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Research Article

Ameliorative Effects of Gibberellic Acid and *Bacillus subtilis* on Two Salt-Stressed Genotypes of Okra [*Abelmoschus esculentus* (L.)Moench] Plant

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ABSTRACT

Increased salinity contributes majorly to the environmental threats, poor growth and productivity of okra plants worldwide. Induced salt-stressed effects on okra plant were mitigated significantly ($P< 0.05$) with the use of combined treatments of gibberellic acid and *Bacillus subtilis*. In this study the okra (NHe 47-4 and Clempson genotypes) seeds were pre-soaked with 0.4, 0.5, or 0.6 mM of gibberellic acid, and control (0) in distilled water respectively for 12 h in the dark. The seeds were air-dried at room temperature and germinated in 10 kg of soil treated with 0, 100, or 200 Mm NaCl in polyethene bags. After two weeks of seed germination, three seedlings per pot were inoculated with *Bacillus subtilis*. Results showed that the effects of gibberellic acid and *Bacillus subtilis* ameliorated the harmful effects of salinity stress and concomitantly increased the concentrations of minerals (magnesium, potassium, calcium, and phosphorous), proline, soluble sugar and soluble protein of okra plant in all salinity levels. Antioxidant enzymes activity in salt-stressed okra plant were increased especially at 200 mM NaCl in both genotypes with exception of superoxide dismutase (SOD) in Clempson genotype that showed little or no activity relative to salt-treated control groups. The increase in radical-scavenging ability of 2,2-diphenyl-1-picrylhydrazyl (DPPH), as well as total phenolic and flavonoids potentials of salt-stressed okra plant in this study, was associated with increase antioxidant enzymes activity.

Keywords: Gibberellic acid; *Bacillus subtilis*; Salinity stress; Okra; Antioxidant enzymes; Compatible solutes

INTRODUCTION

Okra is one of the commonly utilized vegetables by the majority of the world's population [1]. Okra could be categorized as an annual or perennial crop. It grows up to 2 m high with 10 to 20 cm leaves length. It is the most used vegetable crop to prepare traditional meals by all tribes in Nigeria. Studies have shown the susceptibility of okra plant to environmental stress conditions like drought [2], and salinity [3]. Salinity stress inhibits plant growth, development and yield at large, due to mechanisms such as nutrient imbalance [4]. According to the report of Beltran and Manzur [5], large portion of farmland worldwide is actually under the influence of salinity stress, and many irrigated lands are affected by salt stress [6]. In particular, increased levels of salinity in plant tissues retard its development and growth by altering various physiological processes, which includes change in rate of photosynthesis, open and closing of stomata, ions balance, water status and mineral nutrition [7]. Salinity stress results in ionic and osmotic stresses which in turn affect growth of plants at both cellular and physiological levels [8]. High concentration of saline solution interfere with the uptake of essential macronutrients ions (K^+, Ca^{2+}, Mg^{2+}) in plants [9]. The scientific goal is to develop salinity stress-tolerant plants. However, efforts with limited success have been met on salt tolerance in plants [10]. Co-treatments of plant rhizobacteria and plant hormones can rather enhance tolerance levels of plants to salinity. This will serve as an alternative technique to breeding and genetic manipulation. Indeed, various researches findings had shown positive influence of rhizobacteria on a variety of crop plants [11-14] in saline conditions. Plant rhizobacteria have been substantially implicated to increase antioxidant enzymes activity in salt-stressed plants [15]. Among these plant rhizobacteria, *Bacillus subtilis* is considered to be involved in plant tolerance. Inoculation of *Bacillus subtilis* ameliorated salinity stress effects in salt-stressed artichoke plant [16]. Jasmonic acid confers beneficial effects on plants under various stresses [17]. Among plant hormones, gibberellic acid (GA_3) has gained tremendous attentions of scientists [18,19].

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Gibberellic acid applications in plants have been shown to improve salt tolerance and enhance endogenous level of salicylic acid [20]. Kaur et al. [21] showed high seedling growth rate of plants in saline conditions through gibberellic acid application. The application of plant rhizobacteria or phytohormones as the eco-friendly strategy for salt tolerance in sustainable agriculture has been reported by various studies [22-24]. However, there is paucity of information on the co-treatments of *B. subtilis* and gibberellic acid (GA_3) on okra plant under salt stress conditions. Therefore, in this study we examined the combined effects of *B. subtilis* and gibberellic acid on salt-stressed okra.

MATERIALS AND METHODS

Plant materials

The okra seeds (Clemson and NHe 47-4 genotypes) were purchased at the National Horticultural Research Institute (NIHORT) in Ibadan, Nigeria. The two genotypes are widely used in Nigeria as a breeding line but their ability to tolerate salinity stress has not been established. Pretreatment of seeds was done in various concentrations (0.4, 0.5 or 0.6 mM) of gibberellic acid respectively for 12 h in the dark. The seeds were air-dried at room temperature after the salt solutions had been removed. The seeds of each genotype were sown in 10 kg polyethene bags of soil with analytical properties of pH 7.10, Exch. acidity 0.34 mol. kg^{-1}, clay 12.30%, silt 13.90%, sand 65.40%, organic carbon 4.732%, nitrogen 0.25%, phosphorous 20.00 $mg.kg^{-1}$, exchangeable potassium 1.33 $mol.kg^{-1}$, sodium 0.89 $mol.kg^{-1}$, calcium 45.65 $mol.kg^{-1}$, magnesium 13.34 $mol.kg^{-1}$. A screen house at the Biochemistry Department, University of Ibadan, Nigeria was used to conduct the experiment. The seeds were sown in soil that had been treated with a solution of NaCl based on allotted salinity concentrations (100 or 200 mM) or without salinity (control), in the factorial experiment and was laid out in a completely randomized designed, and treatments replicated three times.

After seed germination, the seedlings were wet with tap water thrice a week until soil water attained field capacity.

Bacillus subtilis cell culture

Bacillus subtilis was isolated from the rhizosphere soil of healthy okra plants at the Agronomy Department, University of Ibadan, and cultured on nutrient agar at the Pharmaceutical Microbiology Department, University of Ibadan, Nigeria. The culture was centrifuged at 6200 rpm for 10 min at 4°C. Distilled water was used to wash the pellets obtained and then re-suspended in sterilised distilled water to an optical density of 0.8 at 600 nm (approximate cell density of 1×10^8 $CFUmL^{-1}$). Two weeks after germination, each seedling was inoculated with 30 mL of cell suspensions of 1×10^8 CFU mL^{-1}.

After 45 days of growth, all experimental plant leaves were harvested, freeze-dried for three weeks at room temperature and ground to powder using a mortar and a pestle before the biochemical assays.

Extraction protocol for mineral elements

The samples were digested in an oven at 600°C for 4 hr, the ashes and the crucibles were previously decontaminated with a solution of 10% nitric acid for a night, and rinsed in the distilled water. Then, 10 mL of 5% nitric acid was added to the sample, and this mixture was heated until complete dissolution of the ash. This was filtered and the filtrate was put into 25 mL volumetric flask, and then the volume was made up to the required level using distilled water.

Cationic contents

The calcium, magnesium and potassiumion concentrations were determined according to AOAC [25] method, using an atomic absorption spectrophotometer flame (Bulks Scientific® model AA 240). The concentrations of Na^+ in the samples were determined by Flame Photometry according to AOAC [25] method. The total phosphorus content was determined as described by the spectrophotometry method.

Photosynthetic pigments estimation

The Lichtenthaler and Wellburn [26] method was applied for the photosynthetic pigments assay of the okra leaves. Approximately 250 mg of okra fresh leaves was homogenized with 85% acetone for about 5 min. The sample was mixed using a magnetic stirrer; centrifuged and the absorbance taken at 663, 646, and 470 nm. The concentrations of the pigments were estimated and expressed as mg g^{-1} fresh weight.

Compatible solutes determination

Free proline determination: Proline concentration in the leaf tissues was measured using Bates et al. [27] method. The standard (0-50 µg/mL) used was proline, and the reading of proline concentration was done at 520 nm and the value expressed as milligram/gram dry weight.

Total soluble protein determination

The spectrophotometric technique of Desingh and Kanagaraj [28] was employed for soluble protein concentration determination in the plant sample using Folin-Ciocalteau reagent. A 10% of trichloroacetic acid (5 mL) was mixed with 0.5 g leaf powdered sample. The reaction was centrifuged for 10 min at 2000 rpm. Then about 5 mL of the upper layer was added to 0.1 M NaOH (5 mL), with the addition of Biuret reagent (8 mL). The standard used was BSA (Bovine Serum Albumin). The standard and the sample dilutions were incubated for 30 min at 25°C before the reading was taken at 530 nm using UV/VIS Spectrophotometer.

Total reducing sugars determination

The method of El-Shihaby et al. [29] was used to determine the reducing sugar concentration in the okra plant. Briefly, 0.5 g of dried powder of okra was extracted by using 80% ethanol (10 mL) at 50°C, and a reaction was set up at about 15-30 min. of the extraction. Then 1 mL of the extracted solution was estimated at 530 nm using UV/VIS Spectrophotometer. Reducing sugar concentration was expressed as mg g^{-1} on dry weight. The glucose was applied as a standard.

Total phenol content determination

The spectrophotometric method was employed to determine total phenolic content in the sample [30]. A 1 mL of Folin-ciocalteu's phenol reagent was added to 1 mL of the sample. A

10 mL of 7% Na_2CO_3 solution was added to the mixture after 5 min. and thoroughly mixed with distilled water (13 ml) using a magnetic stirrer. The reaction was stored for 90 min at 25 °C in the dark. The reading was then taken at 750 nm using UV/VIS Spectrophotometer. Gallic acid was used as the standard by using its standard curve for determining the phenolic concentration.

Determination of total flavonoids content

The Park et al. [31] method was applied to determine the total flavonoid concentration in the sample. The mixture of the reaction contained 0.15 mL of 0.3 M aluminum chloride ($AlCl_3$), 0.15 mL of 0.5 M sodium nitrite ($NaNO_2$), 3.4 mL of 30% methanol, and 0.3 mL of the sample extract. The addition of 1 M sodium hydroxide (1 mL) was done after 5 min. Incubation was done for about half an hour at 25°C. Quercetin served as the standard by using its standard curve for determining the flavonoids concentration. Then, the reading was taken at 510 nm using UV/VIS Spectrophotometer.

Enzyme extractions and assays

Tejera et al. [32] method was applied to prepare crude enzyme extract. A 1 g of sample was soaked in a potassium phosphate buffer solution (0.1M, pH 6) with 0.5 m Methylene diaminetetra acetic acid. Centrifugation of the sample extract was done at 15000 rpm for 20 min. Analysis of the enzymes was done in the supernatant.

Superoxide dismutase assay

The superoxide dismutase activity was assayed using the procedure described by Kumar et al. [33]. A 1 mL of sample was taken at 25 to 500 µg mL^{-1} concentrations and mixed with 0.5 mL of buffer solution of potassium phosphate (50 mM, pH 7.6) with 0.1 mL of nitro blue-tetrazolium (NBT) (0.5 mM) and 0.3 mL of riboflavin (50 mM). A fluorescent lamp was used to initiate the reaction, following incubation for 20 min at 25°C. The activity of superoxide dismutase was measured at 560 nm using UV/VIS Spectrophotometer. The standard used was ascorbic acid. The enzyme activity was expressed as Units mg^{-1} protein.

Ascorbate peroxidase assay

Ascorbate peroxidise activity was estimatedusing the method outlined byYoshimura et al. [34]. The mixture of the reaction contained a buffer solution of potassium phosphate (50 mM, pH 7.0) with 0.1 mM of hydrogen peroxide, 0.5 mM ascorbic acid and 200 µL of enzyme extract. The mixture was incubated for 5 min at 25°C. The activity of ascorbate peroxidase was measured at 550 nm using UV/VIS Spectrophotometer. The enzyme activity was expressed as units mg^{-1} protein.

Polyphenol oxidase assay

The Oktay et al. [35] method was used to assay for polyphenol oxidase activity with slight modifications. The mixture of the reaction contained buffer solution of 0.1 M potassium phosphate at pH 6.0 with enzyme extract (0.5 mL) and 0.1 M catechol (1.0 mL). The reaction medium was incubated at 25 °C for 5 min. After which, 1 mL of 2.5 N H_2SO_4 was added to stop the reaction. The reading was taken at 495 nm using UV/VIS Spectrophotometer.

Scavenging activity of DPPH

The method described by Zhang and Hamauzu [36] was used to determine DPPH (1,1-diphenyl-2- picrylhydrazyl) radical scavenging ability. The reaction contained 1 mM of DPPH in ethanol with 1 mg/ml of extract solution at room temperature for an hour. The absorbance against the corresponding blank solution was taken at 517 nm using UV/VIS Spectrophotometer. DPPH radical scavenging capacity was measured by following the equation below.

$$\text{DPPH radical scavenge (\%)} = \left(1 - \frac{AC}{AD}\right) \times 100 \quad (1)$$

Where AC and AD mean solution absorbance with the extract and DPPH solution absorbance respectively.

Statistical analysis

The data collected were subjected to three-way analysis of variance (ANOVA) using the Statistical Package for the Social Sciences (SPSS). Sample means were compared using Tukey-Kramer multiple comparison procedure at $P<0.05$.

RESULTS

Minerals concentration

Tables 1a and 1b and 2a and 2b, show that the single treatment of *B. subtilis* or GA_3 at different concentrations respectively, as well as the combined treatments of *B. subtilis* at different concentrations of GA_3 respectively, enhanced the levels of potassium, calcium, magnesium and phosphorus ions in the two genotypes of salt-stressed okra plant. However, the levels of sodium ion in the tissues of salt-stressed okra plant in the two genotypes were significantly reduced either in single treatment of gibberellic acid, *B. subtilis* or in combined form as compared to the control and negative controls groups (100 and 200 mMNaCl).

Photosynthetic pigments

Table 3 and Table 4 indicate that single treatment of *B. subtilis* and gibberellic acid induced a rise in the concentrations of chlorophylls a and b inall the NaCl-treated plants when compared with the normal control and NaCl-treated groups. But the significant increase in chlorophylls a and b were observed in the combined treatments of gibberellic acid and *Bacillus subtilis* at all concentrations of gibberellic acid as compared to the control and NaCl-treated groups in NHe 47-4 and Clempson genotypes respectively. Whereas, under the combined effects of gibberellic acid and *Bacillus subtilis*, Clempson genotype responds more positively with increase photosynthetic pigments contents than the NHe 47-4 genotype.

Proline accumulation

Figure 1 shows higher proline accumulation in okra (NHe 47-4 and Clempson genotypes) plants under salinity stress, with respect to the control group, and similar trends were reported in the groups treated with only gibberellic acid or *B. subtilis* as well as interaction of gibberellic acid, *Bacillus subtilis* and salinity relative to the NaCl-treated group in genotype NHe 47-4 (Figure 1a). A similar result was observed in the Clempson genotype (Figure 1b) but at moderate and severe levels of salinity no noticeable increase in the proline level across all the treated genotypes.

Table 1A: The gibberellic acid or *Bacillus subtilis* effect on mineral (mg/g dw) elements of salt-stressed okra plant NHe 47-4 genotype.

Treatment	NaCl (mM)	Na	Mg	Ca	K	P
0 mM	0	16.34 ± 0.18	104.65 ± 1.20	135.73 ± 13.45	158.77 ±3 .21	111.78 ± 1.80
	100	222.17 ± 0.22	96.40 ± 9.30	135.98 ± 20.75	246.48 ± 18.50*	106.42 ± 3.02
	200	330.60 ± 0.04*	81.04 ± 0.50*	186.78 ± 10.17*	129.00 ± 13.70	103.05 ± 7.90
GA_3 (0.4 mM)	0	21.45 ± 0.04	121.85 ± 11.92	120.81 ± 10.11	222.00 ± 11.19	136.33 ± 1.80
	100	19.59 ± 0.04	102.22 ± 13.71	117.66 ± 21.62	260.33 ±14.40**	211.70±2.17**
	200	31.22 ± 0.04	121.23 ± 10.52	105.69 ± 12.20	237.85 ± 14.94	101.70 ± 8.90
GA_3 (0.5 mM)	0	21.96 ± 0.28	135.84 ± 10.88	130.39 ± 13.34	202.75 ± 3.39	244.41 ± 2.07
	100	17.88 ± 0.08	125.08 ± 13.80	129.88 ± 13.52	258.70 ± 15.80	112.10 ± 1.27**
	200	22.13 ± 0.16	105.08 ± 3.71	118.98 ± 10.05	294.55 ± 14.09	205.56 ± 14.50
GA_3 (0.6 mM)	0	20.50 ± 0.24	161.65 ± 0.94	167.19 ± 3.90	404.00 ± 14.41	217.39 ± 12.69
	100	16.16 ± 10.28	120.98 ± 15.00	119.22±10.75**	309.20 ± 14.41	117.20 ± 3.54
	200	22.89 ± 0.16	125.65 ± 2.98	131.73 ± 0.19	251.85±15.76**	215.45 ± 18.90
B. subtilis	0	28.73 ± 4.22	106.33 ± 0.10	113.08 ± 14.64	307.75 ± 3.31	126.93 ± 2.77
	100	27.20 ± 3.16	109.78 ± 3.70	124.24 ± 10.03	364.15 ±14.53**	200.21 ± 12.02
	200	21.06 ± 2.22	114.24 ± 0.30	135.43 ± 0.56	292.24 ± 13.30	100.21 ± 17.80

Values represent means ± SE (n=3). **Significant difference (P ≤ 0.05) to gibberellic acid and *B. subtilis* treated groups.

Table 1B: The gibberellic acid and *Bacillus subtilis* effects on mineral (mg/g dw) elements of salt-stressed okra plant NHe 47-4 genotype.

Treatment	NaCl (mM)	Na	Mg	Ca	K	P
GA_3 (0.4 mM) ± *B. subtilis*	0	32.30 ± 3.25	222.24 ± 111.85	141.18 ± 1.34	256.32 ± 12.82	128.98 ± 1.93
	100	15.76 ± 3.18	141.29 ± 12.13	134.77 ± 11.85	237.40 ± 14.65	117.90 ± 0.36
	200	18.36 ± 0.22	137.65 ± 13.70**	125.88 ± 13.52	396.31±12.97**	156.90 ± 7.89
GA_3 (0.5 mM) ± *B. subtilis*	0	22.98 ± 0.14	193.77 ± 2.27	205.55 ± 12.22	459.53 ± 2.17	224.71 ± 0.93
	100	15.72 ± 0.18	174.23 ± 3.12	175.20 ± 1.20	437.85 ± 7.60	160.12 ± 11.70
	200	19.25 ± 0.14	156.76 ± 3.58**	150.05 ± 0.24	362.85 ± 3.55	120.12 ± 14.70
GA_3 (0.6 mM) ± *B. subtilis*	0	26.12 ± 0.21	204.17 ± 4.45	170.38 ± 13.68	204.17 ± 4.45	121.32 ± 11.58
	100	19.50 ± 0.16	148.44 ± 4.23	105.51 ± 13.52	178.44 ± 4.23	187.24 ± 12.84
	200	14.35 ± 0.13	139.89 ± 0.29	156.23 ± 10.56	169.89 ± 0.29	207.14 ± 17.84**

Table 2A: The gibberellic acid or *Bacillus subtilis* effect on mineral (mg/g) elements of salt-stressed okra plant Clempson genotype.

Treatment	NaCl (mM)	Na	Mg	Ca	K	P
0 (mM)	0	21.20 ± 0.33	70.00 ± 10.70	85.73 ± 8.45***	105.78 ± 9.10***	74.11 ± 12.59
	100	133.87 ± 0.16*	105.47 ± 3.80*	105.34 ± 7.61	33.24 ± 6 .83	81.60 ± 2.50
	200	181.51 ± 0.33*	55.66 ± 5.00	182.85 ± 0.75	94.20 ± 3.00	103.60 ± 4.17
GA_3 (0.4 mM)	0	20.48 ± 0.26	55.69 ± 7.71	50.34 ± 8.52	164.64 ± 5.80	73.61 ± 2.60
	100	17.93 ± 0.17	122.06 ± 5.00**	103.97 ± 0.56**	295.10 ± 6.94**	132.32 ± 12.99
	200	21.76 ± 0.24	112.39 ± 0.37**	102.26 ± 0.74**	160.20 ± 5.20	204.33 ± 2.13**
GA_3 (0.5 mM)	0	41.08 ± 0.20	154.48 ± 1.20	109.22 ± 7.42	165.70 ± 4.90	281.69 ± 4.13
	100	13.47 ± 0.20**	144.65 ± 1.20	120.10 ± 3.89	167.17 ± 3.49	140.22 ± 3.38
	200	22.87 ± 0.34	108.23 ± 5.00	117.47 ± 0.19	267.19 ± 4.90**	109.75 ± 3.48**
GA_3 (0.6 mM)	0	30.95 ± 0.29	105.39 ± 14.45	107.19 ± 4.30	267.82 ± 15.28	179.70 ± 3.09
	100	29.16 ± 6.15	104.62 ± 13.58	109.22 ± 9.75	129.85 ± 15.69	133.75 ± 12.56
	200	22.55 ± 0.11	110.54 ± 5.00	114.22 ± 0.75	359.23 ± 5.60**	209.74 ± 1.91
B. subtilis	0	38.08 ± 0.27	132.98 ± 5.00	113.99 ± 3.52	150.20 ± 6.87	179.05 ± 4.05
	100	39.92 ± 0.33	127.83 ± 4.45	141.73 ± 0.19	221.21 ± 5.47	206.25 ± 3.25
	200	42.85 ± 0.34	199.85 ± 2.50	166.25 ± 0.56	309.21 ± 4.50**	220.19 ± 3.34**

Data are means ± SE (n=3). *, **, and *** are significant differences at P ≤ 0.05 to the normal control, positive control groups (GA_3 or *B. subtilis*) and negative control groups (100 and 200 mMNaCl) respectively determined by Tukey-Kramer multiple range test.

Figure 2a shows an increase soluble protein accumulation with increasing levels of salinity stress across all treatments in the NHe 47-4 genotype relatives to the control group, whereas in Clempson genotype, an increase soluble protein accumulation was observed

Table 2B: The gibberellic acid and *Bacillus subtilis* effectson mineral (mg/g) elements of salt-stressed okra plant Clempson genotype.

Treatment (mM)	NaCl (mM)	Na	Mg	Ca	K	P
GA_3 (0.4 mM) ± *B. subtilis*	0	20.85 ± 1.32	143.44 ± 5.65	168.45 ± 4.55	338.29 ± 17.01	147.32 ± 3.21
	100	17.05 ± 2.34	130.39 ± 7.79	100.80±4.64**	291.40 ± 15.31	205.82 ± 4.24**
	200	12.3 ± 3.24	129.65 ± 1.20	121.27 ± 0.19	217.40±15.31**	118.35 ± 4.24
GA_3 (0.5 mM) ± *B. subtilis*	0	23.80 ± 0.28	132.81 ± 13.18	126.34 ± 14.51	332.80 ± 2.37	192.65 ± 5.01
	100	26.08 ± 0.28	120.57 ± 13.96	139.18 ± 13.35	283.25 ± 3.47	200.12 ± 4.28
	200	13.30±0.33**	125.35 ± 1.20	146.02 ± 20.38	234.25 ± 2.45**	211.29 ± 14.20**
GA_3 (0.6 mM) ± *B. subtilis*	0	33.55 ± 0.21	102.12 ± 12.23	133.21 ± 3.83	122.12 ± 2.23	253.48 ± 17.16
	100	27.20 ± 0.17	139.96±114.21**	170.34 ± 4.64**	155.96 ± 4.21	162.15 ±21.45**
	200	19.39 ± 0.27	136.98 ± 15.00**	125.57 ± 0.75	134.98 ± 5.00	290.27 ± 21.12

Values represent means ± SE (n=3). **Significant difference (P ≤ 0.05) to gibberellic acid and *B. subtilis* treated groups.

Table 3: The gibberellic acid and *Bacillus subtilis* effects on photosynthetic pigments of salt-stressed okra plant NHe 47-4 genotype.

Treatments	NaCl (mM)	Chlorophyll a (mg/g fw)	Chlorophyll b (mg/g fw)	Carotenoids (mg/g fw)
0 mM	0	2.18 ± 0.38	3.10 ± 0.14	5.53 ± 0.44
	100	1.14 ± 0.16	2.61 ± 0.14	0.72 ± 0.66
	200	0.15 ± 0.01*	0.06 ± 0.12*	0.74 ± 0.24
GA_3 (0.4 mM)	0	2.13 ± 0.34	3.59 ± 0.28	6.34 ± 0.33
	100	1.38 ± 0.16	3.80 ± 0.22	3.35 ± 0.14
	200	1.45 ± 0.08	2.17 ± 0.17	3.40 ± 0.11
GA_3 (0.5 mM)	0	3.25 ± 0.14	3.31 ± 0.25	3.25 ± 0.12
	100	2.55 ± 0.28	3.15 ± 0.26	3.50 ± 0.35
	200	2.56 ± 0.02***	3.06 ± 0.32	3.13 ± 0.21
GA_3 (0.6 mM)	0	2.70 ± 0.22	2.85 ± 0.51	4.69 ± 0.62
	100	2.16 ± 0.28	1.23 ± 0.08	3.35 ± 0.16
	200	2.79 ± 0.33	1.99 ± 0.25	5.13 ± 0.38
B. subtilis	0	2.15 ± 0.01	13.14 ± 0.01	4.25 ± 0.41
	100	2.98 ± 0.02**	14.20 ± 0.02	3.38 ± 0.01
	200	2.69 ± 0.04***	9.77 ± 0.04	2.40 ± 0.04
GA_3 (0.4 mM) + *B. subtilis*	0	3.54 ± 0.02	1.71 ± 0.02*	5.89 ± 0.01*
	100	3.10 ± 0.21**	2.55 ± 0.16	3.75 ± 0.13
	200	3.36 ± 0.26***	1.33 ± 0.22	2.58 ± 0.22
GA_3 (0.5 mM) + *B. subtilis*	0	3.43 ± 0.16	2.23 ± 0.25	5.90 ± 0.16 *
	100	3.50 ± 0.27**	2.00 ± 0.31	4.28 ± 0.32**
	200	3.76 ± 0.22***	2.95 ± 0.22	3.08 ± 0.32
GA_3 (0.6 mM) + *B. subtilis*	0	2.87 ± 0.04	3.54 ± 0.04	5.19 ± 0.05
	100	2.18 ± 0.25	3.62 ± 0.18	4.31 ± 0.22**
	200	2.10 ± 0.14	3.18 ± 0.18	3.30 ± 0.14

Data are means ± SE (n=3). *, **, and *** are significant differences at P ≤ 0.05 to the normal control, negative control (100 mMNaCl) and negative control (200 mMNaCl) respectively determined by Tukey-Kramer multiple range test.

only in 0.5 mM of gibberellic acid at 200 MmNaCl treated groups as well as in combined effects of gibberellic acid and *B. subtilis* in 0.5 and 0.6 mM of gibberellic acid at 200 mMNaCl respectively relatives to other groups (Figure 2b).

Reducing sugar accumulation

In both genotypes, levels of reducing sugar accumulation were significantly higher in the gibberellic acid-treated or *B. subtilis* inoculated plants than untreated control plants (Figure 3), whereas significant increase of reducing sugar accumulation contents was observed in combined effects of gibberellic acid and *B. subtilis* in both genotypes NHe 47-4 and Clempson (Figures 3a and 3b).

Antioxidant activities

Total phenolic and flavonoids contents: Figure 4a shows that pre-soaked seeds and seedling inoculated with *B. subtilis* had no significant effect on phenolic content of the salt-stressed okra plant. But in Figure 4b, a significant increase in the phenolic content was noticed only in groups treated with the combined effects of gibberellic acid and *B. subtilis* at 200 mM NaCl in NHe 47-4 genotype. A similar effect was observed in flavonoids content in okra plant treated with each concentration of gibberellic acid and *B. subtilis* or in combination (Figures 5a and 5b).

Superoxide dismutase (SOD) activity: In Figure 6a, the interaction

Table 4: The gibberellic acid and *Bacillus subtilis* effects on photosynthetic pigments of salt-stressed okra plant Clemson genotype.

Treatments	NaCl (mM)	Chlorophyll a (mg/g fw)	Chlorophyll b (mg/g fw)	Carotenoids (mg/g fw)
0 mM	0	4.26 ± 0.02	2.30 ± 0.02	4.83 ± 0.42
	100	1.24 ± 0.20	1.35 ± 0.34	1.06 ± 0.29
	200	0.43 ± 0.34*	0.86 ± 0.67*	0.25 ± 0.65
GA_3 (0.4 mM)	0	3.26 ± 0.21	3.25 ± 0.17	1.35 ± 0.27
	100	1.25 ± 0.02	1.12 ± 0.02	1.24 ± 0.02
	200	1.29 ± 0.03	3.37 ± 0.33***	0.99 ± 0.02
GA_3 (0.5 mM)	0	3.23 ± 0.29	2.00 ± 0.15	1.41 ± 0.11
	100	1.13 ± 0.34	5.40 ± 0.33	1.35 ± 0.27
	200	1.12 ± 1.96	1.20 ± 0.18	0.49 ± 1.19
GA_3 (0.6 mM)	0	3.25 ± 0.94	18.34 ± 0.50	1.35 ± 0.37
	100	2.11 ± 0.56	3.25 ± 0.03	1.32 ± 0.03
	200	3.25 ± 0.11	1.45 ± 1.62	1.04 ± 0.20
B. subtilis	0	2.25 ± 1.36	0.63 ± 0.91	1.49 ± 0.36
	100	2.24± 0.56	1.48± 0.56	0.74± 0.75
	200	1.58 ± 0.88	1.26 ± 0.38	0.49 ± 0.52
GA_3 (0.4 mM) + *B. subtilis*	0	4.28 ± 0.12	4.55 ± 0.12*	3.54 ± 0.50
	100	3.29 ± 0.75**	2.95 ± 0.19	2.96 ± 0.74**
	200	3.18 ± 0.24***	3.70 ± 0.17***	2.29 ± 0.26
GA_3 (0.5 mM) + *B. subtilis*	0	4.55 ± 0.32	2.56 ± 0.34	3.50 ± 0.47
	100	3.21 ± 0.53**	3.26 ± 0.51**	3.45 ± 0.67**
	200	4.24 ± 0.03***	3.28 ± 0.03***	1.40 ± 0.01
GA_3 (0.6 mM) + *B. subtilis*	0	4.53 ± 0.32	2.45 ± 0.34	3.15 ± 0.24
	100	1.27 ± 0.38	1.90 ± 0.40	2.07 ± 0.23***
	200	3.96 ± 0.20***	3.72 ± 0.40***	1.56 ± 0.68

Data are means ± SE (n=3). *, **, and *** are significant differences at P ≤ 0.05 to the normal control, negative control (100 mM NaCl) and negative control (200 mM NaCl) respectively determined by Tukey-Kramer multiple range test.

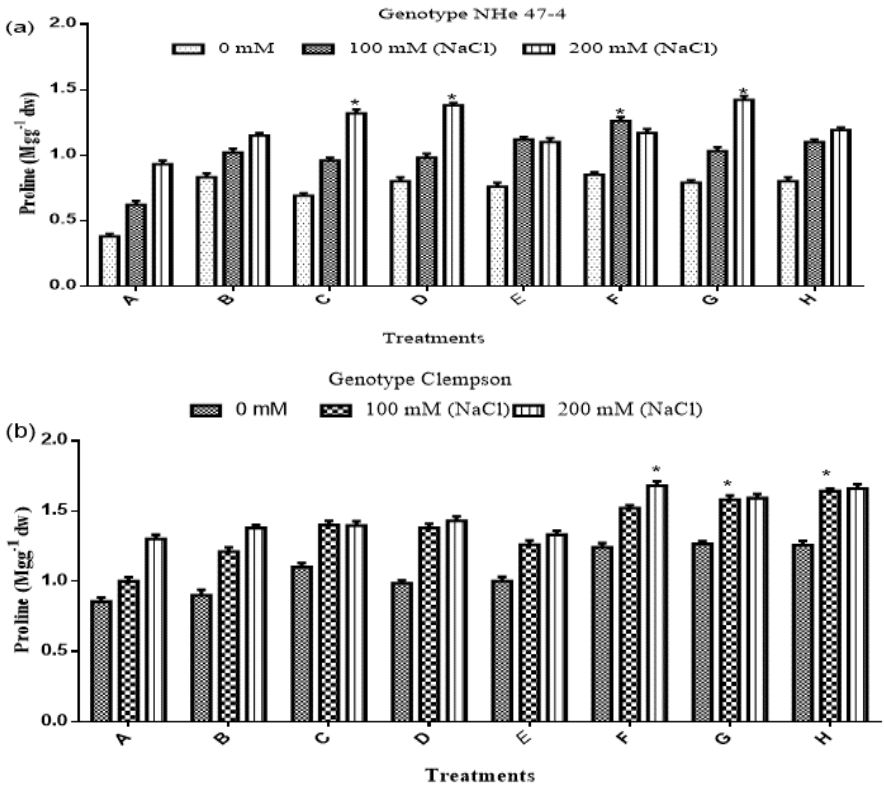

Figure 1: Effects of gibberellic acid and *B. subtilis* on proline accumulation of okra (NHe 47-4 and Clempson genotypes) plant under salinity stress. In each group, mean values ± SE (n=3). *Significant difference (P < 0.05) relatives to the salt-stressed control and the normal control groups. Where A=Controls (Normal control and Negative control groups), B, C, and D=0.4, 0.5, and 0.6 mM of gibberellic acid respectively, E=*Bacillus subtilis*, F, G, and H=0.4, 0.5, and 0.6 mM of gibberellic acid and *Bacillus subtilis* respectively.

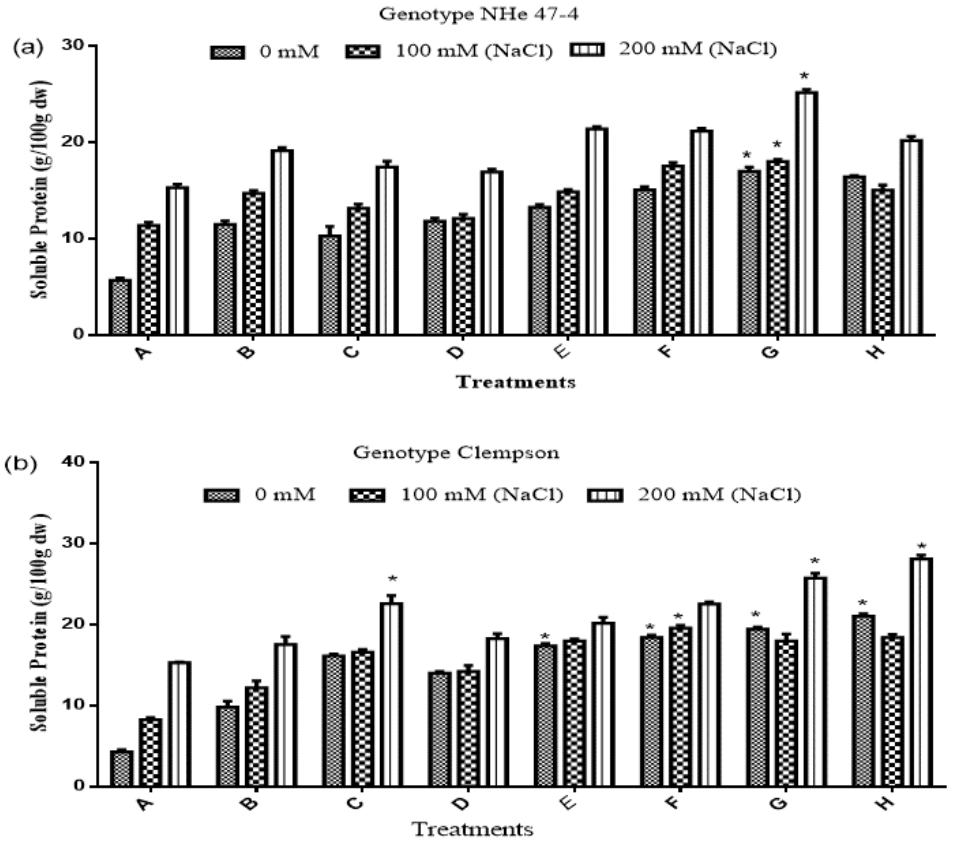

Figure 2: Effects of gibberellic acid and *B. subtilis* on soluble protein accumulation of okra (genotypes NHe 47-4 and Clempson) plant under salinity stress. In each group, mean values ± SE (n=3). *Significant difference ($P < 0.05$) relatives to the salt-stressed control and the normal control groups. Where A=Controls (Normal control and Negative control groups), B, C, and D=0.4, 0.5, and 0.6 mM of gibberellic acid respectively, E=*Bacillus subtilis*, F, G, and H=0.4, 0.5, and 0.6 mM of gibberellic acid and *Bacillus subtilis* respectively.

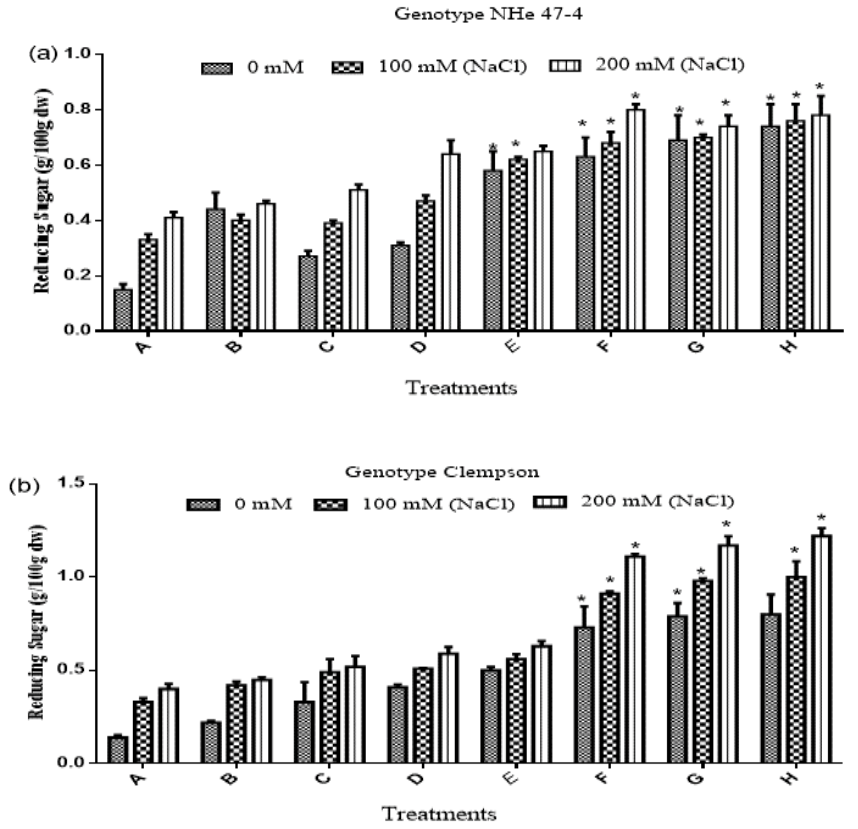

Figure 3: Reducing sugar accumulation of salt-stressed okra (NHe 47-4 and Clempson genotypes) plant under the influence of gibberellic acid and *B. subtilis*. In each group, mean values ± SE (n=3). *Significant difference ($P < 0.05$) relatives to the salt-stressed control and the normal control groups. Where A=Controls (Normal control and Negative control groups), B, C, and D=0.4, 0.5, and 0.6 mM of gibberellic acid respectively, E=*Bacillus subtilis*, F, G, and H=0.4, 0.5, and 0.6 mM of gibberellic acid and *Bacillus subtilis* respectively.

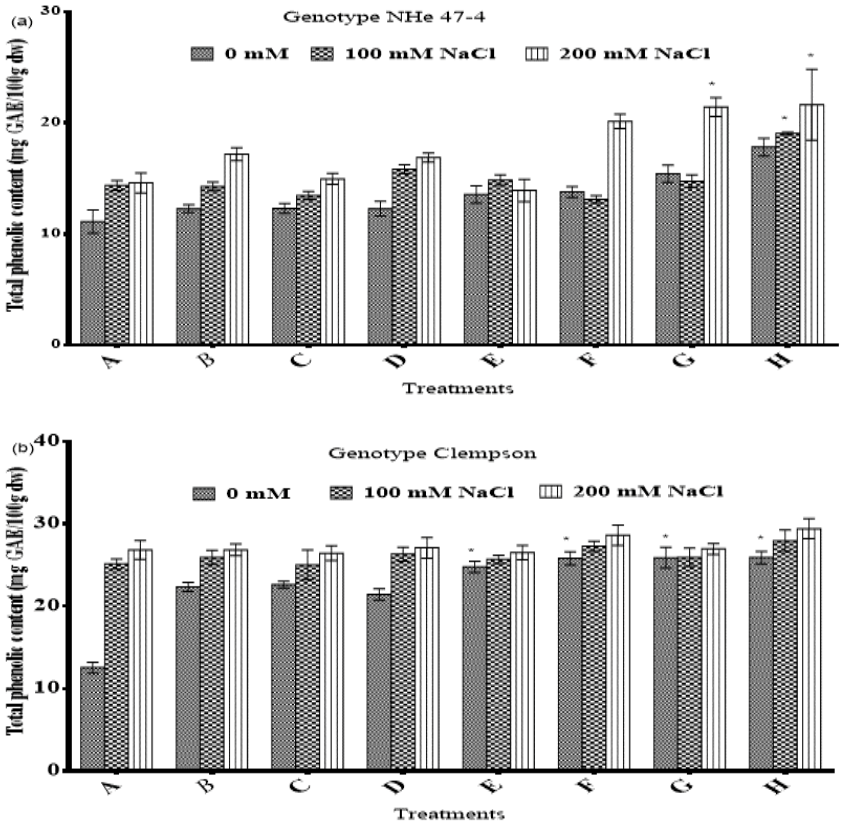

Figure 4: Effects of gibberellic acid and *B. subtilis* on total phenolic content of okra (genotypes NHe 47-4 and Clempson) plant under salinity stress.In each group, mean values ± SE (n=3). *Significant difference ($P < 0.05$) relatives to the salt-stressed control and the normal control groups. Where A=Controls (Normal control and Negative control groups), B, C, and D=0.4, 0.5, and 0.6 mM of gibberellic acid respectively, E=*Bacillus subtilis*, F, G, and H=0.4, 0.5, and 0.6 mM of gibberellic acid and *Bacillus subtilis* respectively.

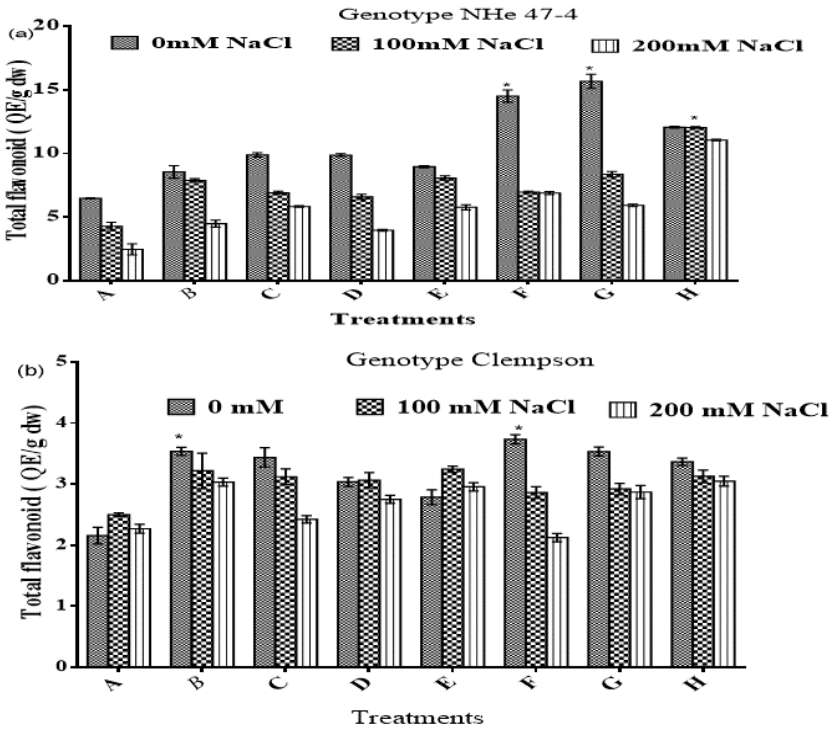

Figure 5: Effects of gibberellic acid and *B. subtilis* on total flavonoids content of okra (genotypes NHe 47-4 and Clempson) plant under salinity stress. In each group, mean values ± SE (n=3). *Significant difference ($P < 0.05$) relatives to the salt-stressed control and the normal control groups. Where A=Controls (Normal control and Negative control groups), B, C, and D=0.4, 0.5, and 0.6 mM of gibberellic acid respectively, E=*Bacillus subtilis*, F, G, and H=0.4, 0.5, and 0.6 mM of gibberellic acid and *Bacillus subtilis* respectively

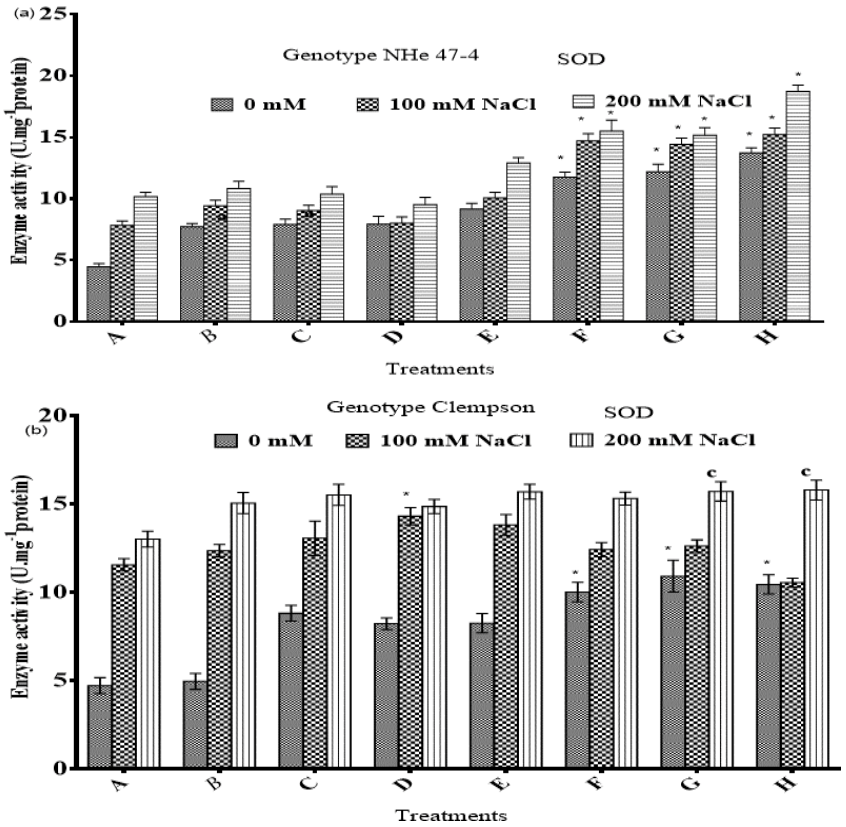

Figure 6: Effects of gibberellic acid and *B. subtilis* on superoxide dismutase activity of okra (genotypes NHe 47-4 and Clempson) plant under salinity stress. In each group, mean values ± SE (n=3). *Significant difference ($P < 0.05$) relatives to the salt-stressed control and the normal control groups. Where A=Controls (Normal control and Negative control groups), B, C, and D=0.4, 0.5, and 0.6 mM of gibberellic acid respectively, E=*Bacillus subtilis*, F, G, and H=0.4, 0.5, and 0.6 mM of gibberellic acid and *Bacillus subtilis* respectively.

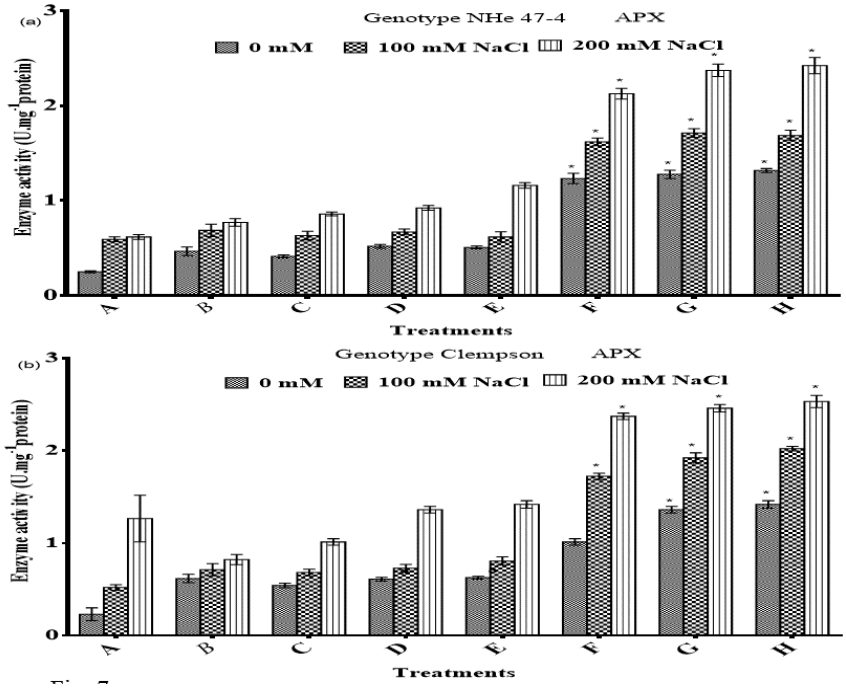

Figure 7: Effects of gibberellic acid and *B. subtilis* on ascorbate peroxide activity of okra (genotypes NHe 47-4 and Clempson) plant under salinity stress. In each group, mean values ± SE (n=3). *Significant difference ($P < 0.05$) relatives to the salt-stressed control and the normal control groups. Where A=Controls (Normal control and Negative control groups), B, C, and D=0.4, 0.5, and 0.6 mM of gibberellic acid respectively, E=*Bacillus subtilis*, F, G, and H=0.4, 0.5, and 0.6 mM of gibberellic acid and *Bacillus subtilis* respectively.

of *B. subtilis* and gibberellic acid showed the greater activity of superoxide dismutase in NHe 47-4 genotype when compared with each concentration of gibberellic acid or *Bacillus subtilis* relative to the control groups. But in Figure 6b, the treatment had no noticeable effect on superoxide dismutase activity in Clempson genotype as compared to the control groups.

Ascorbate peroxidase (APX) activity: In Figure 7, a single treatment with gibberellic acid or *Bacillus subtilis* had no effect on the activity

of APX, but combined effects of *B. subtilis* and gibberellic acid at 200 mMNaCl showed a significant increase activity of APX in both genotypes when compared with control groups (Figures 7a and 7b).

Polyphenol oxidase (PPO) activity: In Figure 8a, no noticeable effect of *B. subtilis* or gibberellic acid treatment on PPO activity, but the interaction of gibberellic acid and *B. subtilis* at all levels of salinity showed greater activities of polyphenol oxidase in NHe 47-4 genotype. But in Figure 8b, increase in the levels of gibberellic acid with *B. subtilis* and salt treatment resulted to an increase activity of polyphenol oxidase when compared with control groups in Clempson genotype.

Radical scavenging capacity of DPPH: In Figure 9a, salt treatment alone or in combination with both gibberellic acid and *B. subtilis* had no effect on *DPPH* percentage inhibition when compared with the control groups in NHe 47-4 genotype. However, in Figure 9b, the combined effects of gibberellic acid and *B. subtilis* showed higher *DPPH* percentage inhibition as compared to the control group in Clempson genotype.

DISCUSSION

In this study, we examined the effects gibberellic acid and *B. subtilis* on salt-stressed okra (NHe 47-4 and Clempson genotypes) plant to ascertain whether pre-soaked seeds in gibberellic acid and seedling inoculated with *B. subtilis* could alleviate the effects of salinity. Our results revealed increased in the levels of phosphorous, potassium, calcium, and magnesium ions concentrations in *B. subtilis*-inoculated and gibberellic acid pretreated plants than the normal control and salt-stressed groups in both genotypes Tables 1a and 1b and 2a and 2b, which supports the fact that the *B. subtilis* and gibberellic acid could reduce ions toxicity and maintain ionic homeostasis and increase plants nutrients uptake, as reported by Dodd and Perez-Alfocea, [37]. The declined levels of sodium ion in okra tissues in response to the treatments protect okra tissues from the effects of salinity in the two genotypes. The increase photosynthetic pigment contents observed in Tables 3 and 4 under the combined effects of gibberellic acid and *B. subtilis* in this study may be due to the growth promoting effects of GA_3 and or *B. subtilis*, which increases photosynthetic efficiency, and in turn improved the plant biomass (Figures 2 and 3). Our result is similar to the findings of Mohamed and Gomaa [38] who observed increased chlorophyll *a*, *b* and carotenoid contents in leaves of salt-stressed radish plants following the inoculation with strains of *B. subtilis* and *Pseudomonas fluorescens*. More also, organic solutes (proline, soluble protein and reducing sugar) levels were found to be increased in salt-stressed okra plant treated with either gibberellic acid or *B. subtilis*, but significant increase in organic solutes levels in okra plant was observed in combined treatments of gibberellic acid and *B. subtilis*. These results are in agreement with those of Esan and Olaiya [39], who observed improvement of okra plant under saline conditions following seeds pre-treatment with salicylic acid, and Younesi and Moradi [40] who observed the same result through the synergistic effects of plant rhizobacteria and *Arbuscularmycorrhizal*

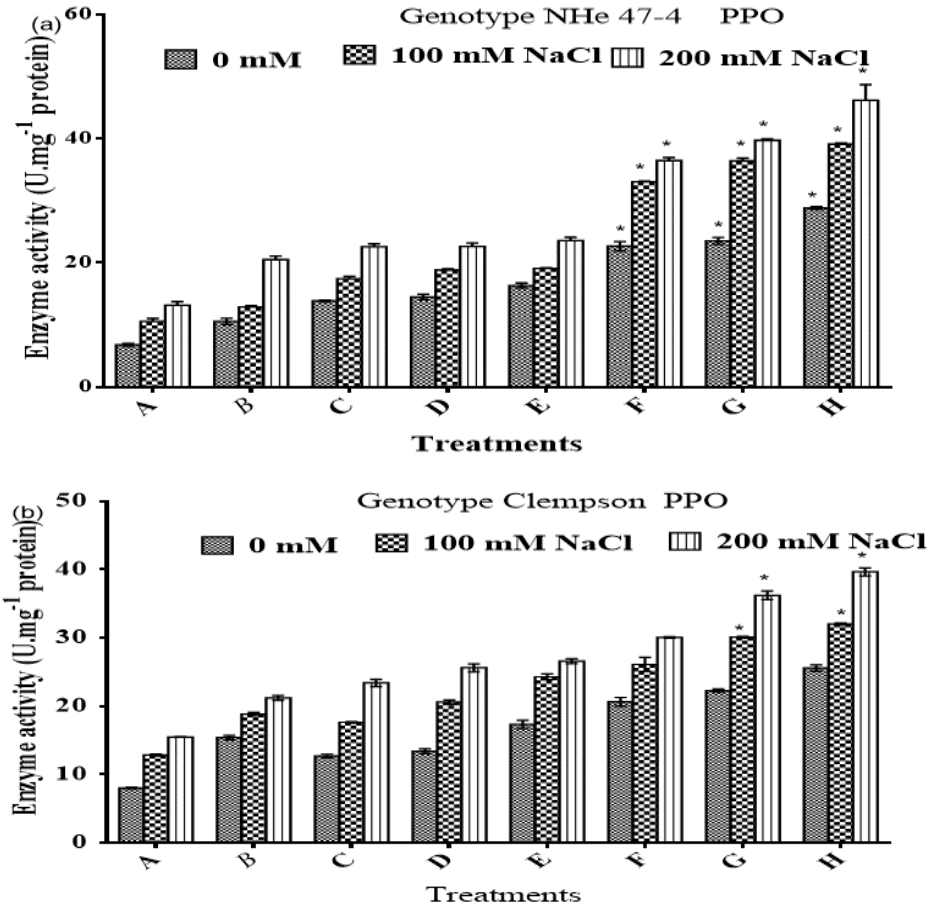

Figure 8: Effects of gibberellic acid and *B. subtilis* on polyphenol oxidase activity of okra (genotypes NHe 47-4 and Clempson) plant under salinity stress. In each group, mean values ± SE (n=3). *Significant difference ($P < 0.05$) relatives to the salt-stressed control and the normal control groups. Where A=Controls (Normal control and Negative control groups), B, C, and D=0.4, 0.5, and 0.6 mM of gibberellic acid respectively, E=*Bacillus subtilis*, F, G, and H=0.4, 0.5, and 0.6 mM of gibberellic acid and *Bacillus subtilis* respectively.

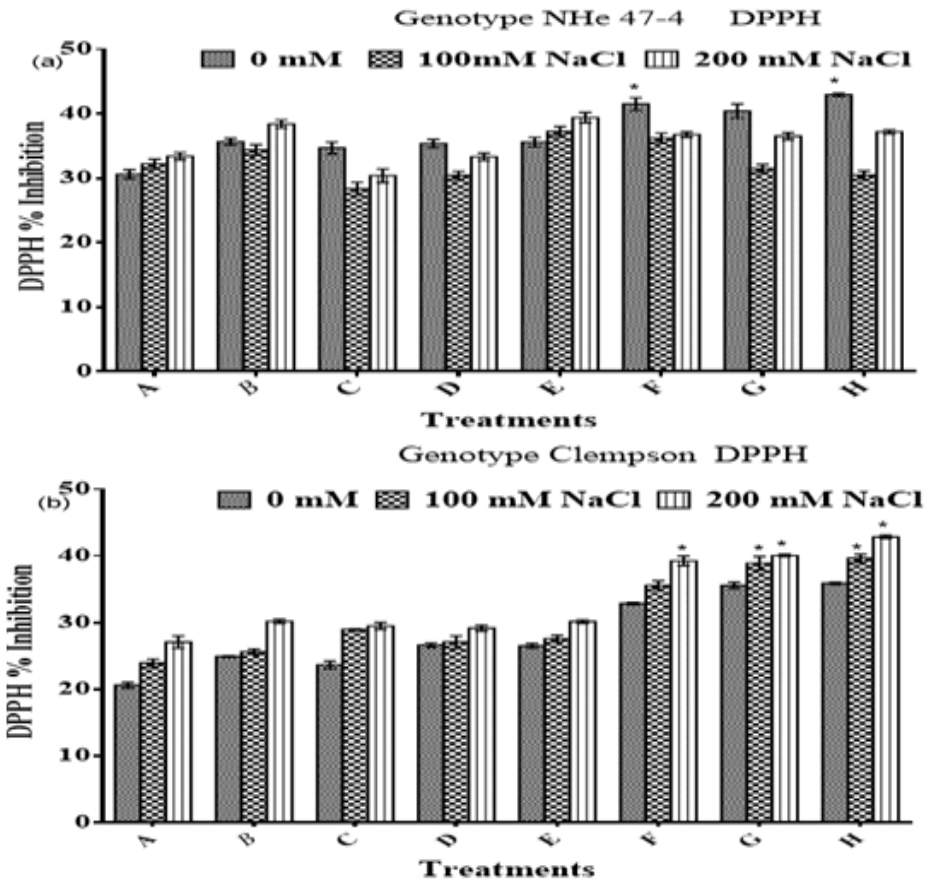

Figure 9: Effects of gibberellic acid and *B. subtilis* on DPPH radical scavenging activity of okra (NHe 47-4 and Clempson genotypes) plant under salinity stress. In each group, mean values ± SE (n=3). *Significant difference ($P < 0.05$) relatives to the salt-stressed control and the normal control groups. Where A=Controls (Normal control and Negative control groups), B, C, and D=0.4, 0.5, and 0.6 mM of gibberellic acid respectively, E=*Bacillus subtilis*, F, G, and H=0.4, 0.5, and 0.6 mM of gibberellic acid and *Bacillus subtilis* respectively.

fungi. Osmotic adjustment in plant tissues due to the organic solutes accumulation in salinity stress was also reported by Miller et al. [41], Amin et al. [42].

The antioxidant enzymes activities studied in the okra plant were significantly increased with increasing level of sodium chloride. More also, the okra plant enzymes activity activation by salinity stress under gibberellic acid or *B. subtilis* treatments showed salt tolerance of okra plant. Similar results were reported by Esan et al. [39], who observed improvement of okra plant on oxidative stress marker and antioxidant potential under saline conditions following seeds pre-treatment with indole acetic acid and salicylic acid, and Mittova et al. [43] who reported that plants modulate free radical's species via the induction of certain anti-oxidative enzymes activity. The interaction of gibberellic acid, *B. subtilis* on salt-stressed okra plant in this study enhanced the total phenolic, flavonoids, and DPPH potential of okra plant. The increase in total phenolic and flavonoids potential and DPPH radical-scavenging capacity may possibly due to the stimulation of antioxidant enzymes activities, which alleviates the adverse effects of free radicals generated in the plant.

Salt stress contributes to the disturbance of major metabolic processes which results to quality and quantity reduction in plants. Salinity stress causes overproduction of reactive oxygen species (ROS) in plants tissues that often lead to oxidative stress [44]. Evidence from various studies suggests that salinity stress through free radical's generation damage plants tissues [45]. Reactive oxygen species alter plant cellular components through degradation of protein, mutation of DNA, and lipid peroxidation [46]. Various strategies have been put in place to overcome salinity stress. However, such strategies are not environmentally friendly and also cost-intensive. Therefore, to reduce salinity stress effects on agricultural products, it requires simple and low-cost methods. In this respect, microorganisms could play an important role; they synthesize plant hormones, improve solutes content and increase the activities of antioxidant enzymes component in plants [47].

CONCLUSION

The present work illustrates the importance of using gibberellic acid and *Bacillus subtilis* on salt-stressed okra. In this our manuscript, we observed that the combined treatment of salt-stressed okra with gibberellic acid and *Bacillus subtilis* is more promising in the management of salt-stressed okra than the single treatment with either gibberellic acid or *Bacillus subtilis*. Conclusively, the pre-soaked seeds of okra with gibberellic acid and seedling inoculation with *B. subtilis* enhanced okra plant salt tolerance by protecting cells from oxidative damage by osmotic pressure via improved compatible solutes such as proline, soluble protein and reducing sugar, and also through increase antioxidant defense mechanism with increase photosynthetic pigments. Thus, *B. subtilis* can be multiplied for mass production of genes that are responsible for salt tolerance, which can be used as biofertilizers for farmers to support growth and yield under saline soils. Therefore, combined treatments of gibberellic acid and *B. subtilis* could be recommended as a promising technique to ameliorate salinity stress effects in

okra plant as shown by increased biomass, the content of mineral elements, photosynthetic pigments, and antioxidant component enzymes, as well as antioxidant potential by DPPH. Hence, gibberellic acid and *B. subtilis* could be an outstanding measure to improve crop growth in saline conditions.

COMPETING INTERESTS

No competing of interests declared by the authors.

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