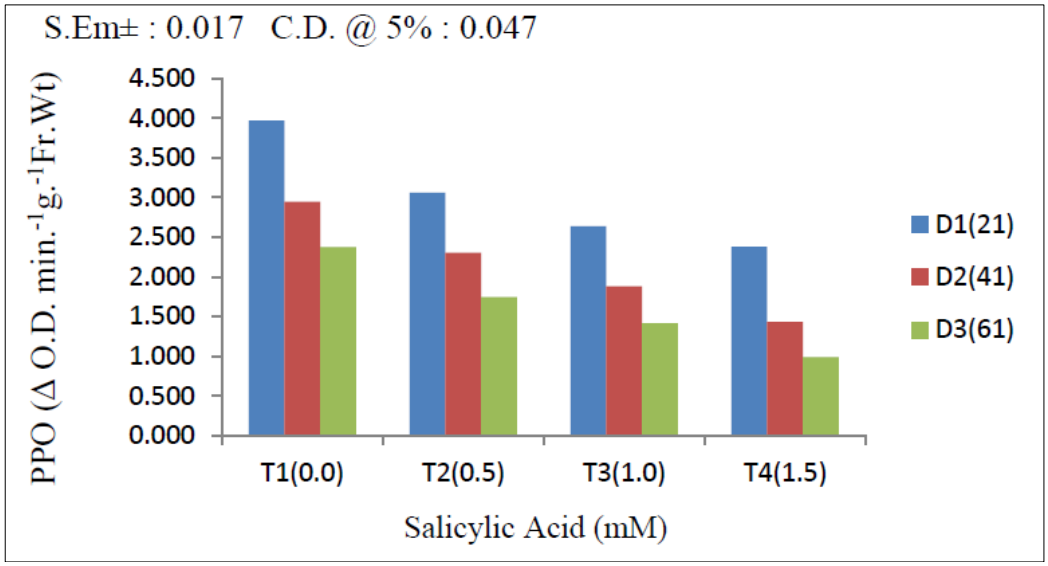

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Fig 2: Interaction effect of (A) Salinity (EC) × Salicylic acid (mM), (B) Salinity (EC) × Growth Stage (DAS) and (C) Salicylic Acid (mM) × Growth Stage (DAS) on Polyphenol Oxidase (Δ O.D. min.$^{-1}$g.$^{-1}$Fr.Wt) in seedling of Black gram.

Among the different stages, mean value of polyphenol oxidase activity content significantly varied between 2.994 and 1.631 Δ O.D. min.$^{-1}$g.$^{-1}$Fr.Wt (Fig. 1 C). The content was decreased from 21 DAS (2.994 Δ O.D. min.$^{-1}$g.$^{-1}$Fr.Wt) to 61 DAS (1.631 Δ O.D. min.$^{-1}$g.$^{-1}$Fr.Wt).

Interaction effect of S × T for polyphenol oxidase activity were revealed significant differences in black gram (Fig. 2 A). The highest value of polyphenol oxidase activity was observed for the S_4T_1 i.e. in plant irrigated with saline water 8 EC combine with salicylic acid 0.0 mM (3.775 Δ O.D. min.$^{-1}$g.$^{-1}$Fr.Wt). The lowest value (0.797 Δ O.D. min.$^{-1}$g.$^{-1}$Fr.Wt) of polyphenol oxidase activity was observed in plant irrigated with saline water 6 EC combine with salicylic acid 0.0 mM (S_4T_4).

Interaction effect of S × D for polyphenol oxidase activity were revealed significant differences in black gram (Fig. 2 B). The highest value of polyphenol oxidase activity was observed for the S_1D_1 i.e. in plant irrigated with saline water 2 EC after at 21 DAS (3.144 Δ O.D. min.$^{-1}$g.$^{-1}$Fr.Wt). The Lowest value of polyphenol oxidase activity was observed for S_1D_3 in plant irrigated with saline water 2 EC after 61 DAS (1.454 Δ O.D. min.$^{-1}$g.$^{-1}$Fr.Wt).

Interaction effect of T × D for polyphenol oxidase activity were revealed significant differences in black gram (Fig. 2 C). The highest value of polyphenol oxidase activity was observed for T_1D_1 i.e. in plant treated with 0.0 mM salicylic acid after at 21 DAS (3.967 Δ O.D. min.$^{-1}$g.$^{-1}$Fr.Wt). The lowest value of polyphenol oxidase activity was observed for T_4D_3 i.e. in plant for control condition 1.5 mM salicylic acid after 61 DAS (0.989 Δ O.D. min.$^{-1}$g.$^{-1}$Fr.Wt).

Qados (2015) also observed that the activities of polyphenol oxidase enzymes increased insignificantly with increasing salt stress, above which the activities of all enzymes were decreased as compared with control plants. Salicylic acid treatment showed a linear increase in enzyme activities in salt stressed and unstressed plants. Tuna *et al.* (2007) [14] noted that the application of salicylic acid in 2 mM dose considerably increased polyphenol oxidase activity.

Peroxidase (EC 1.11.1.7)

POX (EC 1.11.1.7) is the most important among the H_2O_2 scavenging enzymes. The enzyme activity (Δ O.D. min.$^{-1}$g.$^{-1}$Fr.Wt.) analyzed from leaf tissue of black gram collected from plants treated with different concentration of salicylic acid (T_1 to T_4) grown in a pot irrigated with different

concentration of saline water concentration (S_1 to S_4) at different stages (D_1 to D_3) are depicted in Fig. 3 and 4.

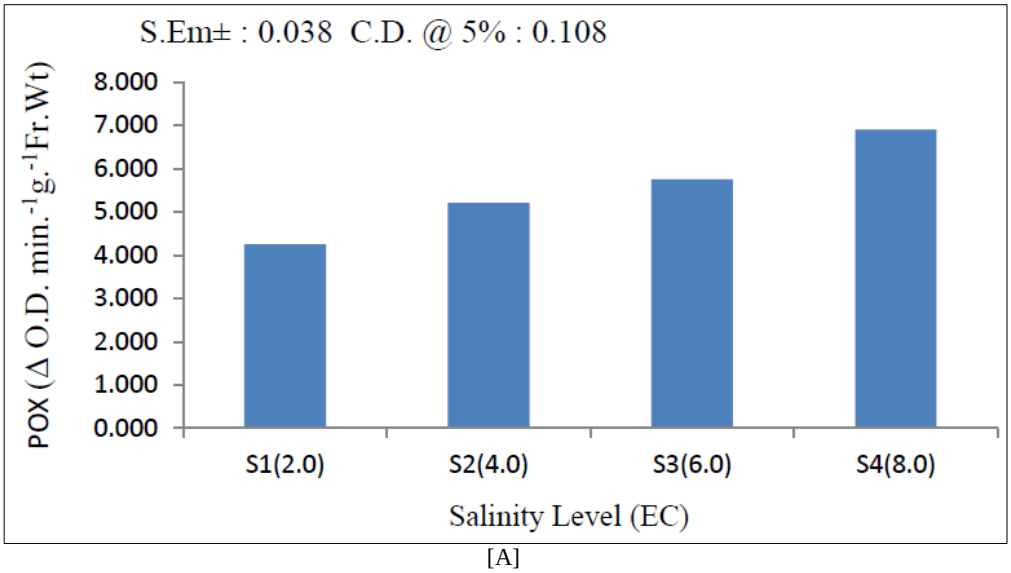
Mean effect of salinity level were found to be significant for peroxidase activity (Fig. 3 A). The S_1 (pot irrigated with saline water 2 EC) showed lowest amount of peroxidase activity (4.255 Δ O.D. min.$^{-1}$g.$^{-1}$Fr.Wt). Among the salinity level, treatment S_4 (irrigated with saline water 8 EC) showed highest amount of peroxidase activity (6.903 Δ O.D. min.$^{-1}$g.$^{-1}$Fr.Wt).

Imposition of different treatments of salicylic acid resulted significant difference for the peroxidase activity. The mean lowest content was noted for the tissues received from pots treated with salicylic acid 0.0 mM (T_1) (4.590 Δ O.D. min.$^{-1}$g.$^{-1}$Fr.Wt).). (Fig. 3 B) and which was followed by T_3-1 mM salicylic acid (5.845 Δ O.D. min.$^{-1}$g.$^{-1}$Fr.Wt.) and T_2-0.5 mM salicylic acid (5.106 Δ O.D. min.$^{-1}$g.$^{-1}$Fr.Wt) irrespective of salinity level and growth stage. The tissues obtain from black gram pots treated with salicylic acid 1.5 mM (T_4) revealed higher amount of mean peroxidase activity (6.585 Δ O.D. min.$^{-1}$g.$^{-1}$Fr.Wt) It suggest the effect of exogenous application of salicylic acid on inducing the peroxidase activity.

Among the different stages, mean value of peroxidase activity significantly varied between 6.336 and 4.682 Δ O.D. min.$^{-1}$g.$^{-1}$Fr.Wt. (Fig. 3 C). The content was decreased from 21 DAS (6.336 Δ O.D. min.$^{-1}$g.$^{-1}$Fr.Wt) to 61 DAS (4.682 Δ O.D. min.$^{-1}$g.$^{-1}$Fr.Wt).

Interaction effect of S × T for peroxidase activity were revealed significant differences in black gram (Fig. 4 A). The lowest value (3.162 Δ O.D. min.$^{-1}$g.$^{-1}$Fr.Wt) of peroxidase activity was observed in plant irrigated with saline water 2 EC combine with salicylic acid 0.0 mM (S_1T_1). The highest value of peroxidase activity was observed for the S_4T_4 i.e. in plant irrigated with saline water 8 EC combine with salicylic acid 1.5 mM (7.816 Δ O.D. min.$^{-1}$g.$^{-1}$Fr.Wt).

Interaction effect of S × D for peroxidase activity were revealed significant differences in black gram (Fig. 4 B). The lowest value of peroxidase activity was observed for S_1D_3 in plant irrigated with saline water 2 EC after 61 DAS (3.426 Δ O.D. min.$^{-1}$g.$^{-1}$Fr.Wt).



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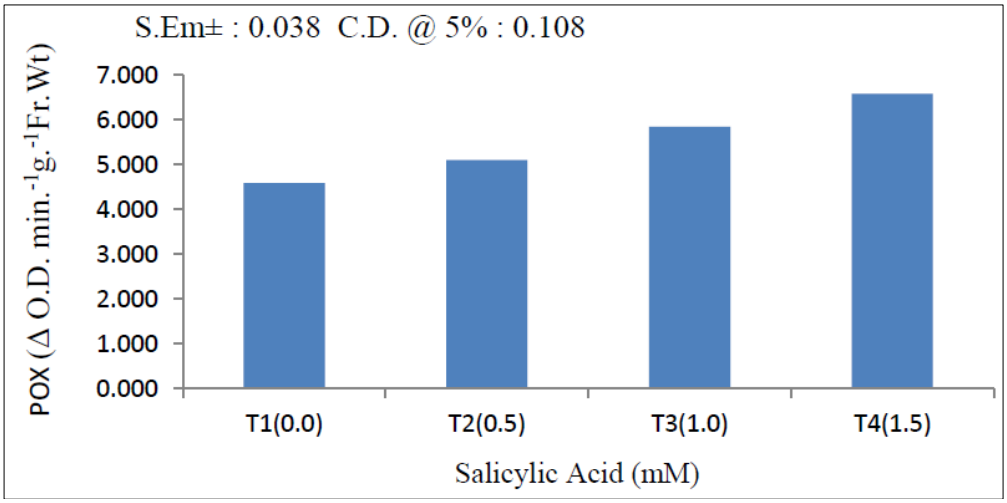

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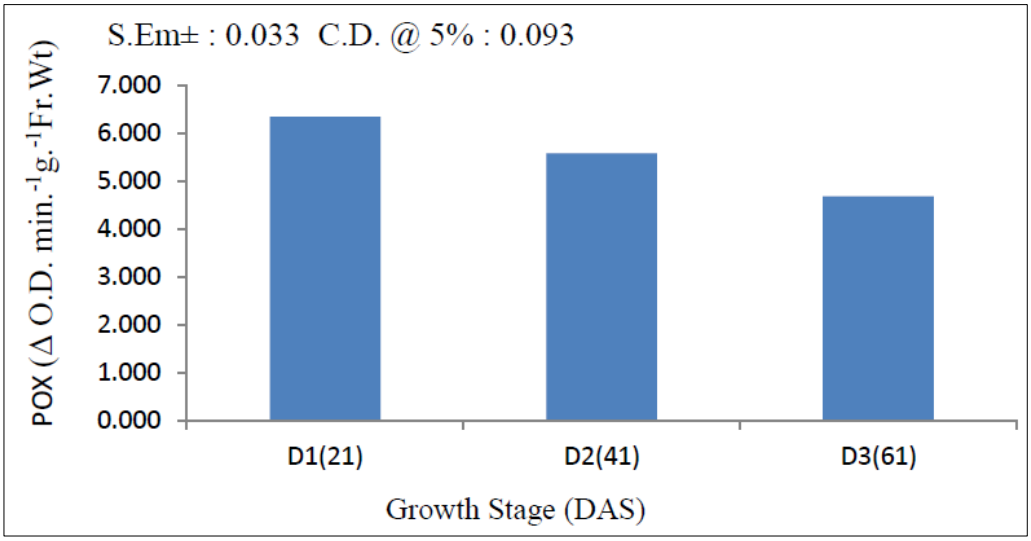

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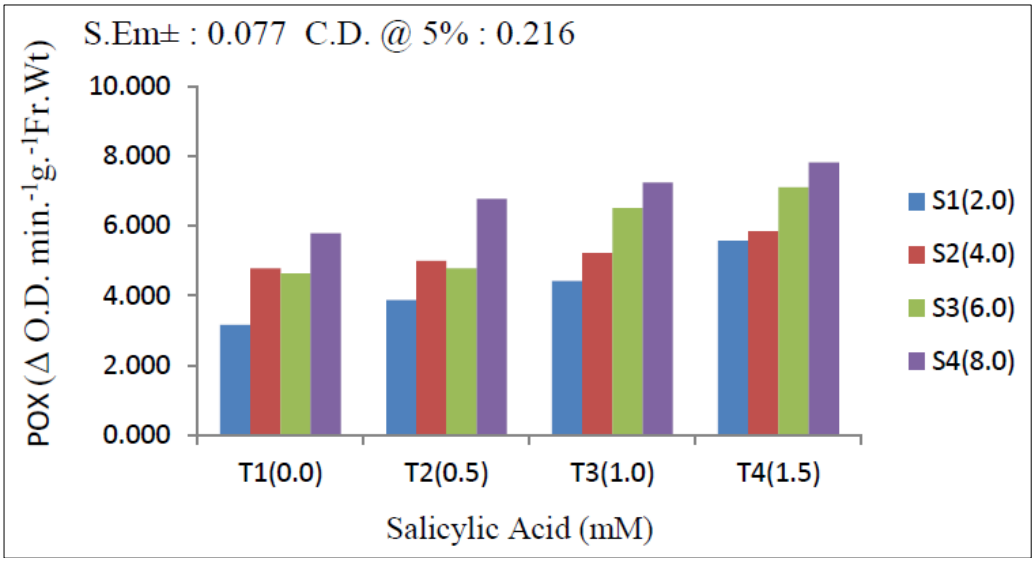
Fig 3: Mean effect of (A) Salinity, (B) Salicylic acid and (C) Growth Stage on Peroxidease (Δ O.D. min.$^{-1}$g.$^{-1}$Fr.Wt) in seedling of Black gram



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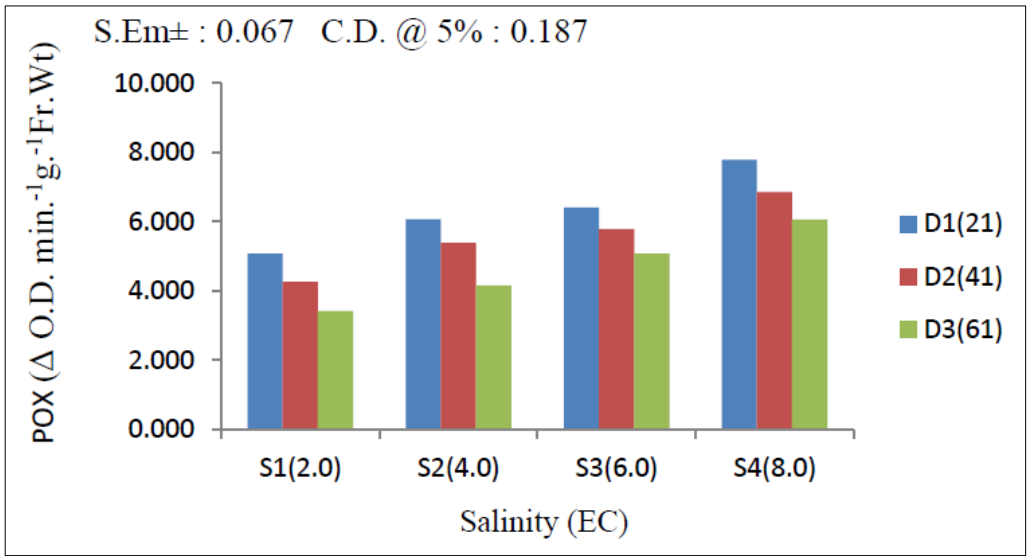

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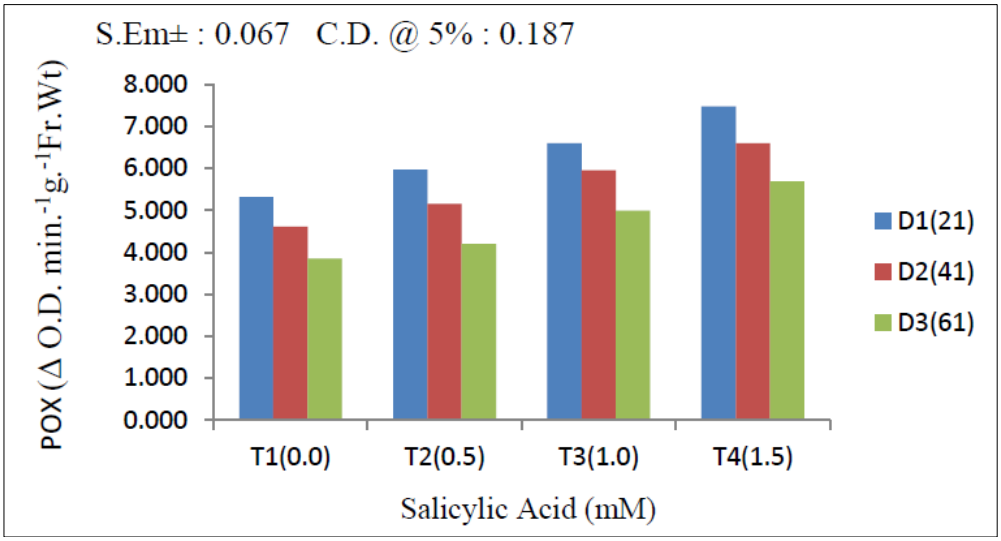


Fig 4: Interaction effect of (A) Salinity (EC) × Salicylic acid (mM), (B) Salinity (EC) × Growth Stage (DAS) and (C) Salicylic Acid (mM) × Growth Stage (DAS) on Peroxidease (Δ O.D. min.$^{-1}$g.$^{-1}$Fr.Wt) in seedling of Black gram

The highest value of peroxidase activity was observed for the S_4D_1 i.e. in plant irrigated with saline water 8 EC after at 21 DAS (7.789 Δ O.D. min.$^{-1}$g.$^{-1}$Fr.Wt).

Interaction effect of T × D for peroxidase activity were revealed significant differences in black gram (Fig. 4 C). The lowest value of peroxidase activity was observed for T_1D_3 i.e. in plant for control condition 0.0 mM salicylic acid after 61 DAS (3.847 Δ O.D. min.$^{-1}$g.$^{-1}$Fr.Wt). The highest value of peroxidase activity was observed for T_4D_1 i.e. in plant treated with 1.5 mM salicylic acid after at 21 DAS (7.476 Δ O.D. min.$^{-1}$g.$^{-1}$Fr.Wt). Result of present experiment were in agreement with following research.

Ghasemzadeh and Jaafar (2013) [3] also studied the effect of foliar salicylic acid (SA) applications on activities of peroxidase (POX) and concluded that the exogenous application of SA increased antioxidant enzyme activities. Sedghi *et al.* (2013) [12] studied effects of salicylic acid on the antioxidant enzymes activity in sunflower. The results showed that all three enzymes activity increased by SA spraying compared to control. Tuna *et al.*, (2007) [14] also noted that application of salicylic acid in 2 mM dose considerably increased Peroxidase activity different effects of SA on antioxidant activity of plants have been reported. Qados (2015) observed that activities of peroxidase enzymes increased insignificantly with increasing salt stress until 4000 ppm, above which the activities of all enzymes were decreased as compared with control plants. In general salicylic acid treatment showed a linear increase in enzyme activities in salt stressed.

Catalase (E.C 1.11.1.6)

CAT (E.C 1.11.1.6) is the most important among the H_2O_2 scavenging enzymes. The enzyme activity (Δ O.D. min.$^{-1}$g.$^{-1}$Fr.Wt.) analyzed from leaf tissue of black gram collected from plants treated with different concentration of salicylic acid (T_1 to T_4) grown in a pot irrigated with different concentration of saline water concentration (S_1 to S_4) at different stages (D_1 to D_3) are depicted in Fig. 5 and 6.

Mean effect of salinity level were found significant for catalase activity (Fig. 5 A). The S_4 (pot irrigated with saline water 8 EC) showed lowest amount of catalase activity (2.426 Δ O.D. min.$^{-1}$g.$^{-1}$Fr.Wt.). Among the salinity level, treatment S_1 (irrigated with saline water 2 EC) showed highest amount of catalase activity (2.682 Δ O.D. min.$^{-1}$g.$^{-1}$Fr.Wt.) while Qados (2015) observed that activities of catalase enzymes increased insignificantly with increasing salt stress until 4000 ppm, above which the activities of all enzymes were decreased as compared with control plants.

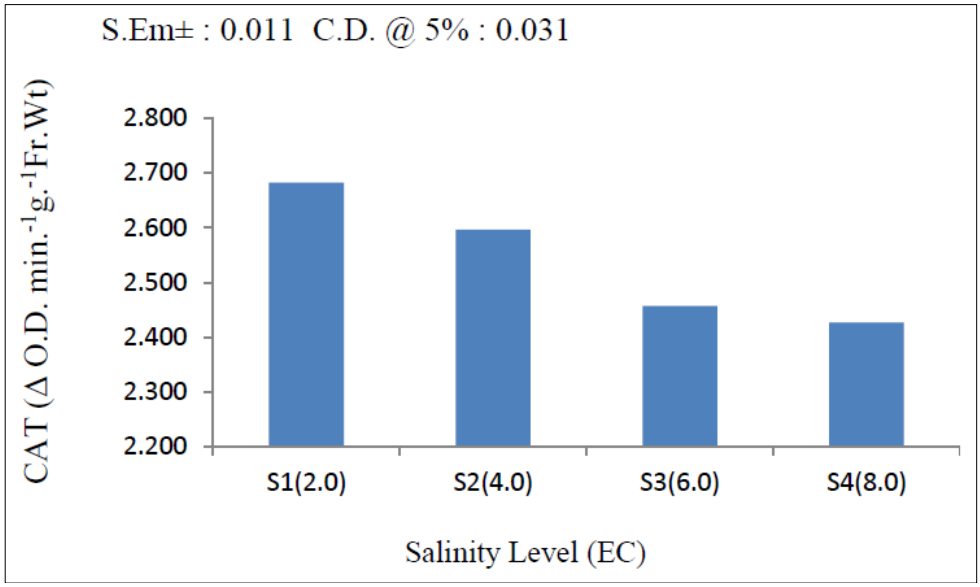
Imposition of different treatments of salicylic acid resulted significant difference for the catalase activity (Fig. 5 B). The mean lowest content was noted for the tissues received from pots treated with salicylic acid 1.5 mM (T_4) (1.885 Δ O.D. min.$^{-1}$g.$^{-1}$Fr.Wt.) and which was followed by T_2-0.5 mM salicylic acid (2.611 Δ O.D. min.$^{-1}$g.$^{-1}$Fr.Wt.) and T_3-1.0 mM salicylic acid (2.255 Δ O.D. min.$^{-1}$g.$^{-1}$Fr.Wt.) irrespective of salinity level and growth stage. The tissues obtain from black gram pots treated with salicylic acid 0.0 mM (T_1) revealed higher amount of mean catalase activity (3.413 Δ O.D. min.$^{-1}$g.$^{-1}$Fr.Wt.).

Among the different stages, mean value of catalase activity significantly varied between 3.222 and 1.992 Δ O.D. min.$^{-1}$g.$^{-1}$Fr.Wt. (Fig. 5 C). The content was decreased from 21 DAS (3.222 Δ O.D. min.$^{-1}$g.$^{-1}$Fr.Wt.) to 61 DAS (1.992 Δ O.D. min.$^{-1}$g.$^{-1}$Fr.Wt.).

Interaction effect of S × T for catalase activity were revealed significant differences in black gram (Fig. 6 A). The lowest value (1.480 Δ O.D. min.$^{-1}$g.$^{-1}$Fr.Wt.) of catalase activity was observed in plant irrigated with saline water 8 EC combine with salicylic acid 1.5 mM (S_4T_4). The highest value of catalase activity was observed for the S_4T_1 i.e. in plant irrigated with saline water 8 EC combine with salicylic acid 0.0 mM (3.718 Δ O.D. min.$^{-1}$g.$^{-1}$Fr.Wt.).

Interaction effect of S × D for catalase activity were revealed significant differences in black gram (Fig. 6 B). The lowest value of catalase activity was observed for S_1D_3 in plant irrigated with saline water 2 EC after 61 DAS (1.843 Δ O.D. min.$^{-1}$g.$^{-1}$Fr.Wt.). The highest value of catalase activity was observed for the S_1D_1 i.e. in plant irrigated with saline water 2 EC after at 21 DAS (3.740 Δ O.D. min.$^{-1}$g.$^{-1}$Fr.Wt.).

Interaction effect of T × D for catalase activity were revealed significant differences in black gram (Fig. 6 C). The lowest value of catalase activity was observed for T_4D_3 i.e. in plant for control condition 1.5 mM salicylic acid after 61 DAS (1.362 Δ O.D. min.$^{-1}$g.$^{-1}$Fr.Wt.). The highest value of catalase activity was observed for T_1D_1 i.e. in plant treated with 0.0 mM salicylic acid after at 21 DAS (4.005 Δ O.D. min.$^{-1}$g.$^{-1}$Fr.Wt.).



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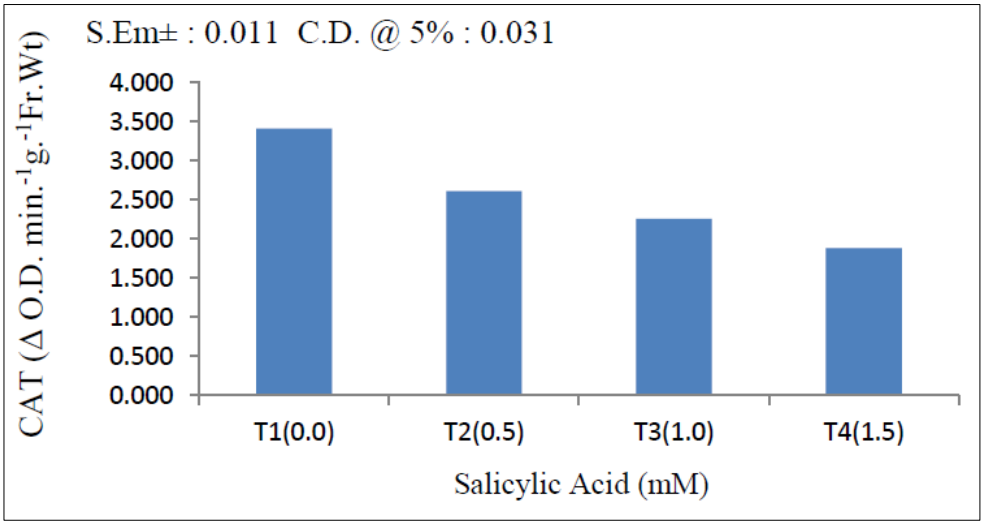

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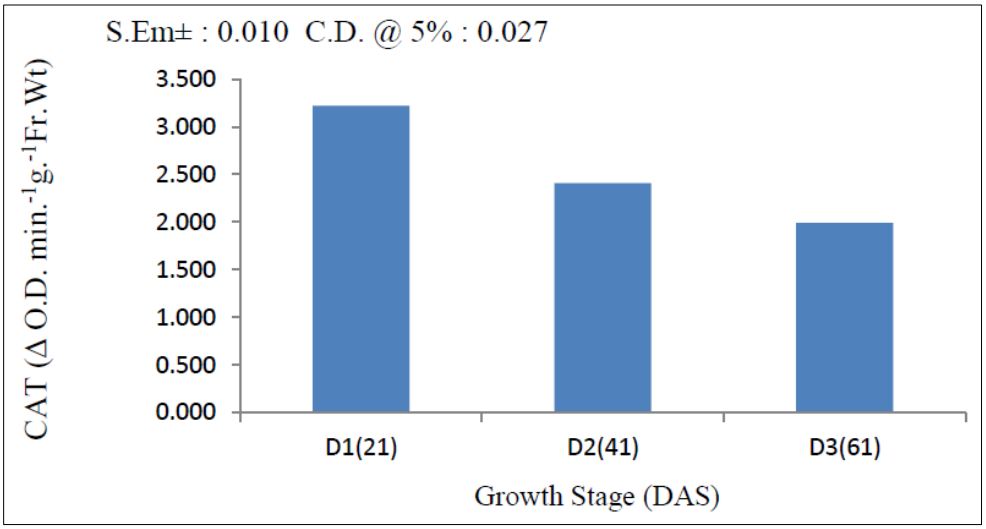

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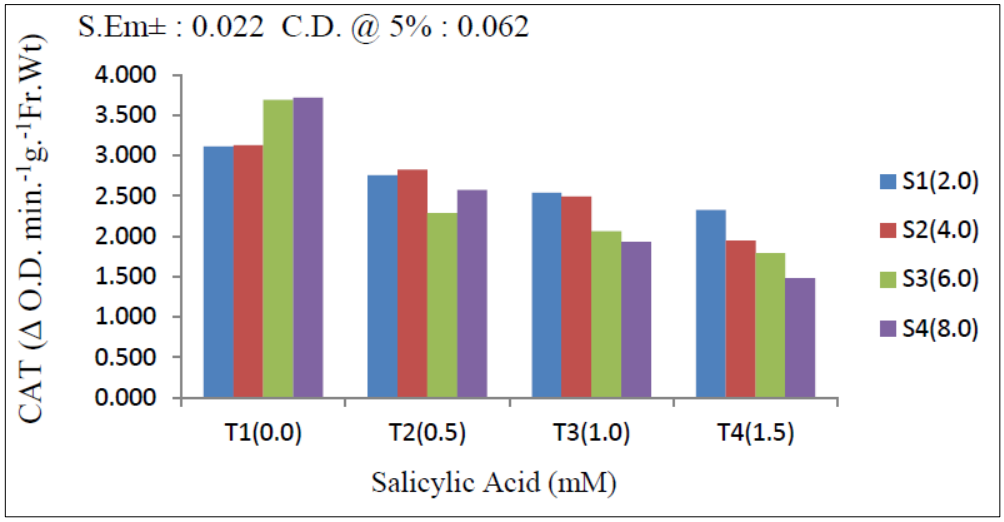
Fig 5: Mean effect of (A) Salinity, (B) Salicylic acid and (C) Growth Stage on Catalase (Δ O.D. min.$^{-1}$g.$^{-1}$Fr.Wt) in seedling of Black gram.



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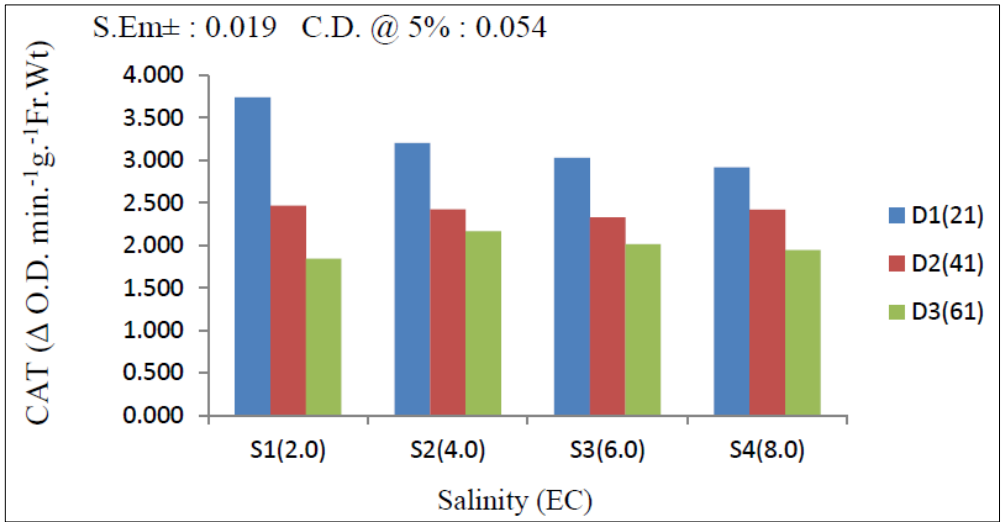

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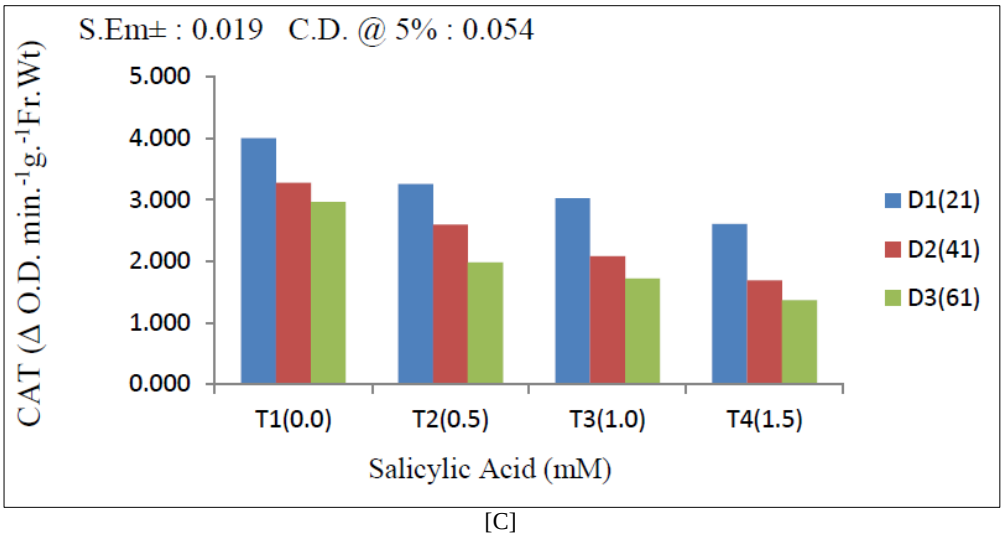

[C]

Fig 6: Interaction effect of (A) Salinity (EC) × Salicylic acid (mM), (B) Salinity (EC) × Growth Stage (DAS) and (C) Salicylic Acid (mM) × Growth Stage (DAS) on Catalase (Δ O.D. min.$^{-1}$g.$^{-1}$Fr.Wt) in seedling of Black gram.

This result were in agreement with Neto *et al.* (2005) [8]. They reported effects of salt stress on the activity of antioxidative enzymes and lipid peroxidation were studied in leaves and roots of two maize genotypes, BR5033 (salt-tolerant) and BR5011 (salt-sensitive), grown under control (nutrient solution) or salt stress (nutrient solution containing 100 mM NaCl) conditions salt stress had no significant effect on catalase (CAT) activity in the salt-tolerant, but it was reduced significantly in the salt-sensitive genotype.

Conclusion

From ongoing discussion experiment concluded changes in antioxidant enzymes activities of peroxidase and polyphenol oxidase in leaf was increased with higher concentration of salt

stress but catalase activity was decrease. Antioxidant enzymes activity showed similar trends but higher dose of salicylic acid decreased the catalase and polyphenol oxidase enzyme activity. Salicylic acid can also be helpful in yield improvement and prevent adverse effect of salt stresses.

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