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Antioxidant enzymes and non-enzymatic antioxidants as defense mechanism of salinity stress in cowpea (*Vigna unguiculata* L. Walp)—Ife brown and Ife bpc

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Abstract

Background: Several mechanisms had been exhibited by plants to mitigate deleterious effects of salinity stress. A screen house experiment was conducted to investigate the effects of salinity stress on the activities of osmolytes (antioxidative and non-antioxidative enzymes) in the leaves of two cowpea (*Vigna unguiculata* L. Walp)—Ife brown and Ife bpc, with the aim of better understanding the biochemical mechanisms of salt tolerance. Salts of sodium chloride (NaCl) and sodium sulfate (Na_2SO_4) at 5, 10 and 15 dS/m concentrations were used for this study. The saline solution was prepared following standard methods. Proline, lipid peroxidase (LP), superoxide dismutase (SOD) and glutathione (GSH) were determined following standard protocols.

Results: Results showed that minimum proline content (12.07 mg/g) and maximum proline determination (16.05 mg/g) were observed in Ife bpc at 5 and at 15 dS/m under NaCl and Na_2SO_4 treatments. The LP content significantly increased in Ife brown at 15 dS/m under NaCl treatment and at 10 dS/m (9.49 mg/g) under Na_2SO_4 salinity. Minimum GSH content (120 µm/g) and maximum glutathione accumulation (138.97 µm/g) were observed in Ife bpc in the stressed cowpea seedlings (5 and 10 dS/m) under NaCl treatment with respect to the control. Also, SOD activities in the leaves of Ife brown increase with increase in salinity stress in both NaCl and Na_2SO_4 treatments.

Conclusions: This study concludes that the accumulation of enzymatic and non-enzymatic antioxidants is capable of detoxifying and scavenging reactive oxygen species, thereby mitigating salinity-induced oxidative damage.

Keywords: Antioxidants, Defense mechanism, Oxidative damage, Salinity, Scavenging

Background

The fundamental mechanism of salinity effect on plant function is the increase in osmotic pressure of the plants environment that inhibits the absorption of water and nutrients (Borzouei et al. 2012). Invariably, salinity inhibits photosynthesis through its effect on stomatal conductance, water and nutrient uptake and decrease in chlorophyll concentration (Khare et al. 2020; Hu et al. 2020). Mechanisms exhibited by plants to mitigate the deleterious effects of salinity include: compartmentalization of ions, synthesis of compatible solutes (osmolytes), induction of plant hormones and alteration of membrane structure (Zainab et al. 2021). During the initial stages of salinity stress, water absorption capacity of root system decreases and water loss from leaves is accelerated due to osmotic stress of high salt accumulation (Zhao et al. 2015; Bhatt 2020). Proline is a water-soluble amino acid classified as a compatible osmolyte, i.e., it does not damage cell structures at high concentrations, but is capable of lowering the cell osmotic potential (Ibrahim et al.

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2018). It performs multiple functions in stress adaptation, recovery and signaling, stabilization of proteins and protein complexes in the chloroplast, cytosol and protection of the photosynthetic apparatus in plants (Ozgur et al. 2013). Bacha et al. (2017) suggested that the application of proline successfully improved stress tolerance in plants. Production of reactive oxygen species (ROS) has been touted as one of the biochemical changes possibly occurring when plants are subjected to salinity stress (Hu et al. 2020). The photosynthetic apparatus is an important site for the production of such radicals which include superoxide (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radicals (Naveed et al. 2020). These ROS disrupt normal cellular metabolism through membrane alteration and damage to lipids, proteins and nucleic acids (Naveed et al. 2020). Plants have developed complex defense mechanism involving a series of enzymatic antioxidants, such as catalase (CAT), glutathione (GSH), superoxide dismutase (SOD), ascorbate peroxidase (APX) and guaiacol peroxidase (GPX), and non-enzymatic antioxidants which include ascorbate, carotenoids, flavonoids and other phenolics to mitigate the deleterious effects of oxidative damage (Bacha et al. 2017). Superoxide dismutase (SOD) plays an important role in scavenging of hydroxyl radicals imposed by oxidative damage (Ozgur et al. 2013; Gharsallah et al. 2016). Thus, SOD is regarded as a key enzyme for maintaining normal physiological function and managing oxidative stress in ROS (Aref and Rad 2012). Glutathione is abundant in several eukaryotic organisms where it performs plethora of functions, which include sulfide transport and regulation of redox potential of intracellular organelles (Rahman et al. 2016; Zainab et al. 2021). It is a potent reductant capable of scavenging ROS directly or in conjunction with other antioxidants (Sharma et al. 2012; Ibrahim et al. 2018). Membrane damage is sometimes regarded as an indicator of lipid destruction under various stresses (Aref and Rad 2012). Malondialdehyde (MDA) is synthesized as a result of degradation of polyunsaturated lipids by ROS. This aldehyde production has been used as an important biomarker to determine the level of oxidative stress (Khare et al. 2020). Therefore, this study investigated the effects of salinity stress on the activities of osmolytes (antioxidative and non-antioxidative enzymes) in the leaves of two cowpea (*Vigna unguiculata* L. Walp)—Ife brown and Ife bpc. This is with the aim of better understanding the biochemical mechanisms of salt tolerance.

Methods

Plant material

Seeds of cowpea (*Vigna unguiculata* L. Walp), namely Ife brown and Ife bpc, were utilized in the experiment. The seeds were collected from International institute of Tropical Agriculture (IITA), Ibadan, Nigeria. They were authenticated at the Herbarium unit of the Federal University of Technology Akure, Nigeria.

Soil and pre-planting analysis

The soil was collected at the school farm of the Federal university of Technology Akure. The following physicochemical properties of the soil were determined: particle size and textural class, organic carbon, organic matter, pH, total nitrogen, available phosphorus, potassium, sodium, calcium, magnesium, effective cation exchange capacity and exchangeable acidity. The soil was later air-dried and transferred into 48 plastic pots of about 21 cm diameter and 17 cm depth. The pots were perforated to enhance drainage during the course of the experiment.

Screen house experiment

The experimental setup was located in the screen house. The essence is to monitor the plant growth and to avoid being destroyed by rodents, contaminations and direct rainfall.

Preparation of salt solution

Salts of sodium chloride (NaCl) and sodium sulfate (Na_2SO_4) were used for this study. The saline solution was prepared following standard methods of Meot-Duros and Magne (2008).

Experimental design

A $2 \times 2 \times 4 \times 3$ randomized complete block design (RCBD) was adopted. The experimental factors include salt type (NaCl and Na_2SO_4), variety (Ife brown and Ife bpc) and salt concentrations {control, 5dS/m, 10dS/m and 15dS/m and replicated three times.

Experimental setup

The experiment was conducted at the screen house of the Department of Biology, Federal University of Technology Akure. About 5 kg of air-dried soils was filled into perforated plastic pots. Five (5) seeds of cowpea varieties (Ife brown and Ife bpc) were sown in each pot and watered regularly until the plants were fully established.

Salinity treatment

Salinization commenced four (4) weeks after planting, and each pot was administered with 200 ml of saline solution every three days till the end of the experimental period.

Non-enzymatic antioxidants assay

Lipid peroxidation

Lipid peroxidation in fresh cowpea leaf samples was determined by estimating the malondialdehyde (MDA)

following standard protocols of (Zeb and Ullah 2016). About 1 g of frozen leaf samples was ground to a fine powder with liquid nitrogen and extracted with ice-cold 50 mM phosphate buffer (pH 7.0). Centrifugation of the homogenate was done for ten minutes, and 0.5 ml of the supernatant was then mixed with 1. 5 ml, 0.5% thiobarbituric acid (TBA). Incubation of the supernatant was done in a water bath at 95 °C for 25 min. The concentration of MDA was determined from the absorbance at 532 nm (correction factor was applied by subtracting the absorbance at 600 nm for unspecific turbidity) by using extinction coefficient of 155 mM^{-1} cm^{-1}.

Proline determination

0.5 g of leaf samples from each variety was homogenized in 3% (w/v) sulfosalicylic acid, and the homogenate filtered was then filtered using Whatman's filter paper following standard methods of Bates et al. (1973). The mixture was heated at 100 °C for 1 h in water bath after acid ninhydrin and glacial acetic acid have been added. The reaction was then stopped by ice bath. The mixture was extracted with toluene, and the absorbance of fraction aspired from liquid phase was read at 520 nm. Proline concentration was determined using calibration curve and expressed as µmol proline g^{-1} FW.

Glutathione (GSH)

The method of Beutler et al. (1963) was adopted in the estimation of the level of reduced glutathione (GSH) in the leaves of cowpea. Sample homogenate (0.2 ml) was added to 1.8 ml of distilled water, and 3 ml of the precipitating agent, sulfosalicylic acid, was mixed with the sample. This was centrifuged at 3,000 g for 4 min. Thereafter, 0.5 ml of the supernatant was added to 4.5 ml of Ellman reagent. A blank was prepared with 0.5 ml of the diluted precipitating agent and 4 ml of phosphate buffer and 0.5 ml of Ellman's reagent. The absorbance of the reaction mixture was taken within 30 min of color development at 412 nm against a reagent blank. The concentration of the GSH was extrapolated from the GSH standard curve.

Antioxidant enzyme assays

Superoxide dismutase (SOD)

Total SOD (EC 1.15.1.1) activity was determined by measuring its ability to inhibit the photochemical reduction of nitrotetrazolium blue chloride (NBT), as described by Giannopolitis and Ries (1977). The reaction mixture (1.5 mL) contained 50 mM phosphate buffer (pH 7.8), 0.1 µM ethylenediaminetetraacetic acid (EDTA), 13 mM methionine, 75 µM NBT, 2 µM riboflavin and 50 µL enzyme extract. Riboflavin was added, and the tubes were subjected to mechanical shaker and later illuminated with two 20-W fluorescent tubes. The reaction was allowed to proceed for 15 min after which the lights were switched off and the tubes covered with a black cloth. Absorbance of the reaction mixture was read at 560 nm. One unit of SOD activity (U) was defined as the amount of enzyme required to cause 50% inhibition of the NBT photoreduction rate, and the results were expressed as units mg^{-1} protein.

Statistical analysis

Data collected were subjected to one-way analysis of variance (ANOVA), and means were separated with Duncan Multiple Range Test (DMRT) at 0.05 probability level (SPSS version 21.0).

Results

Proline accumulations in the leaves of Ife brown (IFB) and Ife bpc (IBPC) under NaCl and Na_2SO_4 salinity are shown in Table 1. Minimum proline content (12.07 mg/g) and maximum proline determination (16.05 mg/g) were observed in Ife bpc at 5 and at 15 dS/m under NaCl and Na_2SO_4 treatments. There was significant difference in the proline content of Ife brown and Ife bpc at 15 dS/m under both treatments. Also, there was significant difference in the maximum proline (14.85 mg/g) that was recorded in Ife brown at 5 dS/m relative to the control.

Lipid peroxidase (LP) accumulations in the leaves of Ife brown (IFB) and Ife bpc (IBPC) under NaCl and Na_2SO_4 salinity are shown in Table 2. Maximum lipid peroxidation was recorded in Ife bpc at 5 dS/m under Na_2SO_4 treatment. However, minimum MDA content was also noticeable in Ife brown at 10 dS/m under Na_2SO_4 salinity. The LP content significantly increased in Ife brown at 15 dS/m under NaCl treatment and at 10 dS/m (9.49 mg/g) under Na_2SO_4 salinity. Meanwhile, Ife bpc did not record

Table 1 Proline accumulations in the leaves of Ife brown (IFB) and Ife bpc (IBPC) under NaCl and Na_2SO_4 salinity stress

Treatment	Conc (dS/m)	Biochemical parameters (mean ± SE)	
		IFB proline (mg/g)	IFBPC proline (mg/g)
NaCl	5.00	13.06 ± 0.14b	16.05 ± 0.04d
	10.00	14.15 ± 0.40bc	12.69 ± 0.03b
	15.00	14.53 ± 0.42 cd	12.07 ± 0.04a
Na_2SO_4	5.00	14.85 ± 0.21d	15.76 ± 0.00e
	10.00	13.75 ± 0.19b	14.38 ± 0.04d
	15.00	12.94 ± 0.24a	13.34 ± 0.04c
Distilled H_2O	0.0	13.77 ± 0.05b	14.26 ± 0.43d

Means followed by different letters in the column are significantly different ($P < 0.05$) from one another using Duncan's new multiple range test (DNMRT)

Table 2 Lipid peroxidase (LP) accumulations in the leaves of Ife brown (IFB) and Ife bpc (IBPC) under NaCl and Na_2SO_4 salinity stress

Treatment	Conc (dS/m)	Biochemical parameters (mean ± SE)	
		IFB lipid peroxidase (mg/g)	IFBPC lipid peroxidase (mg/g)
NaCl	5.00	1.29 ± 0.02a	1.63 ± 0.03a
	10.00	5.37 ± 0.29c	5.91 ± 0.06d
	15.00	6.02 ± 0.25d	5.33 ± 0.03c
Na_2SO_4	5.00	1.40 ± 0.03a	9.49 ± 0.01f
	10.00	1.10 ± 0.03a	4.58 ± 0.16b
	15.00	6.82 ± 0.06e	8.70 ± 0.03e
Distilled H_2O	0.0	3.20 ± 0.17b	1.63 ± 0.13a

Means followed by different letters in the column are significantly different ($P < 0.05$) from one another using Duncan's new multiple range rest (DNMRT)

any induction of lipid peroxidase at 5 dS/m under NaCl treatment.

Glutathione (GSH) contents in the leaves of Ife brown (IFB) and Ife bpc (IBPC) under NaCl and Na_2SO_4 salinity are shown in Table 3. Minimum GSH content (120 µm/g) and maximum glutathione accumulation (138.97 µm/g) were observed in Ife bpc in the stressed cowpea seedlings (5 and 10 dS/m) under NaCl treatment with respect to the control. Maximum GSH (134 µm/g) was also recorded in Ife brown at 15 µm/g under NaCl treatment. The minimum GSH content (125.75 µm/g) in Ife brown was observed at 5 dS/m under NaCl treatment. There were significant differences in the GSH accumulations for Ife brown at 15 dS/m under NaCl and Na_2SO_4 stresses. Significant increase was also recorded in Ife bpc at 5 dS/m under NaCl and Na_2SO_4 treatments.

Superoxide dismutase (SOD) activities in the leaves of Ife brown (IFB) and Ife bpc (IBPC) under NaCl and Na_2SO_4 salinity are shown in Table 4. SOD activities in

Table 3 Glutathione (GSH) contents in the leaves of Ife brown (IFB) and Ife bpc (IBPC) under NaCl and Na_2SO_4 salinity stress

Treatment	Conc (dS/m)	Biochemical parameters (mean ± SE)	
		IFB GSH (µm/g)	IFBPC GSH (µm/g)
NaCl	5.00	125.75 ± 0.11b	138.97 ± 0.11d
	10.00	130.42 ± 0.12d	123.65 ± 0.13ab
	15.00	132.29 ± 0.21e	120.64 ± 0.12a
Na_2SO_4	5.00	128.52 ± 0.13c	138.29 ± 0.12d
	10.00	126.04 ± 0.26b	131.49 ± 0.12c
	15.00	123.81 ± 0.23a	125.30 ± 0.87b
Distilled H_2O	0.0	127.37 ± 1.36bc	131.91 ± 2.76c

Means followed by different letters in the column are significantly different ($P < 0.05$) from one another using Duncan's new multiple range test (DNMRT)

Table 4 Superoxide dismutase (SOD) activities in the leaves of Ife brown (IFB) and Ife bpc (IBPC) under NaCl and Na_2SO_4 salinity stress

Treatment	Conc (dS/m)	Biochemical parameters (mean ± SE)	
		IFB SOD (%)	IFBPC SOD (%)
NaCl	5.00	38.87 ± 0.18b	45.24 ± 1.37d
	10.00	42.11 ± 0.27c	42.86 ± 0.01cd
	15.00	48.81 ± 0.20d	28.67 ± 0.06b
Na_2SO_4	5.00	36.16 ± 2.38c	38.19 ± 1.37ab
	10.00	45.76 ± 0.19d	26.59 ± 1.38a
	15.00	51.10 ± 0.01e	21.06 ± 0.02c
Distilled H_2O	0.0	51.50 ± 2.89e	46.03 ± 4.20d

Means followed by different letters in the column are significantly different ($P < 0.05$) from one another using Duncan's new multiple range test (DNMRT)

the leaves of Ife brown increase with increase in salinity stress in both NaCl and Na_2SO_4 treatments. There were significant differences in the SOD activities for Ife brown at 10 dS/m under NaCl and Na_2SO_4 treatments. Significant differences were also recorded in Ife bpc at 15 dS/m under NaCl and Na_2SO_4 treatments. SOD activities in the stressed seedlings of both varieties were found to be lower compared to the control seedlings.

Discussion

The structural stability and osmotic adjustment of proline during stress had been an important indicator of oxidative damage in plants (Michael and Krishnaswamy 2012). Miranda et al. (2014) previously reported proline accumulations in response to various stresses in leaf in many studies. The observed maximum proline accumulations in IBPC under NaCl and Na_2SO_4 are in agreement with these findings. Cellular architecture releases compatible osmolytes to maintain cell turgor and osmoregulation. Compatible osmolytes act as stabilizer by cushioning the effect of oxidative damage to macromolecules by salinity stress (Gharsallah et al. 2016). The increased MDA content in IFB under both treatments possibly indicates that IFB is capable of maintaining high degree of cell membrane homeostasis under high salt stress. Osmotic adjustment remained an integral factor and an important physiological character associated with salinity tolerance. This mechanism had attracted much attention and generated much debate during the past years. The observed minimum MDA content recorded in Ife brown (IFB) at 10 dS/m under Na_2SO_4 stress possibly demonstrates that the IFB displays higher antioxidative ability, thereby reflecting higher salinity tolerance. This is in agreement with the findings of Sharma et al. (2012). Ozgur

et al. (2013) reported that increase in reactive oxygen species (ROS) and lipid peroxidation is indicators of the decrease in antioxidant defenses. Osmotic adjustment simply involves the differential accumulation of compatible solutes in a cell in response to a decrease in solute potential of the cell's environment (Rahman et al. 2016). Oxidative damage necessitated by salinity stress is mitigated by a combined action of both enzymatic and non-enzymatic antioxidant systems. These include α-tocopherol (α-toc), β-carotenes, carotenoids, ascorbate (ASC), reduced glutathione (GSH), enzymes including superoxide dismutase (SOD), peroxidase (POD), ascorbate peroxidase (APX), catalase (CAT), polyphenol oxidase (PPO) and glutathione reductase (GR). Superoxide radicals are produced in various physiological processes under conditions of abiotic stress (Das et al. 2014). The observed increase in SOD activities in IFB under NaCl treatment is an indication that SOD is capable of mitigating effect of oxidative stress in cowpea seedlings. An increase in the activities of antioxidative enzymes under salt and water stress possibly indicates an increased production of reactive oxygen species (ROS). Findings from this research are in agreement with Karuppanapandian et al. (2011), who reported that increased production of ROS is capable of developing mechanisms to reduce oxidative damage in plants. It is widely perceived that the biosynthesis of many secondary metabolites in plants is an important part of the defense response to stress conditions (Isah 2019). The increase in glutathione activities in response to salinity stress in IFB may possibly be connected with increased stress tolerance (Khare et al. 2020). Stress tolerance is a complex phenomenon involving coordinated response that includes ion sequestration, metabolic adjustment and antioxidative defense (Abogadallah 2010). Salinity-induced alterations and disruptions in the physiology and biochemistry of the plant may elicit metabolic disturbances that trigger an abundant production of the product of oxidative damage (Naveed et al. 2020).

Conclusions

The increased proline content in IBPC confers structural stability to cellular machinery by acting as osmolyte capable of detoxifying reactive oxygen species (ROS) toward ameliorating salinity-induced damage in cowpea seedlings. The increased lipid peroxidation content in IFB suggests that the variety is capable of acting as osmoprotectant against membrane damage by salinity stress. The accumulation of superoxide dismutase (SOD) and glutathione (GSH) in IFB clearly demonstrates that enzymatic and non-enzymatic antioxidants are capable of scavenging reactive oxygen species, thereby mitigating salinity-induced oxidative damage in cowpea.

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Authors' contributions
The author read and approved the final manuscript.

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Declarations

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Not applicable.

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