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Differential Antioxidative and Osmolytic Responses to Salt (NaCl) Stress among Seven Bhendi (*Abelmoschus esculentus* (L.) Moench) Varieties

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ABSTRACT: Antioxidative enzyme activities and foliar contents of proline and glycine betaine along with lipid peroxidation rates and electrolytic leakage were determined in seven bhendi (*Abelmoschus esculentus* (L.) Moench) varieties (Jagruti, Mahyco, NS-810, Rasi-5, Sonal, US-7109 and ZOH-303) subjected to salt stress of different concentrations (0, 40, 80 and 120mM) as a basal dose and sampling was done in leaves on 30th Days After Treatment (DAT). The activities of antioxidant enzymes which include superoxide dismutase (SOD, EC 1.15.1.1), catalase (CAT, EC 1.11.1.6), glutathione reductase (GR, EC 1.6.4.2), ascorbate peroxidase (APX, EC 1.11.1.11) and peroxidase (POD, EC 1.11.1.7) were significantly high in the salt stressed leaves. Higher antioxidant enzyme activities were observed in the leaf extracts of Mahyco while the lowest activities were recorded with Sonal. Lower rates of membrane lipid peroxidation and electrolytic leakage were noticed in the leaves of NS-810 under salt stress. Quantitative differences were also noticed in foliar proline and glycine betaine contents among seven bhendi varieties in response to salt stress. The leaves of Mahyco and NS-810 accumulated more proline and glycine betaine under salt stress while lower content in Sonal and US-7109. Our data demonstrate that among seven bhendi varieties, Mahyco and NS-810 have efficient antioxidative characteristics which could provide better protection against oxidative stress in leaves under salt stressed conditions.

Keywords: Antioxidative enzymes, Electrolytic leakage, Lipid peroxidation, *Abelmoschus esculentus*, osmolytes, Salt stress.

I. INTRODUCTION

Plants experience a multiple of stress, of which salt stress is important one which affects tremendously the physiology of plants [1]. *Abelmoschus esculentus* (L.) Moench, is an economically important vegetable crop. As other major crops in India, bhendi is also subjected to environmental stresses, particularly salinity. The stresses most commonly associated with water deficits are drought, high salinity and low temperature [2]. When CO_2 fixation is limited because of stomata closure caused by water deficit, the rate of active oxygen formation increases in chloroplasts because an excess of excitation energy that is not dissipated by the protective mechanisms, is used to form reactive oxygen species (ROS) such as hydrogen peroxide (H_2O_2), superoxide ($.O_2^-$), hydroxyl radicals (.OH) and singlet oxygen ($^1O_2$) [3]. Plants possess defense antioxidant mechanisms, which can overcome this oxygen toxicity and delay the deleterious effects of free radicals and these ROS attack lipids, proteins and nucleic acids, causing lipid peroxidation, protein denaturation and DNA mutation [4]. The enzymatic antioxidative system includes superoxide dismutase (SOD), which catalyze the reaction from superoxide ($.O_2^-$) to H_2O_2 and catalase (CAT), guaiacol-type peroxidases and enzymes of the ascorbate-glutathione cycle, e.g. ascorbate peroxidase (APX), peroxidase (POD) and glutathione reductase (GR), which function to detoxify the H_2O_2 produced [5]. The rate of lipid peroxidation level in terms of MDA can be used as an indication to evaluate the tolerance of plants to oxidative stress as well as the sensitivity of plants to salt stress [6]. Cell membrane stability is frequently related to salt tolerance in plants and electrolytic leakage is usually used as an indicator of membrane injuries in salt treated plants [7]. Accumulation of protective solutes like proline and glycine betaine is an unique plant response to environmental stresses, particularly to salt stress [8]. The main objective of the present study was to compare the antioxidative characteristics and osmotic protectents among seven bhendi varieties under salt stress with an aim to screen for certain efficient varieties, which could be used for producing better yields even under adverse environmental conditions.

II. MATERIALS AND METHODS

The certified Bhendi (*Abelmoschus esculentus* (L.) Moench) seeds (Varieties: Jagruti, Mahyco, NS-810, Rasi-5, Sonal, US-7109 and ZOH-303) were procured from Tamil Nadu Agriculture University, Coimbatore. Seeds with uniform size were selected and the plants were raised in pots containing red and clay soil and pH of the soil was 7.2 with EC of 0.2 dsm^{-1}. After 20 days, seedlings were thinned and three plants of uniform vigor were maintained in each pot. Plants were grown under natural climatic conditions. The maximum irradiance (PAR, 400-700 nm) available during growth was 1800-2000 µmol $m^{-2}s^{-1}$ on a clear day. Daily maximum and minimum temperatures were 29-33°C and 20-22°C,

respectively. Plants were watered for the first 20 days after germination. The seedlings were divided into four groups. One group of seedlings were maintained under non-salinized conditions which served as control plants. The watering solution for control plants consists of tap water and one-fourth strength of Hoagland nutrients [9]. Other three groups were salinized by irrigation daily to soil capacity (500 ml d^{-1}) with the nutrient medium containing 40 mM, 80 mM and 120mM NaCl. 40mM consider as a low salinity level, 80mM consider as a medium salinity level and 120mM salinity consider as a high salinity level. All the plants used in this study were of comparable size. Sodium chloride (NaCl) used in this study was Laboratory AR grade Assay 99.8%, (Universal Laboratories Pvt. Ltd. Mumbai). Salt treatment was continued until each plant received the required mM NaCl. Care was taken for individual plants in each group received the pre-calculated concentrations of NaCl in full. Additional pots with plants were also maintained for control, as well as each salinity treatment for need of plant material. Young and fully matured leaves were taken from control and salinity treated plants on 30th Days after Treatment (DAT), for all the experiments described below.

Enzymes are extracted from leaf tissues using an ice-cold mortar and pestle, 60mg polyvinyl polypyrrolidone and 1 ml of following optimized extraction media: SOD (100mM K-phosphate buffer, pH 7.8, 0.1 mM EDTA and 0.1% Triton X-100); CAT, GR (100mM K-phosphate buffer, pH 7.0 and 0.1mM EDTA); APX (50 mM K-phosphate buffer, pH 7.0 and 1 mM ascorbate) and Peroxidase (POD) (50 mM K-phosphate buffer, pH 7.0). The resulting slurry was centrifuged at 15000Xg for 15min at 4℃. The supernatants were collected and used for the assays of protein content [10] and enzyme activities.

The activity of SOD (EC 1.15.1.1) was assayed by measuring its ability to inhibit the photochemical reduction of nitro-blue tetrazolium (11). The activity of CAT (EC 1.11.1.6) was estimated by measuring the rate of decomposition of H_2O_2 (12). GR (EC 1.6.4.2) activity was measured by oxidized GSH–dependent oxidation of NADPH (13). APX (EC 1.11.1.11) activity was estimated by monitoring the decline in absorbance at 240nm (14). POD (EC 1.11.1.7) activities were determined with guaiacol at 470nm (extinction coefficient 25.2mM cm^{-1}) (15).

Lipid peroxidation rates were determined by measuring the malondialdehyde (MDA) equivalents [16]. Electrolytic leakage was recorded by measuring the total inorganic ions leaked out in the leaves during salinity stress [17]. Proline and glycine betaine contents were estimated in leaf extracts under salinity stress [18, 19].

For statistical analysis, five samples were taken for each treatment from five individual plants. Student's t-test and Analysis of Variance (ANOVA) were applied for analyzing significant differences between the control and treated plants ($P<0.05$).

III. RESULTS AND DISCUSSION

Salinity is a common abiotic stress that severely limits crop growth and development, productivity and causes the continuous loss of arable land, which results in desertification in arid and semi-arid regions of the world [20, 21]. The high salt content lowers osmotic potential of soil water and consequently the availability of soil water to plants. The salt–induced water deficit is one of the major constraints for plant growth in saline soils. In addition, many nutrient interactions in salt–stressed plants can occur that may have important consequences for growth [22]. Reactive oxygen species (ROS) are produced as a normal product of plant cellular metabolism and salt stress lead to excessive production of ROS causing progressive oxidative damage and ultimately cell death [23]. Adaptation to salt-stress may depend on different mechanisms including the capacity to maintain high levels of antioxidants and/or through the induction of antioxidant enzymes. Figs. 1-4 and Table 1 shows antioxidant enzyme activities (SOD, CAT, GR APX and POD) in leaf extracts of seven different bhendi varieties under well watered and salt stressed conditions. Activities of all the enzymes were increased in seven bhendi varieties under salt stressed condition. However, significantly high activities of all the antioxidant enzymes were recorded in Mahyco and NS-810. For instance, maximum enhancement of SOD activity was observed in Mahyco by 69% (382.26 Units/mgpro/min) followed by NS-810 by 56% (276.35 Units/mgpro/min) under 120mM salinity treatment compared to respective control plants, while lower enhancement of SOD activity was noticed in Sonal by 24% (153.12Units/mgpro/min) followed by US-7109 by 28% (164.75Units/mgpro/min). Catalases and peroxidases (APX and POD) play an essential role in scavenging from the H_2O_2 activity [24]. The combined action of CAT and SOD converts the toxic superoxide radical ($O_2^{\bullet-}$) and hydrogen peroxide (H_2O_2) to water and molecular oxygen (O_2), thus averting the cellular damage under unfavorable conditions like salt stress [25]. The activity GR which was relatively high in salt-stressed plants might be able to increase the rate of $NADP^+$/NADPH, thereby causing availability of $NADP^+$ to accept electrons from photosynthetic electron transport chain and to facilitate the regeneration of oxidized ascorbate [26]. Higher activities of antioxidant enzymes in Mahyco and NS-810 clearly demonstrate that more efficient antioxidant system functioning significantly high rates when compared to ZOH-303, Rasi-5, Jagruti, US-7109 and Sonal.

Lipid peroxidation can be described generally as a process under which oxidants such as free radicals attack lipids containing carbon-carbon double bond(s), especially polyunsaturated fatty acids (PUFAs) [27]. One of the consequences of uncontrolled oxidative stress (imbalance between the prooxidant and antioxidant levels in favor of prooxidants) is cells, tissues, and organs injury caused by oxidative damage and high levels of free radicals or reactive oxygen species (ROS) can inflict direct damage to lipids [28]. The two most prevalent ROS that can affect profoundly the lipids are mainly hydroxyl radical ($HO^{\bullet}$) and hydroperoxyl ($HO^{\bullet}_2$). Lipid peroxidation and electrolytic leakage under salt-stress were more in Sonal and US-7109 varieties compared to Mahyco, NS-810, ZOH-303, Rasi-5 and Jagruti.

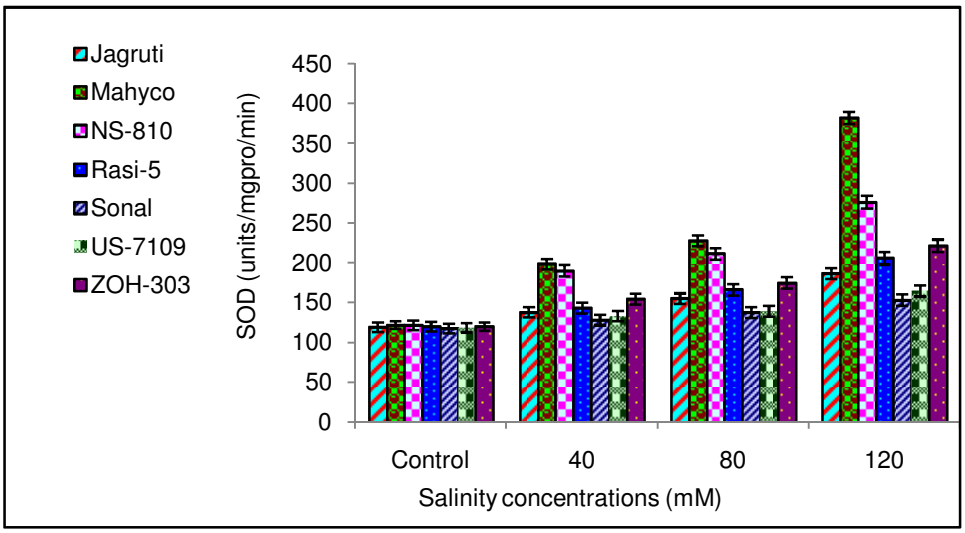

Fig. 1. Changes of superoxide dismutase activity on 30th DAT in leaves of Bhendi varieties under varying levels of salt (NaCl) stress. Each value represents mean ± SE of five independent determinations ($p<0.05$).

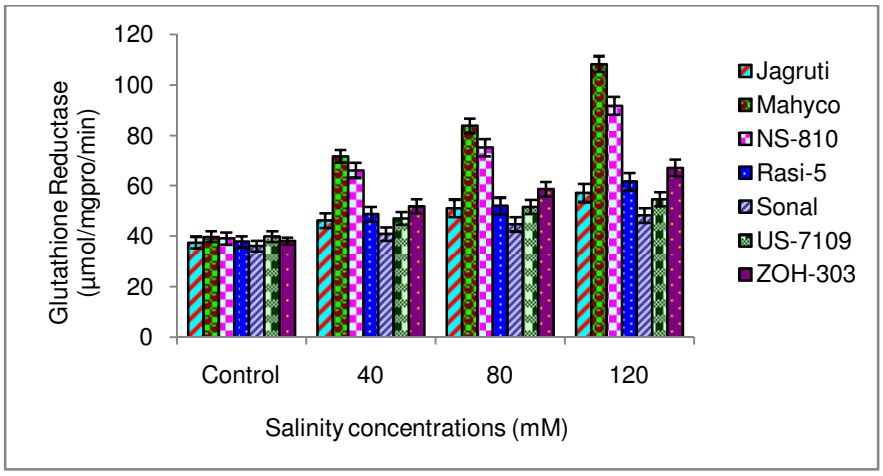

Fig. 2. Levels of glutathione reductase activity in leaves of Bhendi varieties under varying salt (NaCl) concentrations on 30th DAT. Each value represents mean ± SE of five independent determinations ($p<0.05$).

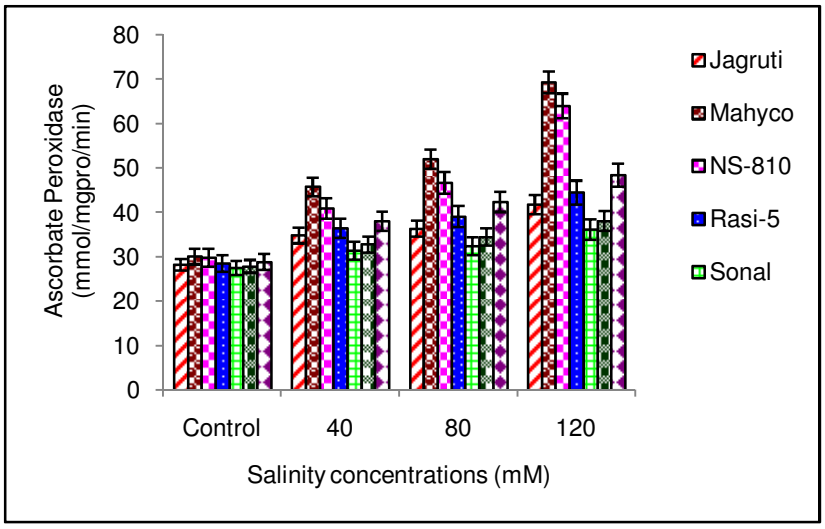

Fig. 3. Levels of ascorbate peroxidase activity in leaves of Bhendi varieties on 30th DAT under varying levels of salt (NaCl) stress. Each value represents mean ± SE of five independent determinations ($p<0.05$).

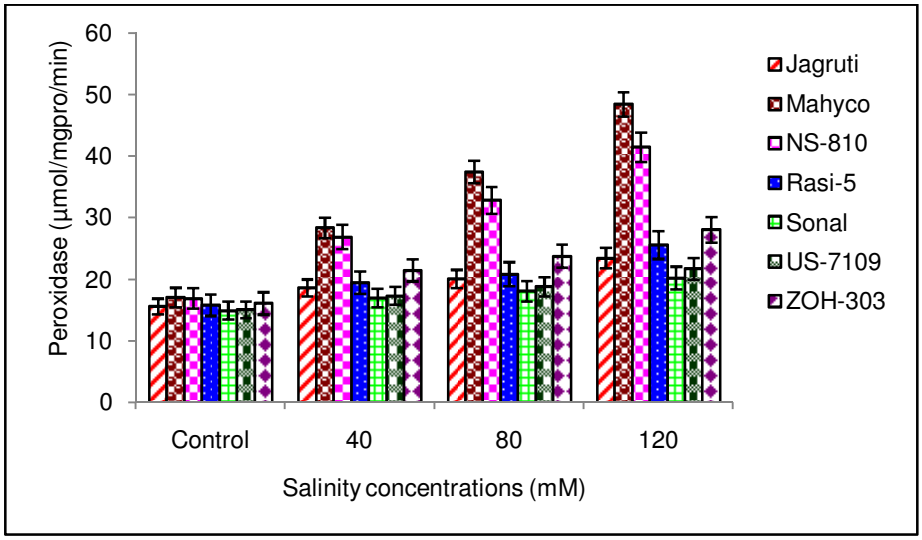

Fig. 4. Variation of peroxidase activity in leaves of Bhendi varieties on 30th DAT under varying salt (NaCl) concentrations. Each value represents mean ± SE of five independent determinations ($p<0.05$).

Table 1: Changes of Catalase activity (m mol/mg pro/min) in leaves of Bhendi varieties on 30th DAT under varying levels of salt (NaCl) stress. Each value represents mean ± SE of five independent determinations ($p<0.05$).

Varieties	Salinity Concentrations (mM)			
	Control	40	80	120
Jagruti	14.34±5.85	17.42 ±6.42	19.50 ±6.67	21.04 ±6.92
Mahyco	16.97±5.02	26.75±6.66	34.50±6.85	44.62±7.49
NS-810	16.27±6.15	25.00±6.93	30.25±7.42	36.25± 7.98
Rasi-5	15.04±6.10	19.20±6.84	22.32±7.23	24.12±7.87
Sonal	13.29±5.98	14.65±6.57	16.33±6.78	17.59±7.22
US-7109	13.86±5.94	15.96±6.48	17.60±6.73	21.74±6.98
ZOH-303	15.52±5.07	20.85±6.75	23.65±6.99	27.15±7.75

F_1 =9.515748 and F_2 =15.33051 (F_1 and F_2 values indicates significant differences between the treatments and varieties respectively at 5% level)

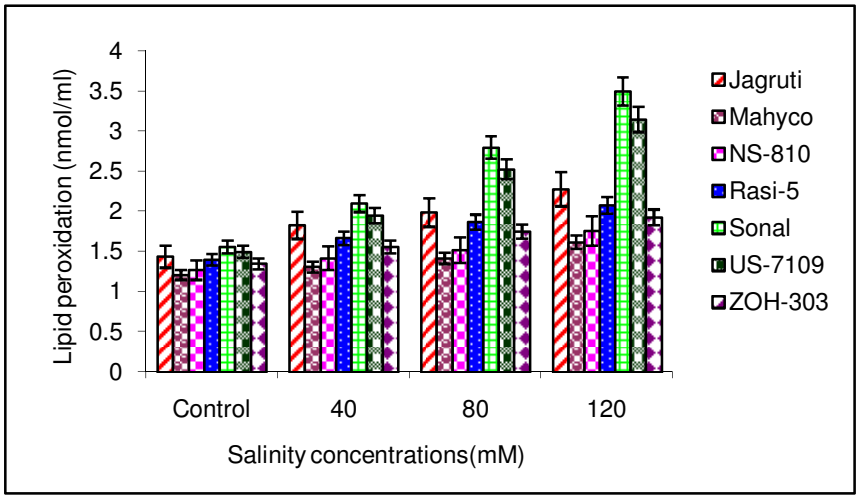

Fig. 5. Variation of lipid peroxidation in response to varying salt (NaCl) stress on 30th DAT on leaves of Bhendi varieties. Each value represents mean ± SE of five independent determinations ($p<0.05$).

Higher MDA content was observed in the variety Sonal followed by US-7109 and it was 3.50 nmol/ml and 3.15nmol/ml, respectively, under high salinity (120mM), while lowest MDA content was observed in Mahyco (1.62nmol/ml) followed by NS-810 (1.76nmol/ml). Lipid peroxidation is a destructive chain reaction and it can directly damage the structure of membrane [29].

Salt stress affected the activity of plasmamembrane ATPase activity and peroxidation of membrane lipids is an indication of membrane damage and leakage under salt stress conditions [30]. The electrolyte leakage is mainly caused by the efflux of K^+ and so-called counter ions (Cl^-, HPO_4^{2-}, NO_3^-, citrate^{3-}, malate^{2-}) that move to balance the efflux of positively charged potassium ions [27].

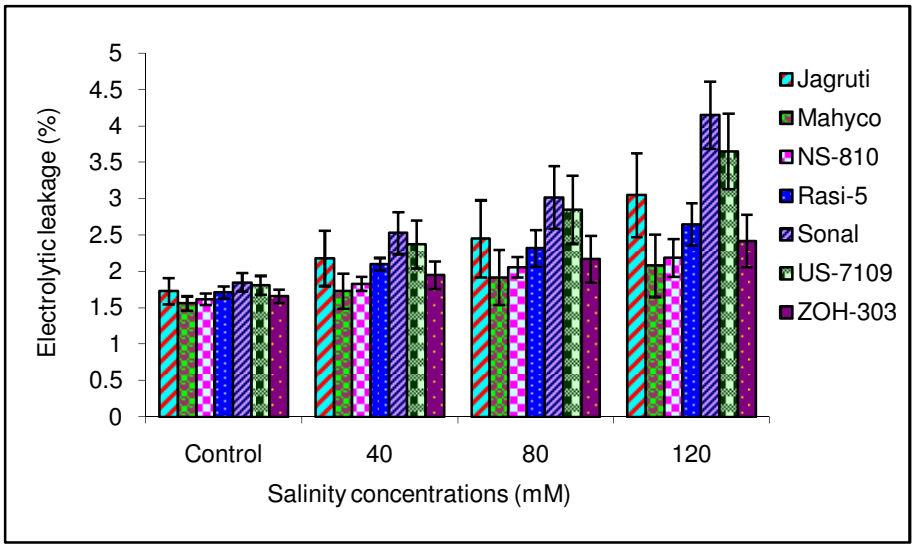

Fig. 6. Effect of different salt (NaCl) levels on electrolytic leakage in the leaf extracts of Bhendi varieties on 30th DAT. Each value represents mean ± SE of five independent determinations ($p<0.05$).

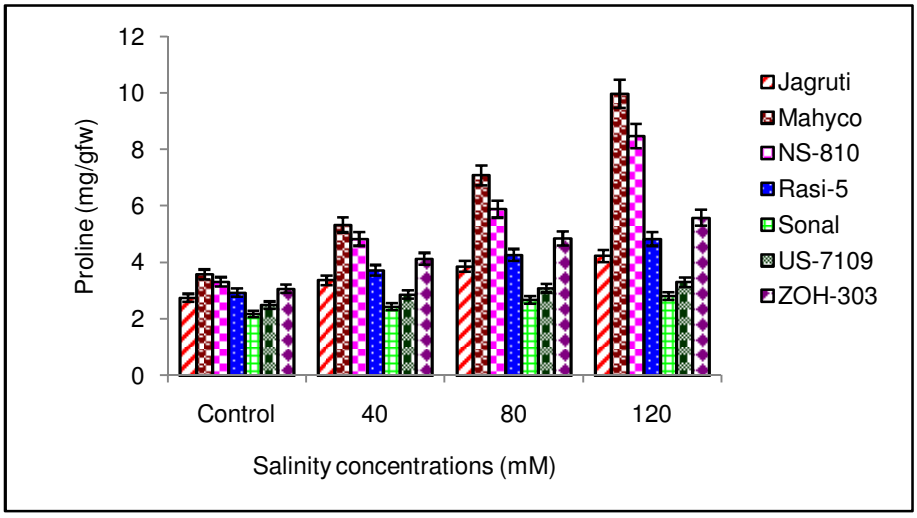

Fig. 7. Changes of proline content in leaves of Bhendi varieties on 30th DAT under varying levels of salt (NaCl) stress. Each value represents mean ± SE of five independent determinations ($p<0.05$).

Our results strongly suggest that salt stress can induce membrane lipid peroxidation resulting membrane fluidity leading to enhanced electrolytic leakage (Figs. 5, 6). In the present study, lower percentage of electrolytic leakage was noted in Mahyco (2.08%) followed by NS-810 (2.19%) relative to control plants (1.56% and 1.62%, respectively). Our data also indicate that the degree of cell membrane lipid peroxidation were relatively less in Mahyco and NS-810 under salt stress conditions.

Biochemical studies have shown that plants under salt stress accumulate a number of metabolite, termed compatible solutes, because they do not interfere with the plant metabolism [31]. Accumulation of proline could be due to *de novo* synthesis, decreased degradation, lower utilization, or hydrolysis of proteins. Extensive intercellular proline transport occurs between the cytosol, chloroplasts and mitochondria due to its compartmentalized metabolism. Although all functions of proline in stress tolerance are still a matter of debate, it is suggested that proline contributes to stabilization of sub-cellular structures, scavenging free radicals, and buffering cellular redox potential [32]. Glycine betaine (*N,N,N*-trimethylglycine) is a quaternary ammonium compound that occurs naturally in a wide variety of plants and is a dipolar but electrically neutral molecule at physiological pH [33]. Quantitative estimation of proline and glycine betaine, among the bhendi varieties were depicted in Fig. 7 and Table 2.

Mahyco and NS-810 accumulated significantly high amounts of proline and glycine betaine, while lowest in Sonal and US-7109. For instance, under higher salinity, maximum proline content was observed in Mahyco (9.99mg/gfw) followed by NS-810 (8.49 mg/gfw), while lowest proline content was noted in Sonal followed by US-7109 and it was 2.83mg/gfw and 3.32mg/gfw, respectively. Proline and glycine betaine are known to serve as nitrogen and carbon source which can be used as during recovery from the stress [34].

Table 2: Accumulation Glycinebetaine of (mg/gfw) in leaves of Bhendi varieties on 30th DAT subjected to varying levels of salt (NaCl) concentration. Each value represents mean ± SE of five independent determinations (p<0.05).

Varieties	Salinity Concentrations (mM)			
	Control	40	80	120
Jagruti	4.03±0.085	5.27±0.096	5.84±0.27	6.37±0.30
Mahyco	4.87±0.20	7.80±0.32	9.21±0.44	12.25±0.52
NS-810	4.50 ±0.46	6.85±0.53	7.92±0.62	10.40±0.89
Rasi-5	4.14±0.32	5.58±0.44	6.28±0.56	6.83±0.65
Sonal	3.20±0.084	3.61±0.28	3.96±0.37	4.25±0.46
US-7109	3.59±0.090	4.35±0.23	4.73±0.32	4.86±0.38
ZOH-303	4.26±0.24	5.86±0.38	6.75±0.47	7.50±0.56

F_1 = 11.2091 and F_2 =14.64445 (F_1 and F_2 values indicates significant differences between the treatments and varieties respectively at 5% level)

These compatible solutes also involved in cell osmoregulations and protects the photosystem II (PS II) complex by stabilizing the association of the extrinsic PS II complex proteins under salt stress. [35]. Enhanced synthesis of proline under drought or salt stress has been implicated as a mechanism to alleviate cytoplasmic acidosis and maintain $NADP^+$: NADPH at values compatible with metabolism [36]. Glycine betaine accumulates in response to stress in many crops, including spinach, barley, tomato, potato, rice, carrot, wheat and sorghum [37, 38].

IV. CONCLUSION

Our data in this study clearly demonstrate that among the seven bhendi varieties, Mahyco and NS-810 are superior with respect to the antioxidative defense system and osmotic protectant. Selection of bhendi varieties with genetic traits like antioxidants and accumulation of osmotic substances might be certainly useful in assessing the adaptive responses of bhendi to salt stress. This study can be also useful in bhendi breeding programs or transgenic bhendi research to generate plants with high antioxidant performance even under salt stressed regimes.

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Conflict of Interest. No

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