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An analysis of the effects of various levels of salicylic acid on the protective enzyme activity of red beet

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Abstract
Enzymes are biological catalysts that increase the rate of chemical reactions without being consumed or altered. Studies show that foliar application of salicylic acid (SA) can alter enzyme activity in plants. The objective of this study was to investigate the effects of different concentrations of SA (0.4, 0.8, 1.2, 1.6, 2.0, 2.4, 2.8, and 3.2 mM) on antioxidant enzyme activity in *Beta vulgaris* L. plants. Activity of Enzymes like Ru BP Case (Ribulose-1,5-Bisphosphate Carboxylase), SOD (Superoxide Dismutase), Catalase, Peroxidase (POD) and Polyphenol Oxidase (PPO) were measured. The plants were grown in pots, and after the seedling thinning, salicylic acid treatments were applied. Concentrations of 2.8 and 3.2 mM SA caused a significant increase in polyphenol oxidase activity. No significant changes were recorded for catalase activity at any concentration of SA. Different concentrations of salicylic acid had different effects on enzyme activities in beet. Among all the enzymes, PPO shows negative correlation with all other enzymes. Higher concentrations of SA decreased SOD and POD activities, but at lower concentrations (0.4 to 1.2 mM SA) increased enzyme activities of Ru BP Case, Superoxide Dismutase, Peroxidase effectively. Therefore, Lower concentration of SA up to 1.2 mM is optimum to enhance growth, maximum concentration induce stress which affects growth of beet.

Keywords: antioxidant enzymes, enzyme activity, foliar application, salicylic acid

Introduction
Garden beet, table beet, or beetroot (*Beta vulgaris* L.) is a bi-annual plant with leafy stems that belongs to the Chenopodiaceae family. Since ancient times, the red beet has been used as a source of nutrition. Beetroots and leaves have long been used in traditional medicine to treat a variety of ailments, including fever and constipation, which the ancient Romans used to treat [1]. Use of beetroot as an antiproliferative, blood building tonic, an inhibitor of calcium oxalate crystal and diuretic, anticoagulant also influences the release of nitric oxide in humans, reduce cholesterol, and increase HDL levels [2, 3, 4]
Plant growth regulators can improve physiological efficiency by affecting photosynthesis, flower and fruit formation, and overall productivity [5]. Foliar application of elements can greatly influence plant characteristics and yield than soil application [6]. Among all Plant hormones/phytohormones applied to the plants, SA effectively influences plant growth and responds to environmental cues. At very low doses, phytohormones act as endogenous signals that regulate various physiological functions. With some exceptions, SA is usually present in the form of conjugates. Salicylic acid (SA) is a phenolic derivative that can be found in a variety of plant species. SA is biosynthesized from the shikimate pathway [7]. SA plays a controversial role in plant growth and development, depending on its concentration, plant growth conditions, and developmental stages. SA directly affects plant thermogenesis, growth, flower induction, and ion exchange [8]. It also affects stomatal movement, ethylene biosynthesis and reverses the effects of Abscisic Acid on leaf abscission [9]. By consuming H_2O_2 in the cytosol, vacuole, cell wall, and extracellular space, peroxidase plays an important role in the biosynthesis of lignin and the defense against biotic stresses [10]. SA can influence catalase and peroxidase activities positively or negatively depending on H_2O_2 concentration [11]. Polyphenol oxidase catalyzes the oxygen-dependent oxidation of phenols to quinines, is found in all angiosperms, and is thought to be involved in pest and pathogen defense [12]. SA binds to catalase specifically and inhibits its activity [13]. SA's growth-promoting effects could be attributed to hormonal changes or improvements in photosynthesis, transpiration, stomatal conductance, antioxidant enzyme activities, and osmoregulation [14, 15, 16, 17]. Exogenous application of SA can increase plant tolerance to environmental stresses by regulating the activities of intracellular antioxidant enzymes like Superoxide Dismutase and Peroxidase [18, 19]. Therefore, the present investigation was undertaken to study the impact of foliar application of salicylic acid on the enzyme activity and plant growth in red beet.

Material and method
The seed of beet variety Detroit dark red was obtained from a Kalash seed company in Jalna, Maharashtra, to investigate the effects of different concentrations of salicylic acid treatments. The foliar treatment included a series of increasing salicylic acid concentrations of 0.4, 0.8, 1.2, 1.6, 2.0, 2.4, 2.8, and 3.2 mM and control with

replicates. RuBPCase (Ribulose-1,5-Bisphosphate Carboxylase), SOD (Superoxide Dismutase), catalase, peroxidase and polyphenol oxidase were measured at the time of harvesting using the fully expanded leaf.

Enzyme extraction
A frozen leaf sample (25 mg) was homogenized in 5.0 ml. 50 mM sodium phosphate buffer (pH 7.8) using a pre-chilled mortar and pestle; the samples were then centrifuged at 15,000x g for 20 minutes at 4 °C. For analysis, the supernatant was kept at 4 °C and used for SOD, CAT, POD and PPO enzyme assay.

SOD (Superoxide Dismutase)
The superoxide dismutase activity was estimated using the modified Giannopolitis and Ries method [20]. In a 2.0 mL reaction mixture, 50 mM sodium phosphate buffer with 0.1 mM EDTA, 12 mM methionine, 75 mM Nitro blue tetrazolium chloride (NBT), 50 mM Na_2CO_3, and 100 mL enzyme extract were used, with 100 mL buffer being used instead of enzyme extract in the blank reaction mixture. Following that, 200 µL of 0.1 mM Riboflavin was added to each mixture. The tubes were shaken and irradiated for 15 min under the fluorescent light (15 W). Each solution's absorbance was measured at 560 nm. Under the experimental conditions, one unit of SOD represents the quantity of enzyme that inhibited NBT reduction by 50%.

Catalase
The catalase activity was determined according to Zhang [21]. The assay mixture for catalase contained 100 µL of H_2O_2 (300 mM), 100 µL of enzyme extract, and 2.8 mL of 50 mM potassium phosphate buffer with 2 mM EDTA (pH 7.0). The Catalase activity was determined by measuring the decrease in absorbance at 240 nm caused by the disappearance of H_2O_2.

Peroxidase
The peroxidase activity was determined according to Zhang [21]. 100 µL enzyme extract, 100 µL guaiacol (1.5%, v/v), 100 µL H_2O_2 (300 mM), and 2.7 mL 25 mM potassium phosphate buffer with 2 mM EDTA (pH 7.0) made up the 3 mL peroxidase reaction mixture. At 470 nm, the increased rate of absorbance was spectrophotometrically measured.

Polyphenol Oxidase
The following method described by Tagele [22] was used to determine polyphenol oxidase activity. The reaction mixture contained of extract (2 mL), 3 mL of 0.1M sodium phosphate buffer at pH 7.0, and 1 mL of 0.01M catechol. At each one-minute interval, the mixture was incubated for 5 minutes at 28°C, and absorbance was measured immediately against a substrate blank at a wavelength of 495 nm. The activity of polyphenol oxidase was expressed as µM. g^{-1} D.W. [23].

RuBPCase (Ribulose-1, 5-Bisphosphate Carboxylase)
The experiment was performed using the sage method [24]. For RuBPCase the frozen leaf disc (from fully expanded green leaf) was ground to a fine powder for each sample in a pre-cooled mortar and pestle. Then, the leaf powder was homogenized in a 4.0 ml extraction buffer, pH 8.0, at 0-4°C. The buffers were prepared CO_2 free prior to the addition of $NaHCO_3$. The sample extract was quickly transferred to two 1.8-ml microcentrifuge tubes and centrifuged for 15 seconds at 8000x g after it had been ground. In subsequent assays, the supernatant was used. Rubisco's initial activity was measured immediately after extraction and final activity after a 12- to 15-minute incubation at 25°C in a buffer containing 10 mM $NaHCO_3$ and 20 mM $MgCl_2$ to fully carbamylate Rubisco. Rubisco activity was determined by measuring the rate of NADH oxidation at 340 nm on a Shimadzu UV-1900 spectrophotometer[25]. Rubisco carboxylase activity was measured in µmol CO_2 m^{-2} leaf area s^{-1}.

Statistical analysis
Software SPSS 16.0 was used to analyze data to calculate the mean value, standard deviation, and least significant difference (LSD) in the beet for each treatment and control. To test their significance, simple correlation coefficients were calculated using the bivariate correlation method (Pearson's correlation coefficient) at the probability levels of 0.05 and 0.01.

Result and discussion
SOD (Superoxide dismutase)
Metal-containing enzymes called superoxide dismutase catalyze the dismutation of superoxide radicals to oxygen and hydrogen peroxide. Because the production of oxygen radicals can be exacerbated by environmental stress, SOD has been proposed as a key factor in plant stress tolerance. Salicylic acid treatments and their effects on SOD activity have been shown in Fig., 2. The significant increase in SOD activity, i.e., 1363 ± 2.0 and 1374 ± 6.1 Units g^{-1} D.W. was recorded at 0.4 and 0.8 mM SA treatment over the control (1341 ± 9.5) at $P<0.05$ level.

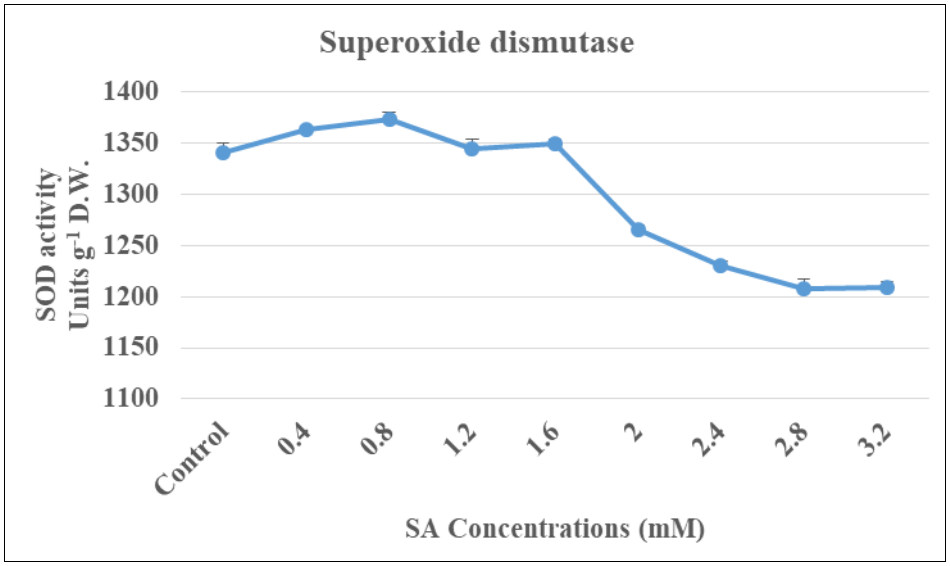

Fig 2: Study on SOD activity Under Increasing Concentrations of Salicylic Acid. Values are given in mean ± S.D. Bars in each group show significant difference at $p < 0.05$

In the present study the maximum enzyme activity recorded at 0.8 mM SA, on the contrary the enzyme activity decreases as SA concentration increases. All of the highest concentrations of salicylic acid treatments, ranging from 2.0 to 3.2 mM, resulted in a significant decrease in SOD activity. Yusuf [26] reported that SA application increased the activity of SOD under stress, same trend was observed in our study but at higher levels of SA application decreases SOD activity. In maize pretreatment with SA activated the antioxidant enzymes, the study by Janda [27]. The increased activity of antioxidant enzymes like SOD, CAT, and APX after treatment with SA is linked to the metabolism of H_2O_2 caused by cold, resulting in cold stress resistance in banana seedlings [28]. SA maintained redox homeostasis by balancing reactive oxygen species generation and detoxification by inducing adaptive responses and antioxidant enzyme accumulation like SOD, CAT, POD, and osmolytes such as proline [29].

Catalase

Catalase is an enzyme that catalyzes the decomposition of H_2O_2 into water and oxygen and is found in nearly all living organisms exposed to oxygen. It is a crucial enzyme for protecting cells from oxidative stress caused by reactive oxygen species (ROS). Catalase activity was determined for all salicylic acid treatments with the control, as represented in Fig., 3. Red beet had different responses in catalase activity at different levels of SA; however, at the $P<0.05$ level, no significant difference was recorded among all the salicylic acid concentrations but maximum activity was recorded at 0.8 mM SA. Catalase and Peroxidase, which use ascorbate as a hydrogen donor, convert H_2O_2 to oxygen and water; SA inhibits CAT, resulting in H_2O_2 accumulation30, [31]. On the other hand, other studies found that after SA treatment, CAT activity increased [32].

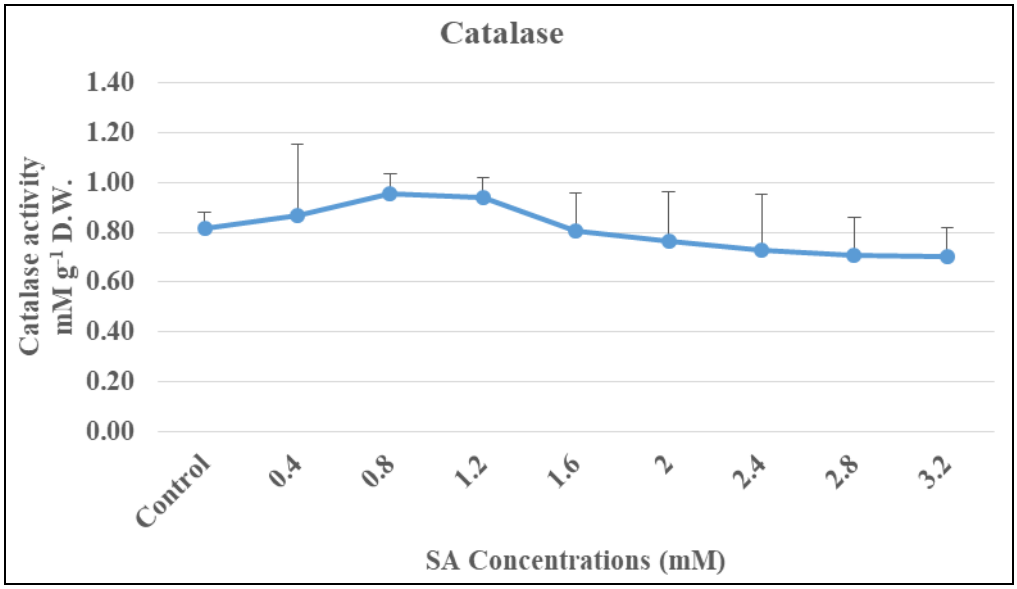

Fig 3: Study on Catalase activity Under Increasing Concentrations of Salicylic Acid. Values are given in mean ± S.D. Bars in each group show significant difference at $p < 0.05$

Peroxidase

Peroxidase uses the free radical mechanism to catalyze an oxidation-reduction reaction that converts several compounds into oxidized or polymerized products. Salicylic acid treatment and its effects on peroxidase activity are shown in Fig.4. The significantly increased peroxidase activity (1.22 ± 0.2 *mM g*$^{-1}$ *D.W.*) was recorded till 0.8 mM salicylic acid treatments which was highest. Salicylic acid treatments showed a significantly decreased peroxidase activity from 2.8 mM SA concentration. Antioxidant activity may be increased due to the SA being involved in the regulation of important plant physiological processes. This finding was in line with those of Mittler, Hussein and Jaiswal [33, 34, 35]. A study by Karalija and Paric [36] foliar application of salicylic acid recorded increased leaf area, secondary metabolites, and peroxidase activity of *Ocimum basilicum* L. In Capsicum annuum L. Different levels of SA had different effects on enzyme activities concentrations 3 mM of SA decreased enzyme activities [37].

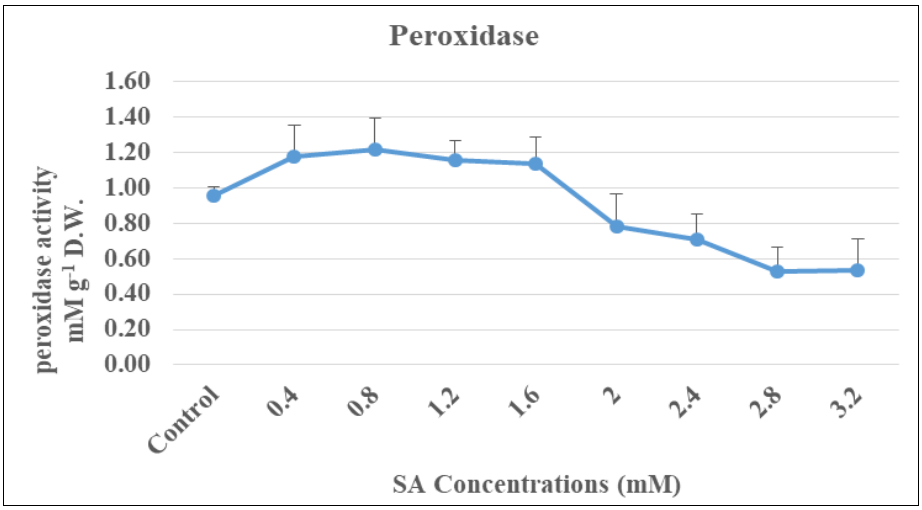

Fig 4: Study on POD activity Under Increasing Concentrations of Salicylic Acid. Values are given in mean ± S.D. Bars in each group show significant difference at p < 0.05

Polyphenol oxidase

Polyphenol oxidase is a copper-containing metalloprotein that catalyzes the conversion of phenolic compounds to quinones, which result in brown pigments in injured tissue. The observations for the various concentrations of salicylic acid treatment and their effects on polyphenol oxidase activity have been depicted in Fig., 5. Increased polyphenol oxidase activity, i.e., 1.81 ± 0.2 and 1.88 ± 0.3 *μM g*$^{-1}$ *D.W.* was recorded at salicylic acid treatments of 2.8 and 3.2 mM compared to control, i.e., 1.15 ± 0.2 *μM g*$^{-1}$ *D.W.*, at the P<0.05 level. Corn had the highest peroxidase, polyphenol oxidase, and lowest malondialdehyde activity after a 0.5 percent SA foliar application under a water deficit stress [38]. Chandra [39] reported two SA (1.4 mM SA, pH 6.5) applications followed by inoculation with *Rhizoctonia solani* resulted in increased isoforms 7 and 10 of polyphenol oxidase and isoform 4 of peroxidase. On the contrary, at lower concentrations, salicylic acid (0.5 and 1 mM) decreased PPO activity in the presence of 200 mM NaCl in wheat seedlings [40]. SA spraying at 100 mL/L decreased concentrations of polyphenol oxidase in basil under Lead Stress [41]. SA can decrease polyphenol oxidase activity at lower concentrations. In present study, the enzyme like SOD, CAT, POD and RuBPCase decreased with increased concentration of salicylic acid from 2.4 mM treatment/application, but PPO shows reverse trend compared to above mentioned enzymes PPO increases with increasing SA concentration

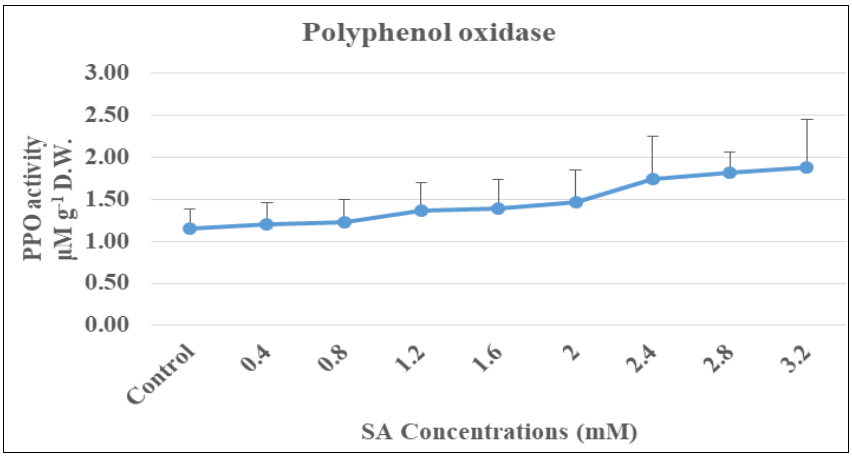

Fig 5: Study on PPO activity Under Increasing Concentrations of Salicylic Acid. Values are given in mean ± S.D. Bars in each group show significant difference at p < 0.05

RuBPCase (Ribulose-1, 5-Bisphosphate Carboxylase)

RuBPCase (Ribulose 1, 5-bisphosphate carboxylase/oxygenase) is the keystone of atmospheric CO2 fixation. It catalyzes the addition of CO_2 to enolized ribulose 1,5-bisphosphate (RuBP), resulting in the formation of 3-phosphoglycerate, which is then converted to sugars. Salicylic acid treatments and their effects on RuBPCase activity in Fig., 1. The significant increase in initial RuBPCase activity (141 ± 6.1 µ mole m^{-2} leaf area s^{-1}) was recorded at 1.2 mM SA treatment over the control (115 ± 5.3) at P<0.05 level. The increase in total RuBPCase activity, i.e., 174 ± 13.0 and 175 ± 13.2, was recorded at 0.8 and 1.2 mM SA treatment. There was no significant reduction in initial/total RuBPCase activity at highest concentrations of salicylic acid treatments. SA has been shown to influence plant growth, production, and physiological and biochemical activation [42]. Stevens [15] studied the SA influencing the content and activity of rubisco catalyzing the carbon dioxide in salt-stressed plants. Application of SA (10^{-2}, 10^{-5} M, and 0.1 mM) improved the ratio of photosynthesis in *B. juncea* and tomatoes and rubisco activity in maize [43, 15, 26].

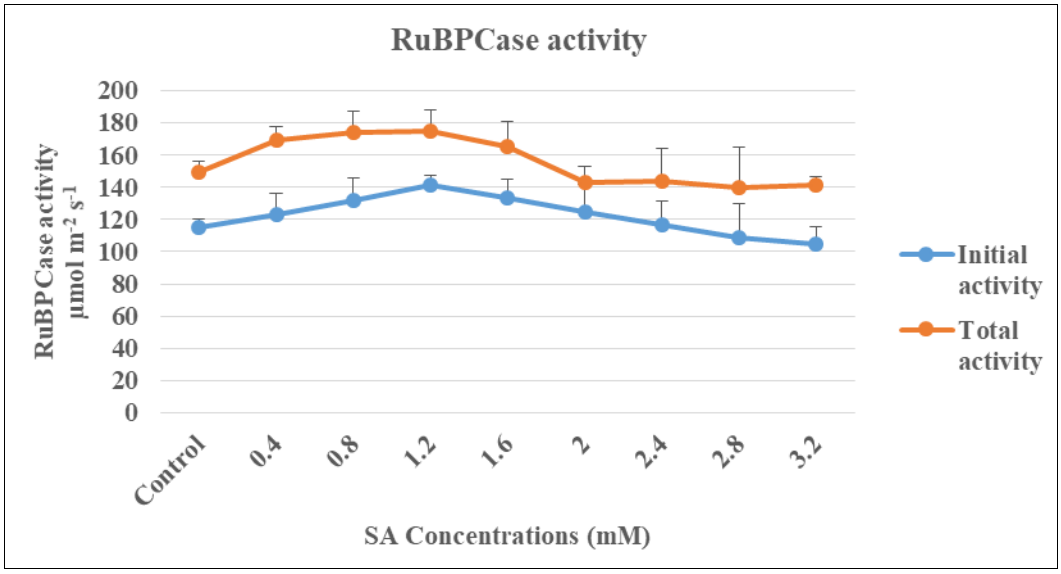

Fig 1: Study on RuBPCase activity Under Increasing Concentrations of Salicylic Acid. Values are given in mean ± S.D. Bars in each group show significant difference at p < 0.05

Correlation studies between enzyme activity parameters

The interrelationships between antioxidant enzymes (SOD, CAT, POD and PPO) and photosynthetic enzymes (RuBPCase.) was determined by correlation studies to find out association of enzymes under SA application. The correlation studies among enzyme activity parameters of the beet revealed significantly (P < 0.01) strong positive correlation of superoxide dismutase with peroxidase (r = 0.903), catalase (r = 0.516) and Ribulose-1,5-Bisphosphate Carboxylase (r = 0.695) (Table 1).

While polyphenol oxidase shows negative correlations with superoxide dismutase (-0.612, P < 0.01), peroxidase (r = -0.460, P < 0.05) and Ribulose-1,5-Bisphosphate Carboxylase (r = -478, P < 0.05). Furthermore, correlation between catalase and polyphenol oxidase was non-significant (Table 1). Positive correlations between proline, SOD, CAT, and POD activities in the studied varieties at low SA concentrations (10^{-5} M) suggest that increases in CAT and POD activities accompanied SOD activity due to the high demands of H_2O_2 quenching [44]. According to Sairam and Srivastava [45], the severity of drought response is determined by the species, the plant's developmental and metabolic state, and the duration and intensity of the stress. The inhibition of CAT, an H_2O_2-scavenging enzyme, by SA is important in the generation of ROS [31]. In barley, a different pattern of antioxidants was observed in the presence of abiotic stress in both SA-primed and SA-free conditions [46].

Table 1: Correlation studies between enzyme activity parameters.

	SOD	POD	CAT	PPO
POD	0.903**			
CAT	0.516**	0.624**		
PPO	-0.612**	-0.460*	-0.312	
RuBPCase	0.695**	0.717**	0.608**	-478*

****** Correlation is significant at the 0.01 level. ***** Correlation is significant at the 0.05 level.

SOD: Superoxide Dismutase, POD: Peroxidase, CAT: Catalase, PPO: Polyphenol Oxidase, RuBPCase: Ribulose-1, 5-Bisphosphate Carboxylase.

Conclusion

The application of SA can increase the antioxidant activity in beet compared to untreated plants. The current findings revealed that an among all the concentration of salicylic acid, 0.8 mM was best treatment to enhance

antioxidant and photosynthetic activity in the form of RuBPCase activity which can directly affects the plant growth and metabolism, while a higher concentration of SA reduced antioxidative enzyme activity and photosynthetic activity. The biochemical process was affected by the higher 2.8 and 3.2 mM SA concentrations, which resulted in reduced photosynthetic pigments and leaf browning, which could be due to increased PPO activity. Among all the enzymes, PPO shows negative correlation with all other enzymes. Higher concentrations of SA decreased SOD and POD activities, but at lower concentrations (0.4 to 1.2 mM SA) increased enzyme activities of RuBPCase, Superoxide Dismutase, Peroxidase effectively. Therefore, Lower concentration of SA up to 1.2 mM is optimum to enhance stress tolerance, growth, maximum concentration induce stress which affects growth of beet.

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