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Salt Stress Elevates Endogenous Phytohormones and Activates Antioxidative Defense System in Leaves of *Spinacia oleracea* (Spinach)

C. O. Olaiya[1*], K. O. Karigidi[2], E. E. Idanegbe[1] and O. S. Fagbami[1]

[1]Department of Biochemistry, University of Ibadan, Ibadan, Nigeria.
[2]Biochemistry Program, Department of Chemical Sciences, Ondo State University of Science and Technology, Okitipupa, Nigeria.

Authors' contributions

This work was carried out in collaboration between all authors. Author COO designed the study, performed part of the statistical analysis and wrote the first draft of the manuscript. Author KOK managed the analyses of the study. Author EEI managed the experimental process. Author OSF managed the literature searches. All authors read and approved the final manuscript.

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ABSTRACT

Aims: Salinity stress is one of the major environmental stresses and continues to limit the growth and productivity of most food crops. Therefore, the aim of this research was to evaluate the effects of salinity stress on endogenous phytohormones and antioxidative defense system of *Spinacia oleracea*.
Study Design: Salinity stress was induced using three concentrations (50 mM, 100 mM and 150mM) of sodium chloride (NaCl). The treatments were laid out in complete randomized blocks with three replicates each.
Place and Duration of Study: The experiment was conducted in the screen house of the Department of Biochemistry, University of Ibadan, Nigeria for eight weeks.
Methodology: After eight weeks of planting on saline soil, fresh leaves were harvested and biochemical analysis was carried out on the leaves to determine the effect of salinity stress on

**Corresponding author: E-mail: cooolaiya@yahoo.com;*

photosynthetic pigments, minerals, antioxidative enzymes, indole-acetic acid (IAA) and gibberellic acid (GA) using standard methods. Data were statistically analyzed by one way ANOVA and Duncan's multiple range tests were performed to test the differences among treatments at $P<0.05$.
Results: Results showed that treatment with 150 mM NaCl increased the level of IAA, GA, catalase (CAT), superoxide dismutase (SOD) and sodium significantly ($P<0.05$) in *Spinacia oleracea* leaves, while photosynthetic pigments, glutathione peroxidase, magnesium, manganese, potassium and iron were significantly decreased ($P<0.05$).
Conclusion: These results suggest that *Spinacia oleracea* responds to salinity stress via an elevation of CAT, SOD, IAA and GA.

Keywords: Salinity stress; photosynthetic pigments; minerals; antioxidative enzymes; Spinacia oleracea.

1. INTRODUCTION

An environmental factor that limits crop productivity or destroys biomass is referred to as a stress [1]. Among the various abiotic stress factors, soil salinization is the biggest threat to food production [2], while its prominence has been reported in arid and semi-arid regions [3]. Saline soils are defined as those with high concentration of sodium salt especially sodium chloride (NaCl) with an electrical conductivity (EC_e) of more than 4 dSm^{-1}. Soil salinity is expressed in deciSiemen per meter (dSm^{-1}) and can be measured by determining the electrical conductivity of a solution of a soil using electrical conductivity meter. Growing experimental evidences have shown that salt stress affects the integrity of cellular membranes, activity of enzymes and the functioning of the plant photosynthetic apparatus [4]. An important cause of this damage is production of reactive oxygen species (ROS) [5]. Salinity also causes low osmotic potential of soil solution [3] and nutritional imbalances [6]. Finally, in extreme saline conditions, tissue death and ultimately death of the plant occur [7-8]. Despite the great deal of research into salinity tolerance of plants, the adaptive mechanisms utilized by plants to survive salinity stress are still not well understood [9]. This is partly due to the fact that the mechanisms of salt tolerance are so complex that variation occurs not only amongst species but, in many cases, also among cultivars within a single species [6,10]. Nevertheless, many studies have shown a positive correlation between the accumulation of glycinebetaine (quaternary ammonium compound) and proline (amino acid) and stress tolerance in plants [11-12]. There is now conclusive evidence that production of ROS is enhanced in plants in response to different environmental stresses such as salinity and plants containing high concentrations of antioxidants show considerable resistance to the oxidative damage caused by the activated oxygen species [13].

Spinach (*Spinacia oleracea)* is an edible flowering plant in the family of Amaranthaceae, native to central and southwestern Asia. Spinach has a high nutritional value and is extremely rich in antioxidants, especially when fresh. It is a rich source of vitamins, magnesium, manganese, folate, iron, calcium, potassium, folic acid, copper, protein, phosphorus, zinc, niacin, selenium, omega-3 fatty acids [14], flavonoids and total phenolic compounds [15]. Apart from its use as food, traditionally, it is used in treatment and management of diseases of blood and brain, asthma, leprosy and biliousness [16], and in the treatment of urinary calculi [17]. *Spinacia oleracea* has been classified as one of the vegetables that can be grown in high salt concentrations up to 130 mM NaCl [18]. The objective of this research work is to evaluate the effects of salinity stress on *Spinacia oleracea* and possibly deduce its strategy to adapt to salt stress.

2. MATERIALS AND METHODS

2.1 Plant and Soil Materials

Seeds of *Spinacia oleracea* used in this study were obtained from the Rubber Research Institute of Nigeria (RRIN), Iyanomo, Edo State. Soil was collected from soil surface, air dried, ground, passed through a 5 mm mesh screen, thoroughly mixed and analysed. The soil was spread separately over thick plastic sheets and moistened with solutions of 0 mM, 50 mM, 100 mM, and 150 mM NaCl. Ten kilogram soil portions of each lot were filled in polyethylene bags (20 cm x 25 cm) and five seeds were sown in each pot at a depth of about 10 mm. Each treatment was laid out in complete randomized blocks with three replicates. *Spinacia oleracea*

seeds were soaked in distilled water at room temperature for 20 minutes prior to sowing. Watering was done on alternate days with distilled water. After 8 weeks, fresh leaves of the treated and control plants were harvested and bioassayed.

2.2 Estimation of Photosynthetic Pigments

The photosynthetic pigments (chlorophyll a, chlorophyll b, and carotenoids) were estimated by the spectrophotometric method of Metzner et al. [19]. Chlorophyll a, chlorophyll b, and carotenoids were determined using the following equations:

- Chlorophyll a = 10.3 E_{663} – 0.918 E_{645}
- Chlorophyll b = 19.7 E_{645} – 3.870 E_{663}
- Carotenoids = 4.2 E_{452} (0.0264 chl. a + 0.426 chl. b)

Where E = optical density at the given wavelength.

2.3 Estimation of Minerals

Minerals were determined by the method of AOAC [20]. Samples were dried at approximately 80℃ for 12 hours and it was finely ground. About 1 g of the dried plant tissue was weighed into a 100 ml berzeliu beaker and 5 ml HNO_3 and 2 ml $HClO_4$ were added. It was covered and digested by heating to a final volume of 3-5 ml. 10-15 ml of distilled water was added and filtered through an acid-washed filter paper into a 50ml volumetric flask. The filter paper was washed with water and the filtrate was diluted to volume with deionized water. 25 ml of digest was then used to determine Na, K, Mg, Fe, Mn, and Zn. Atomic absorption spectrophotometer (Spectrumlab 23A, Techmel & Techmel, Texas, USA) was used to determine Mg, Fe, Mn, and Zn while K and Na were determined with flame photometer (EEL, Flame Photometer).

2.4 Extraction and Quantification of Phytohormones

Extraction and purification for IAA and GA were done according to the method of Iqbal and Ashraf [21]. The plant material was freeze dried and ground. Ground samples were weighted and extracted with 100% methanol [2.5 ml per gram of fresh weight (gfw)]. The extract was centrifuged at 4,000 g for 10 min at room temperature. The resulting supernatant was transferred to a new tube and evaporated until the volume decreased to less than one-tenth of the initial. One volume of distilled water was added to the residue. The pH was adjusted higher than 9 with 1M KOH to keep IAA and GA, ionized and then partitioned against 100% ethyl acetate. The aqueous and organic phases were separated by centrifugation at 16,000 g for 5 min, and the lower aqueous phase was transferred to a new tube. The pH of the solution was adjusted below 3 with concentrated acetic acid to conserve IAA in protonated form. The acidic sample was partitioned against 100% ethyl acetate and cleared by centrifugation. The upper organic phase was recovered and completely evaporated and then dis solved in 300µL of the mobile phase. Compounds were analyzed on a reversed phase HPLC column under isocratic elution and UV detection in the presence of 1% acetic acid and methanol. IAA and GA were fully detected in 15 minutes with symmetrical peaks.

2.5 Estimation of Antioxidant Enzymes

About 1 g of fresh samples was homogenized with 4 ml of phosphate buffer (pH 7.0), then the homogenate was centrifuged at 12,000 rpm for 16 minutes at 4℃. The supernatant was removed and pellet discarded. The supernatant was kept for enzymes assay.

2.5.1 Catalase (CAT)

Catalase activity was determined according to Aebi [22]. The decomposition of H_2O_2 was followed as a decrease in absorbance at 240 nm in a UV spectrophotometer. The 1 ml reaction mixture contained potassium phosphate buffer (pH 7.0), 250 µL of enzyme extract and 60 mM H_2O_2 to initiate the reaction. The reaction was measured at 240 nm for 3 min and H_2O_2 consumption was calculated using extinction coefficient, 39.4 $mM^{-1}Cm^{-1}$.

2.5.2 Glutathione peroxidase (GPX)

Glutathione peroxidase (GPX) activity was measured using a Fortress diagnostic kit. Necessary preparation was done according to the procedure specified by the manufacturer. The 2.65 ml of the reaction mixture contained 0.05ml of distilled water, 2.5 mls of working reagent (GPX reagent provided R2) and 0.1 ml dilute cumene hydroperoxide. Absorbance was read at 340 nm with initial reading taken and subsequently at 1 and 2 mins.

2.5.3 Superoxide dismutase (SOD)

Superoxide dismutase (SOD) activity was measured using the Fortress kit. The 2 ml assay reaction mixture contained 50 µl of diluted sample, 1.7 ml mixed substrate [0.05 mM/L xanthine and 0.025 mM/L 2-4 iodophenyl-3-4-nitrophenol-5-phenyl tetrazolin chloride (INT)] and 250 µl of xanthine oxidase.

For the standard curve, standard as provided in the kit was used, reaction mixture was incubated for 30 seconds at 37℃ and the increase in absorbance was read at 550 nm for approximately 5 min at 1 min interval.

2.6 Statistical Analysis

Data were expressed as mean ± SD. The significance of the differences between the means of the samples was established by the analysis of variance (ANOVA) and Duncan's multiple range tests [23] were performed to test the differences among treatments at $P<0.05$.

3. RESULTS AND DISCUSSION

Soil salinity is one of the main problems threatening food production, especially in countries where crops should be irrigated [24]. Osmotic adjustment (salt tolerance) in plants subjected to salt stress can occur by the accumulation of high concentrations of either inorganic ions or low molecular weight organic solutes [9]. The results showed that NaCl-induced salinity has a remarkable effect on the biochemical parameters in *Spinacia oleracea* leaves. Salt stress caused significant alteration in the level of IAA and GA. Previous findings have conjectured that endogenous content of plant hormones such as abscisic acid, cytokinins, zeatin, and gibberellins change in response to salt stress [25]. Salt stress at all concentrations used increase the level of GA while IAA was only increased at 150 mM (Table 1). The significant decrease of IAA observed at 50 and 100 mM of NaCl could be a result of the biochemical conversion of auxins to an inactive compound via increased activity of IAA – oxidase [26].

Salt stress significantly increased chlorophyll a and b at salt concentration of 50 mM but they were significantly reduced at 150 mM. The observed increase at low salt concentration suggests higher chlorophyll accumulation which might be due to reduced expression of the chlorophyll degrading enzyme chlorophyllase and a higher chlorophyll stability index [27]. High salt concentration has been reported to reduce the contents of photosynthetic pigments [28]. Salinity breaks down chlorophyll as a result of increased level of the toxic sodium ion [29]. The salt-induced alterations in chlorophyll content could also be due to impaired biosynthesis or accelerated pigment degradation [30]. Carotenoid level was significantly reduced at all salt concentrations (Table 2). Salt-induced reduction of photosynthetic pigments at high salt concentrations might be due to impaired biosynthesis or accelerated pigment degradation by salinity stress [31], breakdown of chlorophyll contents by sodium salts [29] or reduction in chlorophyll concentrations is probably due to the inhibitory effect of the accumulated ions on the biosynthesis of the different chlorophyll fractions [32].

Table 1. The effect of salt stress on the levels of indole-acetic acid (IAA) and gibberellic acid (GA) of *Spinacia oleracea*

	IAA (µg/ml)	GA (µg/ml)
Control	28.26 ± 1.49^{c}	4.20 ± 0.50^{a}
50 mM	20.51 ± 0.89^{a}	4.53 ± 0.73^{a}
100 mM	25.24 ± 1.08^{b}	5.63 ± 0.15^{b}
150 mM	32.16 ± 1.30^{d}	5.50 ± 1.20^{b}

Values were mean±SD of three determinations. Means followed by the same letter within the same column are not significantly different (P<0.05)

Salinity has been reported to affect nutrient uptake, distribution and accumulation of ions in plant tissues [33]. Salt stress significantly affected the mineral contents of *Spinacia oleracea.* Mn, K, Mg and Fe were significantly reduced with the lowest amount found at 150 mM salt concentration while Na and Zn were significantly increased, the increase being also more prominent at 150 mM salt concentration (Table 3). Under saline conditions, Na^+ competes with K^+ for uptake through common transport systems, since Na^+ and K^+ are physico-chemically similar monovalent cations and could be the result of direct competition between K^+ and Na^+ at the site of ion uptake at plasmalemma [34]. Na^+ could also enhance the efflux of K^+ into the growth medium [35]. Thus, elevated levels of cytosolic Na^+ or high Na^+/K^+ ratios exert metabolic toxicity by a competition between Na^+ and K^+ for the binding sites of many enzymes [36] since K^+ activates more than 50 enzymes [37].

Table 2. The effect of salt stress on the photosynthetic pigments of *Spinacia oleracea*

	Chlorophyll a mg g^{-1} fw	Chlorophyll b mg g^{-1} fw	Carotenoid mg g^{-1} fw
Control	0.10±0.00[b]	0.16±0.02[b]	41.8±1.26[c]
50 mM	0.70±0.28[c]	0.45±0.02[c]	24.8±7.30[b]
100 mM	0.65±0.01[c]	0.20±0.01[a]	17.9±3.17[a]
150 mM	0.30±0.01[a]	0.20±0.01[a]	11.4±2.01[a]

Values were mean±SD of three determinations. Means followed by the same letter within the same column are not significantly different (P<0.05)

Table 3. The effect of salt stress on the mineral content (mg g^{-1}) of *Spinacia oleracea*

	Mg	K	Na	Mn	Fe	Zn
Control	0.68±0.10[c]	0.75±0.20[c]	0.84±0.05[a]	0.89±0.51[a]	0.69±0.51[b]	0.53±0.25[a]
50 mM	0.40±0.10[b]	0.62±0.20[b]	0.86±0.15[a]	0.50±0.20[b]	0.86±0.15[c]	0.61±0.20[b]
100 mM	0.34±0.26[a]	0.63±0.10[b]	0.93±0.20[b]	0.47±0.20[b]	0.84±0.10[c]	0.75±0.13[c]
150 mM	0.31±0.20[a]	0.24±0.11[a]	1.71±0.20[c]	0.42±0.15[c]	0.50±0.20[a]	0.70±0.20[c]

Values were mean±SD of three determinations. Means followed by the same letter within the same column are not significantly different (P<0.05)

Table 4. The effect of salt stress on the antioxidative enzymes of *Spinacia oleracea*

	SOD(U/ml)	GPX(U/mg)	CAT(mol/cm)
Control	0.69±0.03[a]	2056.75±77.29[d]	1.18±0.70[a]
50 mM	1.32±0.11[b]	1724.46±285.56[c]	2.32±0.16[b]
100 mM	1.83±0.03[c]	1093.56±249.82[b]	4.37±0.04[c]
150 mM	1.91±0.02[c]	819.67±11.89[a]	7.66±0.23[d]

Values were mean±SD of three determinations. Means followed by the same letter within the same column are not significantly different (P<0.05)

Salinity stress increases the synthesis of ROS and thereby upregulates the activity of the enzymes that detoxify these species [38]. The coordinated activity of these antioxidant enzymes in the different cell compartments achieves a balance between the rate of formation and removal of ROS [39]. In this work, SOD and CAT were significantly increased as a result of sodium-induced salinity at all levels of treatment while GPX was decreased significantly (Table 4).

SOD is a component of plant antioxidation system and can be used as biomarker of environmental stress such as salinity [40]. Increase in SOD and CAT may be linked to activation of enzymes by the production of excess ROS. Also the decrease in GPX activities may result in the cytotoxicity due to blocking of essential functional groups, replacement of essential metals with heavy metals, changes in structure or the integrity of proteins and the interruption of signal transduction pathways of antioxidant enzymes because of poisonous active oxygen species derivatives [41].

4. CONCLUSION

In conclusion, this study demonstrates that *Spinacia oleracea* responds to salinity stress via improved antioxidative defense system and increased biosynthesis of IAA and GA. The study therefore provides information on some biochemical events used to protect against the unsavoury effects of salt stress in *Spinacia oleracea.*

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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