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Moringa oleifera leaf extract improves growth, physio-chemical attributes, antioxidant defence system and yields of salt-stressed *Phaseolus vulgaris* L. plants

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Abstract: Two field experiments are aimed to study the effect of *Moringa oleifera* leaf extract (MLE; 1 extract paste: 30 tap water), used as seed soaking and/or foliar application, on the growth, physio-chemical attributes and yields of common bean (*Phaseolus vulgaris* L.) plants grown on a saline soil (EC = 6.23 – 6.28). The MLE application used as seed soaking or foliar spray significantly increased growth characteristics (i.e., shoot length, number and area of leaves per plant, and plant dry weight), physio-chemical attributes (i.e., RWC% and MSI%, concentrations of total chlorophylls, total carotenoids, total soluble sugars, free proline and ascorbic acid, contents of N, P, K and Ca, and ratios of K/Na, Ca/Na and K+Ca/Na), antioxidant enzymes (superoxide dismutase, ascorbate peroxidase and glutathione reductase) and the yields of green pods and dry seeds, when compared with the controls (seed soaking and foliar spray with tap water). Further, the MLE application used as seed soaking in combination with foliar spray significantly increased all aforementioned parameters compared to the control and the single treatments with MLE (seed soaking or foliar spray). In contrast, there were significant reductions in leaf EL%, Na% and the enzyme catalase. The combined treatment of seed soaking and foliar spray with MLE was found to be highly effective at improving the growth and yields of common bean plants by alleviating the inhibitory effects of soil salinity stress.
Keywords: *Phaseolus vulgaris* L., *Moringa oleifera* leaf extract, salt stress, growth and productivity, physio-chemical attributes.

Introduction

Common bean (*Phaseolus vulgaris* L.) is one of the most important vegetable Fabaceae crops. It is classified as a salt-sensitive plant[1]. Food legumes, including beans, are an important component of the agricultural sectors of developing countries due to their capacity to produce large quantities of protein-rich seed for human nutrition.

Salinity considers one of the major factors affecting the agricultural productivity worldwide, particularly in the arid and semiarid regions. In these regions, soil salinization caused by some effective factors such as low rainfall, poor drainage, poor irrigation water which contains considerable amounts of salts that accumulate in the soil surface layer, poor water management, high evaporation rate, proximity to the sea and/or the capillarity rise of salts from underground water into the root zone due to the excessive evaporation[2,3]. Salinity reduces the ability of plants to utilize water and causes a reduction in the growth and yield, and changes in the plant metabolic processes[4]. Plants grown under saline conditions are stressed basically in three ways: (1)

water deficit caused by reduced water potential in the rhizosphere, (2) Na^+ and Cl^- ions phytotoxicity and (3) nutrient imbalance by the reduction in the uptake and/or shoot transport[5,6].

Moringa oleifera leaf extract (MLE) is considered one of the plant biostimulants, which are substances when applied as seed soaking and/or foliar spray positively modify plant growth and production with alterations in metabolic processes[3,7,8,9] under normal or stress conditions.

Moringa oleifera Lam., a multipurpose tree from Moringaceae family is native to the sub-Hamaylian tract of India and Pakistan[10,11]. The MLE obtained from fresh moringa leaves posses' high antioxidant activity are rich in some plant secondary metabolites and osmoprotectants[3,7,8]. They also found that MLE is also a source of zeatin, a natural derivative of cytokinin, vitamins and several mineral elements, making it a potential natural growth stimulant. Like other biostimulants, the potential of MLE when applied through seed or plant foliage have been shown to improve the plant tolerance to abiotic stresses, including salinity[3,7,8,12]. These reports and others have been shown that MLE application improved crop performance, resulting from vigorous seedling growth, maintained optimum tissue water status, improved membranes stability, enhanced antioxidant levels and activated plant defense system, increased levels of plant secondary metabolites, reduced uptake of undesirable Na^+ and/or Cl^-, and enhanced shoot or leaf K^+[3,13,14]. The MLE act at low or even diluted concentration of 1:30, and the chemical composition of this extract may vary with species, season of collection and extraction procedure used.

The present work was designed with objective to evaluate the potential effects of the exogenous application of MLE on the changes in growth, yields, endogenous physio-chemical constituents and the antioxidant defence system of *Phaseolus vulgaris* L. plants, exposed to moderate soil salinity stress (EC = 6.23–6.28) and to establish a relationship between the changes in physio-chemical constituents accompanied with the antioxidants and the degree of tolerance, in terms of improvement in growth and yields. The hypothesis tested is that exogenous applications of MLE, used as seed soaking and/or foliar spray, will elevate the level of some non-enzymatic antioxidants, antioxidant enzymes and osmoprotectants that will mitigate the stress generated by salinity stress.

Materials and methods

Soil analysis and preparation, plant material and experimental procedures:

Two field experiments were conducted in two successive seasons (2014 and 2015) on a special Farm at Sherif Basha village, Beni Sueif Governorate; 29°06'20.4"N; 31°07'21.6"E, Egypt. In the 2014 season, daily temperatures ranged from 15.2º – 26.8ºC, with an average of 21.0º ± 2.5ºC. The daily relative humidity averaged 58.0 ± 4.2%, and ranged from 31 – 85%. In addition, the daily temperatures ranged from 13.8º – 28.8ºC, with an average of 21.3º ± 2.8ºC, and the daily relative humidity averaged 56 ± 6.6% and ranged from 28 – 84% were for the 2015 season.

Healthy common bean (*Phaseolus vulgaris* L.) cv. Bronco seeds were sown on 27 February 2014, and on 23 February 2015. Seeds were obtained from Horticulture Research Institute, Agricultural Research Centre, Giza, Egypt. They were sown at the equivalent of 95 kg ha^{-1} to achieve the recommended planting density. Seeds were selected for uniformity by choosing those of equal size and of the same color. The selected seeds were washed with distilled water, sterilized in 1% (v/v) sodium hypochlorite for approx. 2 min, washed thoroughly again with distilled water, and left to dry at room temperature (20 ºC). Uniform, air-dried seeds were sown, after their soaking in water or *Moringa oleifera* leaf extract (MLE), in hills in rows spaced 60 cm apart. The hills were spaced 10 – 15 cm apart in 3.0 m × 3.5 m plots. Thinning was done before the first irrigation to produce two plants per hill.

During soil preparation and plant growth, the soil was supplemented with the full dose of NPK fertilizer according to the recommendations of the Egyptian Ministry of Agriculture and Land Reclamation. These recommendations were for 450 kg ha^{-1} of calcium super-phosphate (15.5% P_2O_5), 120 kg ha^{-1} ammonium sulphate (20.5% N), and 60 kg ha^{-1} potassium sulphate (48% K_2O) during seed-bed preparation. An additional 120 kg ha^{-1} of ammonium sulphate and 60 kg ha^{-1} of potassium sulphate were added at the first irrigation, 2 weeks after each sowing. Irrigation water was added to 100% of the reference crop evapotranspiration (ETo),

values from the Beni Sueif Governorate Meteo Station. All other recommended agricultural practices were followed according to the recommendations of the Egyptian Ministry of Agriculture and Land Reclamation.

Soil analysis of the experimental site in each season was carried out[15,16]. The results from physical and chemical analyses of the soils are shown in Table 1. Electrical conductivity (EC) was measured using a conductivity meter and an extract of each soil paste. Soil EC values were 6.23 and 6.28 dS m^{-1} for 2014 and 2015 seasons, respectively. These EC values classed the soils as being moderately saline[17]. The experiment was arranged in a randomized complete block design, with one level of each of water, SA and MLE with three replicate plots per treatment.

Preparation, analysis and applications of moringa leaf extract (MLE):

Fresh leaves harvested from fully matured *Moringa oleifera* trees were air-dried, grinded and extracted. For extraction, ethyl alcohol was added to leaf powder and the mixture was put for 4 hours on a Rotary Shaker. Extract was purified by filtering twice through Whatman No. 1 filter paper. After purification, the extract was subjected to a Rotary Evaporator to fully evaporate the alcohol. Centrifugation at 8,000 × *g* for 15 min was then conducted for supernant. Supernatant was diluted to 30 times and used to seed soaking and foliar spray applications. The extract was analyzed and its chemical constituents are presented in Table 1.

For seed soaking, common bean seeds were soaked in tap water and MLE using seed weight to solution volume ratio 1:5 for 2 h at room temperature (25 °C). After soaking, seeds were given washings with water and re-dried overnight at room temperature. At early morning, treated seeds were sown as mentioned before. Foliar spray of water or MLE was done at early morning with a sprayer (Vol. 20 L) to run-off twice, at 25 and 40 days after sowing. The concentration of MLE, and the number and timing of sprays were based on results from a preliminary pot trial (data not shown). To ensure optimal penetration into leaf tissues, 0.1% (v/v) Tween-20 was added to the foliar sprays as a surfactant.

Table (1): Physical and chemical properties of the experimental soil, and some chemical constituents of *Moringa oleifera* leaf extract (on dry weight basis) in two seasons

Parameter/component	2014 season	2015 season
The experimental soil		
Clay	50.0	49.8
Silt	30.5	30.2
Sand	19.5	20.0
Soil texture	Clay	
pH	7.72	7.76
EC (dS m^{-1})	6.23	6.28
Organic matter%	0.95	0.92
$CaCO_3$ (%)	5.79	5.66
CEC* ($cmol_c$ kg^{-1})	33.8	34.2
Field capacity (%)	28.6	28.2
Available water (%)	12.8	12.4
Available N (mg kg^{-1} soil)	152.6	148.4
Available P (mg kg^{-1} soil)	12.2	11.4
Available K (mg kg^{-1} soil)	144.2	138.9
Available Fe (mg kg^{-1} soil)	21.2	19.3
Available Mn (mg kg^{-1} soil)	11.0	11.5
Available Zn (mg kg^{-1} soil)	4.1	3.9
Moringa oleifera leaf extract (values in mg g^{-1} DW)		
Amino acids	124.7	126.8
Proline	26.09	27.17
Total soluble sugars	151.4	160.3
Ash	111.3	114.9
Calcium	8.756	9.032

Magnesium	6.035	6.121
Potassium	27.68	29.02
Phosphorus	6.122	6.225
Sodium	0.674	0.662
Iron	1.873	1.920
Manganese	0.966	1.020
Zinc	0.453	0.510
Copper	0.208	0.225
Soluble phenols	2.252	2.164
Total carotenoids	2.243	2.462
Total chlorophyll	4.625	5.008
Ascorbic acid	3.247	3.448
Phytohormones (μg g^{-1} DW):		
Indole-3-acetic acid	0.873	0.904
Gibberellins	0.802	0.824
Zeatin	0.936	0.974
Abscisic acid	0.292	0.284

Plant growth analysis and yield estimations:

Fifty-day-old bean plants (n = 9) were removed from each of the nine treatments and the number of leaves $plant^{-1}$ were counted. Shoot lengths were measured using a meter scale, then the leaf areas were measured manually using a graph sheet, where the squares covered by the leaf were counted to note the leaf area. Plants were then placed in an oven at 70 ºC until constant weight to record plant dry weight. At the marketable green pod stage of each experiment, all the green pods on plants of half-rows in each plot were collected and weighed several times. At the end of the experiments (dry seed stage), all dry pods on plants of the other half-rows in each plot were collected, and seeds were then extracted from their pods, air-dried and weighed.

Determination of leaf pigment concentrations:

Total chlorophylls and carotenoids concentrations (in mg g^{-1} FW) were determined[18]. Leaf discs (0.2 g from each replicate-plot of each treatment) were homogenized in 50 ml 80% (v/v) acetone and centrifuged at 10,000 × *g* for 10 min. The absorbance of each acetone extract was measured at 663, 645, and 470 nm using a UV-160A UV-visible recording spectrometer (Shimadzu, Kyoto, Japan).

Determination of membrane stability index (MSI), electrolyte leakage (EL), and relative water content (RWC):

MSI% was determined using duplicate 0.2 g samples of leaf tissue that were placed in test tubes containing 10 ml of double-distilled water[19]. One sample was heated at 40 °C in a water bath for 30 min, and the electrical conductivity of the solution was recorded using a conductivity bridge (C_1). The second sample was boiled at 100 °C for 10 min, and the conductivity was measured (C_2). The MSI% was calculated using the following formula:

$$MSI\ (\%) = [1 - (C_1/ C_2)] \times 100$$

The total inorganic ion leaked from leaves (EL) was determined[20]. Twenty leaf discs were placed in a boiling tube containing 10 ml deionized water and the electrical conductivity (EC_1) was recorded. The contents were then heated to 45 °C − 55 °C for 30 min each in a water bath and the electrical conductivity (EC_2) was recorded. The sample was boiled at 100 °C for 10 min and the electrical conductivity (EC_3) was recorded. EL% was calculated using the following formula:

$$EL\ (\%) = [(EC_2 - EC_1) / EC_3] \times 100$$

Excluding the midrib, fresh 2 cm-diameter fully-expanded leaf discs were used to determine the RWC% as described early[21] with some modifications[22]. The discs were weighed (fresh mass; FM) and immediately floated on double-distilled water in Petri dishes for 24 h, in the dark, to saturate them with water. Any adhering water was blotted dry and the turgid mass (TM) was measured. The dry mass (DM) was recorded after dehydrating the discs at 70°C for 48 h. The RWC% was then calculated using the following formula:

$$RWC\ (\%) = [(FM - DM) / (TM - DM)] \times 100$$

Determination of leaf concentrations of free proline, total soluble sugars and ascorbic acid:

Leaf free proline concentrations (in μg g^{-1} DW) were determined using a rapid colourimetric method[23]. Proline was extracted from 0.5 g of each fresh leaf sample by grinding in 10 ml 3% (v/v) sulphosalicylic acid and the mixture was then centrifuged at 10,000 × *g* for 10 min. Two ml of the supernatant was placed in a test-tube, to which 2 ml of a freshly prepared acid ninhydrin solution was added. The tubes were incubated in a water bath at 90 ºC for 30 min and the reaction was terminated in an ice bath. Each reaction mixture was extracted with 5 ml toluene and vortex-mixed for 15 s. The tubes were allowed to stand for at least 20 min in the dark, at room temperature, to allow separation of the toluene and aqueous phases. Each toluene phase was then carefully collected into a clean test-tube and its absorbance was read at 520 nm. The free proline concentration in each sample was determined from a standard curve prepared using analytical grade proline, and expressed on a DW basis.

Total soluble sugars were extracted and determined[24]. A 0.2 g sample of fresh leaves was homogenized in 10 ml of 96% (v/v) ethanol and washed with 5 ml 70% (v/v) ethanol. The extract was centrifuged at 3,500 × g for 10 min and the supernatant was stored at 4 ^{o}C for measurement. Total soluble sugar concentrations were determined by reacting 0.1 ml of the ethanolic extract with 3 ml of freshly prepared anthrone reagent [150 mg anthrone plus 100 ml of 72% (v/v) sulphuric acid] and placed in a boiling water bath for 10 min. After cooling, the absorbance of the mixture was recorded at 625 nm using a Bausch and Lomb-2000 Spectronic Spectrophotometer.

The extraction and determination of ascorbic acid (AsA) from bean leaf samples were conducted[25]. Plant leaf material (1.0 g) was obtained from each replicate-plot of each treatment, homogenized immediately in liquid N_2 and extracted with 10 ml 5% (w/v) trichloroacetic acid (TCA). The homogenate was centrifuged at 4°C for 5 min at 15,600 × *g*. The supernatant was transferred to a clean reaction vessel and immediately assayed for AsA content in a 1.0 ml reaction mixture containing 50 μl 10 mM DTT, 100 μl 0.2 M phosphate buffer (pH 7.4), 0.5% (v/v) Nethylmaleimide, 10% (w/v) TCA, 42% (v/v) H_3PO_4, 4% (v/v) 2,2'-dipyridyl, and 3% (w/v) $FeCl_3$.

Measuring the antioxidant enzyme activities:

Superoxide dismutase (SOD; EC 1.15.1.1) activity was assessed by monitoring the inhibition of the photochemical reduction of nitro blue tetrazolium (NBT)[26,27,28]. One unit of SOD activity was defined as the amount of enzyme required for the reduction of 50% NBT. The SOD activity was expressed as A_{564} min^{-1} g^{-1} protein.

Catalase (CAT; EC 1.11.1.6) activity was determined by measuring the consumption of H_2O_2[29]. The reaction mixture consisted of 25 mM Tris-acetate buffer, pH 7.0, 0.8 mM Na-EDTA and 20 mM H_2O_2. The enzyme assay was performed at 25 °C. CAT activity was expressed as A_{290} min^{-1} g^{-1} protein.

Ascorbate peroxidase (APX; EC 1.11.1.11) activity was determined[30] by recording the optical density at 290 nm and the activity was expressed as A_{290} min^{-1} g^{-1} protein.

Glutathione reductase (GR; EC 1.6.4.1) activity was measured after monitoring the oxidation of NADPH for three absorbance taken at 340 nm, and the activity was expressed as A_{340} min^{-1} mg^{-1} protein[30].

Protein was estimated in crude enzyme extracts by dye binding assay[31].

Determination of leaf contents of nitrogen (N), phosphorus (P), potassium (K), calcium (Ca) and sodium (Na):

The content of N (%) in dried bean leaf was determined by using micro-Kjeldahl method[32]. The molybdenum-reduced molybdophosphoric blue color method[16], in sulphuric acid (with reduction to exclude arsenate), was used to determine P content (%). Sulphomolybdic acid (molybdenum blue), diluted sulphomolybdic acid, and 8% (w/v) sodium bisulphite-H_2SO_4 solution were used as reagents. Leaf Ca^{2+} content was determined using a Perkin-Elmer Model 3300 Atomic Absorption Spectrophotometer[33]. The contents of K^+ (%) and Na^+ (%) were determined using 0.2 g of dried bean leaf that was digested with sulphuric acid in the presence of H_2O_2[34]. The mixture was then diluted with distilled water. The total leaf contents of Na^+ and K^+ were measured using Flame Spectrophotometry[35].

Statistical analysis:

The values for all parameters were subjected to statistical analysis, following standard procedures[36]. The 'F' test was applied to assess the significance of each treatment at the 5% level of probability ($P \leq 0.05$).

Results

Growth characteristics (i.e., shoot length, number and area of leaves per plant, and plant dry weight) of salt-stressed common bean plants were positively affected by *Moringa oleifera* leaf extract (MLE), used as seed soaking and/or foliar application over two seasons as shown in Table 2. The single MLE applications, used as seed soaking or foliar application significantly increased all growth traits compared to the controls (seed soaking or foliar spray with tap water). Further, combined MLE application (seed soaking + foliar spray) significantly increased all aforementioned growth characteristics compared to the control and the single MLE applications. Combined MLE treatment found to be the best, increasing shoot length, number of leaves per plant, leaf area per plant and plant dry weight by 21.3%, 20.5%, 21.0% and 25.7%, respectively in 2014 season and by 25.3%, 20.9%, 24.2% and 28.1%, respectively in 2015 season compared to the controls.

Table (2): Effect of seed soaking and/or foliar spray with moringa leaf extract (MLE) on some growth traits of common bean (*Phaseolus vulgaris* L. cv. "Bronco") plants grown under soil salinity in two seasons

Treatments		Parameters			
Seed soaking	**Foliar spray**	**Shoot length (cm)**	**Number of leaves plant^{-1}**	**Leaf area plant^{-1} (dm^2)**	**Plant dry weight (g)**
2014 season					
Tap water	Tap water	25.4c	7.21c	9.42c	6.54c
	MLE	27.6b	7.87b	10.30b	7.30b
MLE	Tap water	27.9b	7.99b	10.36b	7.42b
	MLE	30.8a	8.69a	11.40a	8.22a
2015 season					
Tap water	Tap water	24.9c	7.18c	9.44c	6.43c
	MLE	27.4b	7.78b	10.62b	7.24b
MLE	Tap water	27.7b	7.85b	10.70b	7.37b
	MLE	31.2a	8.68a	11.72a	8.24a

†Mean values (n = 9) in each column for each year followed by a different lower-case letter are significantly different at $P \leq 0.05$ by Duncan's multiple range test.

Table (3): Effect of seed soaking and/or foliar spray with moringa leaf extract (MLE) on leaf photosynthetic pigments (mg g^{-1} fresh weight), relative water content (RWC%), electrolyte leakage (EL%) and membrane stability index (MSI %) of common bean (*Phaseolus vulgaris* L. cv. "Bronco") plants grown under soil salinity in two seasons

Treatments		Parameters				
Seed soaking	Foliar spray	Total chlorophylls	Total carotenoids	RWC (%)	EL (%)	MSI (%)
2014 season						
Tap water	Tap water	1.42c†	0.32c	70.1c	12.06a	63.8c
	MLE	1.68b	0.42b	76.7b	9.68b	70.8b
MLE	Tap water	1.70b	0.44b	77.0b	9.46b	71.7b
	MLE	1.98a	0.52a	84.2a	7.22c	79.2a
2015 season						
Tap water	Tap water	1.46c	0.34c	72.0c	12.12a	64.4c
	MLE	1.70b	0.46b	79.4b	10.04b	71.8b
MLE	Tap water	1.74b	0.49b	80.2b	9.84b	71.9b
	MLE	1.96a	0.58a	88.4a	7.18c	80.8a

†Mean values (n = 9) in each column for each year followed by a different lower-case letter are significantly different at $P \leq 0.05$ by Duncan's multiple range test.

All physio-chemical attributes shown in Table 3 (i.e., concentrations of total chlorophylls and total carotenoids, RWC%, EL% and MSI%), Table 4 (i.e., concentrations of total soluble sugars, free proline and ascorbic acid), Table 6 (i.e., contents of N, P, K and Ca) and Table 7 (i.e., ratios of K/Na, Ca/Na and K+Ca/Na), in addition to antioxidant enzymes, and green pod and dry seed yields shown in Table 7 and Table 8, respectively were behaved the same trend of growth characteristics. Combined MLE treatment (seed soaking + foliar spray) found to be the best, generating the best physio-chemical attributes and enzymatic activity in both 2014 and 2015 seasons.

Table (4): Effect of seed soaking and/or foliar spray with moringa leaf extract (MLE) on the shoot concentrations of total soluble sugars, free proline and ascorbic acid (AsA) of common bean (*Phaseolus vulgaris* L. cv. "Bronco") plants grown under soil salinity in two seasons

Treatments		Parameters		
Seed soaking	Foliar spray	Total soluble sugars (mg g^{-1} DW)	Free proline (µg g^{-1} DW)	AsA (µg g^{-1} FW)
2014 season				
Tap water	Tap water	3.12c†	240.2c	11.4c
	MLE	3.52b	322.4b	20.8b
MLE	Tap water	3.56b	341.0b	21.2b
	MLE	3.98a	390.0a	29.2a
2015 season				
Tap water	Tap water	3.41c	259.6c	12.1c
	MLE	3.87b	343.4b	21.4b
MLE	Tap water	3.92b	362.8b	22.1b
	MLE	4.42a	413.4a	31.4a

†Mean values (n = 9) in each column for each year followed by a different lower-case letter are significantly different at $P \leq 0.05$ by Duncan's multiple range test.

Table (5): Effect of seed soaking and/or foliar spray with moringa leaf extract (MLE) on superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX) and glutathione reductase (GR) activities in leaves of common bean (*Phaseolus vulgaris* L. cv. "Bronco") plants grown under soil salinity in two seasons

Treatments		Parameters			
Seed soaking	Foliar spray	SOD (A_{564} min^{-1} g^{-1} protein)	CAT (A_{290} min^{-1} g^{-1} protein)	APX (A_{290} min^{-1} g^{-1} protein)	GR (A_{340} min^{-1} g^{-1} protein)
2014 season					
Tap water	Tap water	4.16c†	79.6a	36.3c	22.5c
	MLE	5.63b	62.9b	48.7b	28.7b
MLE	Tap water	5.86b	61.0b	51.2b	31.5b
	MLE	7.08a	50.4c	77.4a	40.6a
2015 season					
Tap water	Tap water	5.02c	84.3a	44.9c	24.7c
	MLE	6.22b	73.4b	66.4b	32.5b
MLE	Tap water	6.42b	69.5b	69.5b	34.0b
	MLE	7.64a	52.4c	82.4a	50.2a

†Mean values (n = 9) in each column for each year followed by a different lower-case letter are significantly different at $P \leq 0.05$ by Duncan's multiple range test.

Table (6): Effect of seed soaking and/or foliar spray with moringa leaf extract (MLE) on the contents of macro-nutrients (N, P, K and Ca) and sodium (Na) of common bean (*Phaseolus vulgaris* L. cv. "Bronco") plants grown under soil salinity in two seasons

Treatments		Parameters				
Seed soaking	Foliar spray	N (%)	P (%)	K (%)	Ca (%)	Na (%)
2014 season						
Tap water	Tap water	2.35c†	0.25c	2.53c	1.08c	0.64a
	MLE	2.67b	0.29b	2.94b	1.22b	0.54b
MLE	Tap water	2.64b	0.30b	3.02b	1.24b	0.51b
	MLE	2.97a	0.35a	3.34a	1.35a	0.34c
2015 season						
Tap water	Tap water	2.41c	0.24c	2.48c	1.06c	0.62a
	MLE	2.68b	0.29b	3.01b	1.24b	0.50b
MLE	Tap water	2.72b	0.31b	3.10b	1.28b	0.48b
	MLE	2.98a	0.36a	3.44a	1.39a	0.30c

†Mean values (n = 9) in each column for each year followed by a different lower-case letter are significantly different at $P \leq 0.05$ by Duncan's multiple range test.

Similarly, the combined treatment of seed soaking + foliar spray with MLE found to be the best, increasing green pod weight per plant and per hectare and dry seed weight per plant and per hectare by 76.5%, 74.1%, 24.1% and 28.6%, respectively in the first season and by 81.7%, 82.1%. 22.1% and 29.8%, respectively in the second season compared to the controls. The increases in the green pod and dry seed yields and the increases in the growth characteristics of bean plants were accompanied with significant increases in the concentrations of total chlorophylls, total carotenoids, total soluble sugars, free proline and ascorbic acid by 39.4%, 62.5%, 27.6%, 62.4% and 156.1%, respectively in 2014 season and by 34.2%, 70.6.2%, 29.6%, 59.2% and 159.5%, respectively in 2015 season under the best combined treatment of seed soaking + foliar spray with MLE compared to the controls. In addition, this combined treatment significantly increased RWC%, MSI%, N%, P%, K%, Ca% and K+Ca/Na ratio by 20.1%, 24.1%, 26.4%, 40.0%, 32.0%, 25.0% and 144.5%, respectively in the first season and by 22.8%, 25.5%, 23.7%, 50.0%, 38.7%, 31.1% and 182.0%, respectively in

the second season compared to the controls. The activities of antioxidant enzymes such as SOD, APX and GR were also increased significantly by 70.2%, 113.2% and 80.4%, respectively in the first season, and by 52.5%, 83.8% and 103.2%, respectively in the second season as a result of the combined MLE treatment (seed soaking + foliar spray) compared to the controls. In contrast, the best combined treatment of seed soaking + foliar spray with MLE significantly reduced EL%, Na% and catalase activity in bean tissues by 40.1%, 46.9% and 36.7%, respectively in 2014 season and by 40.8%, 51.6% and 37.8%, respectively in 2015 season compared to the controls.

Table (7): Effect of seed soaking and/or foliar spray with moringa leaf extract (MLE) on nutrient relations with Na in common bean (*Phaseolus vulgaris* L. cv. "Bronco") plants grown under moderate soil salinity in two seasons

Treatments		Parameters		
Seed soaking	**Foliar spray**	**K/Na ratio**	**Ca/Na ratio**	**K+Ca/Na ratio**
2014 season				
Tap water	Tap water	3.95c	1.69c	5.64c
	MLE	5.44b	2.26b	7.70b
MLE	Tap water	5.92b	2.43b	8.35b
	MLE	9.82a	3.97a	13.79a
2015 season				
Tap water	Tap water	4.00c	1.71c	5.71c
	MLE	6.02b	2.48b	8.50b
MLE	Tap water	6.46b	2.67b	9.13b
	MLE	11.47a	4.63a	16.10a

†Mean values (n = 9) in each column for each year followed by a different lower-case letter are significantly different at $P \leq 0.05$ by Duncan's multiple range test.

Table (8): Effect of seed soaking and/or foliar spray with moringa leaf extract (MLE) on green pod and dry seed yields of common bean (*Phaseolus vulgaris* L. cv. "Bronco") plants grown under moderate soil salinity in two seasons

Treatments		Parameters			
Seed soaking	**Foliar spray**	**Pods weight plant^{-1} (g)**	**Pods weight ha^{-1} (ton)**	**Seed weight plant^{-1} (g)**	**Seed weight ha^{-1} (ton)**
2014 season					
Tap water	Tap water	39.2c†	5.60c	10.8c	1.47c
	MLE	53.8b	7.76b	12.1b	1.62b
MLE	Tap water	56.5b	7.94b	12.4b	1.68b
	MLE	69.2a	9.75a	13.4a	1.89a
2015 season					
Tap water	Tap water	40.4c	5.69c	11.3c	1.51c
	MLE	57.6b	8.46b	12.6b	1.71b
MLE	Tap water	60.3b	8.72b	12.9b	1.76b
	MLE	73.4a	10.36a	13.8a	1.96a

†Mean values in each column for each year followed by a different lower-case letter are significantly different at $P \leq 0.05$ by Duncan's multiple range test.

Discussion

Soil salinities have shown to reduce the water availability to plant roots that negatively affect the water status of plant tissues. Disturbances in metabolic processes, which lead to decreases in meristematic activity and cell enlargement, coupled with an increase in respiration rate due to the higher energy requirements are also some of the inhibitory effects of soil salinities on growth and productivity of plants[37,38]. In addition, salinity stress causes over-production of reactive oxygen species (ROS) in plant tissues. A balance between the generation and degradation of ROS is required to avoid oxidative injury and to maintain metabolic functions

under stress conditions. In plant tissues, the level of ROS is controlled by an antioxidant system that consists of antioxidant enzymes and non-enzymatic low molecular weight antioxidant molecules, including proline, ascorbic acid and carotenoids[9,39,40]. In the present study, the reduction in plant growth and productivity under the adverse conditions of soil salinity (controls of Tables 2 and 8) could be attributed to the osmotic effect resulting from salt stress that causes increase of growth inhibitors (i.e., abscisic acid), decrease of growth promoters (i.e., indole-3-acetic acid gibberellins) and disturbance of the water balance of saline-stressed plants. These inhibitory effects of salinity lead to stomatal closure, ionic imbalance, reduction in photosynthesis, disturbance in ionic homeostasis, accumulation of toxic ions and consequently inhibition of growth[3,19,40,41].

Seed soaking and/or foliar spray for common bean plants with *Moringa oleifera* leaf extract (MLE; 1 extract paste: 30 water by volume) significantly improved plant growth characteristics and plant productivity as well as physio-chemical attributes under the adverse conditions of the studied soil salinity. It has been shown from the results of this study that the combined treatment of seed soaking + foliar spray with MLE exhibited significant preferences than single MLE treatments (i.e., seed soaking or foliar spray).

Analysis of MLE as shown in Table 1 revealed presence of essential macro- and micro-nutrients such as Ca, Mg, K, P, Fe, Mn, Cu and Zn. The MLE also contains antioxidants including proline, soluble phenolics, total carotenoids and ascorbic acid coupled with amino acids including proline, total soluble sugars and K as osmoprotectants. It is also rich in phytohormones such as indole-3-acetic acid (IAA), gibberellins (GAs) and zeatin as a cytokinin (Table 1). This diverse composition of MLE indicates that this extract can be used as a plant biostimulant. Many researches highlighted MLE role to improve plant growth and development in different crops[3,13,14], which is also evident from results of the present study. Improved seedling growth traits (i.e. shoot length, number and area of leaves per plant, and plant dry weight) by the MLE application might be due to the enhanced mobilization of germination related metabolites/inorganic solutes such as zeatin, ascorbic acid, Ca and K presented in the MLE (Table 1) to the growing plumule and/or the increase in amylase activity and reducing sugars, contributing to early vigor and increased plant growth[42,43]. In addition, the increased MLE content of IAA, GAs and zeatin encouraged plant growth and productivity under salt stress conditions. Seed soaking and/or seedling foliar application with MLE might provide strong and energetic start for earlier emergence and completed other phenological events well in time[14]. The possible reason for this acceleration of growth might be due to the enriched content of MLE of crude proteins and growth promoting hormones, that is, auxins and cytokinins[10,44]. Proteins are essential for the formation of the protoplasm, while growth hormones favored rapid cell division, cell multiplication and cell enlargement. The combined treatment of seed soaking + foliar spray with MLE showed the best results under saline condition, ameliorating the negative effects of salt stress through preventing decreases in growth characteristics, leaf photosynthetic pigments, relative water contents, membrane stability index and nutrient elements and also by inhibiting increases in leaf electrolyte leakage (Tables 3 – 6).

Maintenance of green leaf area and number of leaves per plant (Table 2) maximized photosynthesizing leaves, increasing sink capacity fulfilled through supply of photo-assimilates from stayed green leaves[45] and/or re-translocation of stem reserves of the present study as a result of the application of cytokinin-rich MLE that may be induced cytokinin biosynthesis. Maximum number of photosynthetic active leaves observed from number and area of leaves per plant indicates the delayed senescence and maintaining the chlorophylls in higher concentrations (Tables 2 and 3). Presence of zeatin-like cytokinin in MLE prevents premature leaf senescence and maintains higher leaf area for photosynthetic activity. During late stage of growth, endogenous levels of cytokinin are usually decreased and exogenously-applied cytokinin (found in MLE) can delay this process[46], possibly through activation of cytokinin dependant isopentenyl transferase (*ipt*) biosynthesis, increasing chlorophyll concentrations.

The increased concentration of chlorophyll and increased growth characteristics of bean plants under salt stress were positively reflected in green pod and dry seed yields that might be attributed to more assimilate partitioning to developing edible parts and have been correlated with cytokinin levels (Zeatin) found in MLE[3,47].

An increase in electrical conductivity of the tested soil (Table 1) indicates elevated leakiness of ions due to a loss of membrane integrity. This is an inherent feature of plants which are exposed to stresses such as salinity[48]. In this study, salt stress significantly increased leaf electrolyte leakage (EL), while the single or combined applications of MLE significantly decreased it. This reduction in EL was most effective in tolerating salt stress when MLE used as a combined application of seed soaking + foliar spray. Electrical leakage enables

cell membrane injury to be assessed when plants are subject to salinity stress. Maintaining integrity of cellular membranes under salt stress is considered an integral part of salinity tolerance mechanism[49]. In addition, application of MLE significantly increased RWC compared with the control, with the highest increase in RWC when using MLE seed soaking combined with MLE foliar spray (Table 3). The RWC is a useful measure of the physiological water status of plants[50]. Water stress often results when plants are grown on saline soils. The MLE is reported to increase RWC and water potential tolerance of plants to salt stress[51]. This is probably due to that MLE increased the osmoprotectant concentrations (i.e., total soluble sugars and proline; Table 4), which ultimately helps in maintaining better water balance in the plants.

Soluble sugars are significantly increased in salt-stressed bean plants by the exogenous applications of MLE (Table 4). The MLE contributes to osmotic adjustment and can directly or indirectly modulate the expression of genes involved in metabolic processes, storage functions, and defence[52]. It has been indicated that the oxidative damage generated during salinity stress is due to the imbalance in production of ROS and antioxidant activity alterations[53]. To avoid the damage caused by oxidative stress, plants have developed many antioxidant systems; among non-enzymatic ones, the accumulation of proline is one of the most frequent changes induced by salinity or drought, although there is controversy concerning whether its accumulation is a stress resistance mechanism or a mere indicator of the existence of stress[54]. One of the substrates of the Halliwell–Asada cycle is ascorbic acid (AsA) that also act as a non-enzymatic antioxidant in an isolated way on being involved in the direct reduction of ROS during different types of stress[55] taking part in the control of the H_2O_2 levels. This situation is reflected in the total concentration of AsA in our study, which are increased either with the single treatments or combined one of MLE, and its maximum concentration was noted with the combined application of MLE seed soaking + MLE foliar application, perhaps to overcome $O_2^{\bullet-}$ accumulation, since the AsA can directly eliminate $O_2^{\bullet-}$ and H_2O_2 in a non-enzymatic way[56]. The healthy metabolic state of the stressed bean plants pretreated or treated with MLE resulted in the healthy plant growth (Table 2). This may be attributed to that MLE is excellent source in minerals, amino acids, soluble sugars and some antioxidants (Table 1). These increased proline and AsA antioxidants supported the antioxidant system in bean plants to enable them to tolerate salt stress. The combined MLE treatment (seed soaking + foliar spray) caused increases in the concentrations of proline and AsA which, in turn, protected plants against the generation of ROS and membrane injury, or may resulted in the synthesis of other substances having a protective effect on plants grown under salt stress[57].

Several studies have indicated that the oxidative damage generated during salinity stress is due to the imbalance in production of ROS and antioxidant activity alterations[53]. To avoid the damage caused by oxidative stress, plants have developed many antioxidant systems; among enzymatic ones, SOD constitutes the first line of defence against ROS[58] by reducing the $O_2^{\bullet-}$ radical to H_2O_2. Hydrogen peroxide can serve as that substrate for numerous enzymes such as CAT which in turn though located in the peroxysomes where the H_2O_2 concentration is very high, is absent in the cytosol and chloroplasts, and thus H_2O_2 is eliminated by peroxidases. These include APX, which is considered one of the most important enzymes in the reduction of this reactive molecule[59,60]. The regenerating enzymes DHAR and GR as a fundamental part of the Halliwell–Asada cycle, as they formed part of the regeneration of AsA from DHA using GSH as a reducing power are described[61]. In turn, the reduced glutathione (GSH) consumed can be regenerated from its oxidized form (GSSG) by the reaction of GR[56]. Our data show that all the treatments significantly increased the activities of SOD, APX and GR compared to the controls (Table 5); this being more pronounced in the case of the combined MLE treatment (seed soaking + foliar spray).

The increased accumulation of Na^+ ions in salt-stressed plants can disturb or upset the ionic balance, inducing a nutritional imbalance due to the blockage of other cations such as N, P, K and Ca tested in the present study or anions such as NO_3^- and thereby the induction of nutritional deficiency symptoms[62]. The maintenance of the ionic homeostasis under salt stress is prerequisite to protect the plant against the build-up of toxic ions, with K^+ and Ca^{2+} accumulating and Na^+ reaching the minimum content in bean leaves (Table 5). Thus, the control of Na^+ accumulation and therefore a high K^+/Na^+ and Ca^{2+}/Na^+ ratios (Table 6) may strengthen salinity tolerance[63].

Salt stress tolerance in bean plants, in this study, was improved with the elevated antioxidant system, including non-enzymatic antioxidants (i.e., carotenoids, free proline and AsA) by the application of minerals, AsA, cytokinins, GAs and IAA-containing MLE (Table 1) applied singly (seed soaking or foliar spray) or in combination (seed soaking + foliar spray). *Moringa oleifera* leaves is a rich source in zeatin[42], minerals and

other phytohormones, so the effectiveness of MLE in mitigating salinity stress by better chlorophyll, antioxidants and plant growth might be due to cytokinin mediated stay green effect. The combined MLE as the best treatment supported the bean antioxidant defence system through increase of carotenoids, free proline and AsA concentrations, maintaining tissue water balance and ionic homeostasis.

Conclusion

This study revealed that the inhibitory effects of salt stress on the growth and productivity of common bean plants could be alleviated by the exogenous application of *Moringa oleifera* leaf extract (MLE) that could protect the plants against injuries by salt stress. Soaking bean seeds + foliar spray with MLE (1 extract paste: 30 water by volume) as a combined treatment was the most effective combined treatment in providing bean plants with salt tolerance when grown under moderate soil salinity (EC = 6.23 – 6.28). The reduced concentration of Na^+ ions and the increased concentrations of K^+ and Ca^{2+} ions coupled with the increased concentrations of carotenoids, free proline and AsA as antioxidants and soluble sugars with free proline as osmoprotectants under salt stress by the effective combined MLE treatment supported common bean plants. In addition, this best treatment improved the activity of antioxidant enzymes, reflecting in maintaining cell membrane integrity, tissue water balance and ion homeostasis, and improving plant growth and productivity. As *Moringa oleifera* trees is began to spread worldwide to easily obtain MLE, it could be used in combined application (seed soaking + foliar spray) to prevent growth and productivity losses under salt stress and may have significant practical applications.

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