Advances in Plants & Agriculture Research

Research Article

Open Access CrossMark

Exogenous application of moringa seed extract positively alters fruit yield and its contaminant contents of *Capsicum annuum* plants grown on a saline soil contaminated with heavy metals

Volume 8 Issue 6 - 2018

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Received: August 29, 2018 | **Published:** December 26, 2018

Abstract

Enhancing plant productivity on contaminated soils by using natural supplementations are urgently seeking. Two main field trials were conducted to study the potential effects of moringa seed (MSE; 0.5%) extraction growth and yield, physio-biochemical components, antioxidant defense system, and contamination of pepper plants grown on heavy metals-contaminated saline soil. MSE was applied in two single treatments (i.e., with drip irrigation water; SA or as foliar spray; FS) or in integrative (i.e., MSE-SA + MSE-FS) treatment. The results showed that all single or integrative treatments significantly increased plant growth and yield, leaf contents of leaf photosynthetic pigments, free proline, total soluble sugars, N, P, and K+, ratio of K+/Na+, and activities of CAT, POX, APX, SOD and GR, while significantly reduced contaminants; Na+, Cd, Cu, Pb and Ni contents in plant leaves and fruits compared to the control (free from MSE). Additionally, the integrative MSE-SA + MSE-FS treatment significantly exceeded all single treatments in this concern. The integrative MSE-SA + MSE-FS treatment was the best that it had been recommended for maximizing pepper fruits with minimizing contaminants on heavy metal-contaminated saline soils.

Keywords: pepper, productivity, plant extract, stress, antioxidant system

Introduction

Pepper (*Capsicum annuum* L.) is an economically important vegetable crop due to its nutritional value, antioxidant compounds, bioactive products, and natural colors for human health.[1] Pepper is considered as salt sensitive[2] or moderately salt sensitive[3] based on its growth stage. The salinity threshold level of pepper is averaged 1.5dS m^{-1}.[4] In addition, peppers like most of vegetables cultivated in Egypt are sometimes grown in heavy metals-contaminated soils close to factories and industrial cities. These soils are exposed to heavy metals contamination by which soils are contaminated up to the unpermitted levels,[5] negatively reflecting in plant growth and productivity,[6] and the contaminants will accumulate in plant edible parts (i.e., pepper fruits). Heavy metals enter into soil by many ways, including chemical fertilizers, atmospheric precipitation, and wastes of agricultural industries.[7] The response to heavy metal stress involves a complicated signal transduction network that is activated by sensing the heavy metals and is characterized by the synthesis of stress-related proteins and signaling molecules, and finally the transcriptional activation of specific metal-responsive genes to counteract the stress.[8]

Salinity is one of the major abiotic stresses limiting plant performance (growth and yield), especially in arid and semi-arid regions including Egypt. It causes a reduction in plant performance at varying degrees depending on the salinity level.[9] High salinity causes ion disequilibrium, osmotic (physiological drought) and oxidative stresses in plant tissues,[10] which inhibit the synthesis of photosynthetic pigments and photosynthetic process,[11] inducing an over-reduction of the reaction centers in photo system II that may destruct the photosynthetic machinery if the plant is unable to consume excess energy.[12] Salinity effects are attributed, mainly, to the decrease of soil water potential or the increase of ion concentration in plant tissue to levels that interfere with metabolism.[13] The common consequence of most abiotic stresses, including salinity and heavy metals;[5,14,15] are an overproduction of reactive oxygen species (ROS; 1O2, O2•–, H2O2, and OH–)that are extremely toxic to plants. They caused damages to DNA, proteins, lipids,and chlorophyll.[16] However, plants are well equipped with antioxidant defense systems consisted of antioxidant enzymes (i.e., superoxide dismutase, peroxidase, catalase, glutathione reductase, etc.) and non-enzymatic low molecule antioxidants (i.e., proline, tocopherols, carotenoids, glutathione, ascorbic acid, etc.) to counteract the oxidative stress to protect plants from the oxidative injuries,[17] and the extent of oxidative cellular damage in abiotic-stressed plants is controlled by the capacity of their antioxidant systems.[18–20] In most cases, the endogenous antioxidant defense systems of plants are not enough to maintain healthy growth of plants, therefore, plants need an exogenous support such as plant extracts to increase their tolerance to the stress.[14–24]

Plant extracts contain natural growth-promoting substances such as phytohormones, osmoprotectants, antioxidants, and nutrients, which are important to strengthen the antioxidant defense systems of plants to efficiently face the environmental stresses. Among these extracts, moringa (*Moringa oleifera* L.) seed extract (MSE) that contains plant performance enhancing capabilities. It is rich source in antioxidants and osmo protectants such as proline, soluble sugars, ascorbic acid, glutathione, and selenium. It is also rich in phytohormones including significant amounts of auxins, gibberellins, and zeatin-type cytokinins, and nutrient elements. There are many different plant extracts that are used as effective natural bio stimulants for supporting the plants grown under several environmental stresses.[14,20–25] To our knowledge, however, studies using MSE as a natural bio stimulantisvery scarcely applied. Therefore, the aim of this study was to assess the potential effects of the exogenous MSE supplemented in drip irrigation water

(SA) and/or as foliar spray (FS) on the changes of growth and yield, physio-biochemical components, mineral nutrients and heavy metal accumulation, and the antioxidant defense system of pepper plants grown on heavy metal (Cd, Cu, Pb, and Ni)-contaminated saline (EC=7.73–7.78dS m^{-1}) soil. The hypothesis tested herein is that supplementation of the integrative MSE-SA + MSE-FSwill promote plant growth and productivity through reducing the accumulation of heavy metals and improving the levels of nutrients and osmoprotectants, and the activity of non-enzymatic and enzymatic antioxidants that play crucial roles in alleviating the stress produced by salinity and heavy metals.

Materials and methods

Experimental layout

A preliminary field trial was performed in 2015 season, and two main field trials were conducted in both 2016 and 2017 seasons on special farms at Al-Husayniyah, Sharkyya Governorate, Egypt. Soil samples were randomly selected from the study sites before each agricultural season and analyzed according to Black et al.,[26] and Jackson[27] and the results are shown in Table 1. According to soil analyses results before planting, soil EC values were 7.73 and 7.78dS $m^{-1}$of the soils of both 2016 and 2017 seasons, respectively, indicating that are saline soils.[28] EC analyses for the different soils were conducted in soil paste extract. The saline soil was also contaminated with some heavy metals such as cadmium (Cd), copper (Cu), lead (Pb) and nickel (Ni) that were detected through soil analyses (Table 1) and indicated that it is contaminated soil based on the concentrations of these metals.[29]

Seeds of pepper (*Capsicum annuum* L., cv. Top Star) were provided by Sacata Co., Cairo, Egypt. In both 2016 and 2017 seasons, seeds were sown on 28th February using growing trays and the 45-day-old seedlings were transplanted into the field in plots (3.0×3.50m=10.5m2) on rows spaced 60cm, and the distances among transplants were 15–20cm. Before sowing, all plots of the experiments were fertilized with 100kg potassium sulfate (50% K2O) ha^{-1} and 200 kg calcium superphosphate (15.5% P2O5) ha^{-1}. Nitrogen fertilizer was added at a rate of 250 kg ammonium nitrate (33% N) ha^{-1}. The empirical design of experiments was a completely randomized plots for 4 treatments each with 9 replicates. The details of the 4 treatments are as follows: 1. Control; no extract was added in drip irrigation water + foliar spray was done with tap water, 2. MSE-SA; moringa seed extract was added in drip irrigation water + foliar spray was done with tap water, 3. MSE-FS; no extracts were added in drip irrigation water+ foliar spray was done with moringa seed extract, 4. MSE-SA+MSE-FS; moringa seed extract was added in drip irrigation water + foliar spray was done with moringa seed extract.

Extraction, analyses, and applications of MSE

Each 200g of air-dried ground Moringa oleifera seeds was put in 2L of 80% ethanol and strongly stirred on a shaker for 5h to effectively extract the seed active ingredients. The mixture was then filtered using What man No.2 filter paper and the filtration was evaporated using a big Fan to completely get rid of the alcohol. The extract was reached to 40 L by distilled water. The Moringa oleifera seed extract should be used within 5 h from extraction process otherwise was stored at–20°C and only taken out when it requested to use. MSE was analyzed for main chemical components that are shown in Table 2. MSE (0.5%; 5 g seeds per L) was applied as foliar sprays (FS) three times; 20, 35, and 50 days after transplanting (DAT) to run-off with adding few drops of Tween-20 to the solutions as a surfactant to assure an effective and complete penetration of the spray solutions. In addition, MSE was applied as a Rhizosphere application by adding 200 L extract (0.5%) $ha^{-1}$to drip irrigation water three times with the 2nd, 4th, and 6th irrigation.

Table 1 Physical and chemical properties of the investigated soil for two experimental seasons

Soil characteristic	Value		Unit
	2016	2017	
Soil particles distribution:			
Sand	43.4	43.3	%
Silt	28.7	28.6	
Clay	27.9	28.1	
Textural class	Loam		
Field capacity	15.8	15.2	%
$CaCO_3$	29.6	32.7	g kg^{-1}
Organic matter	6.95	6.42	
pH*	7.32	7.28	
EC**	7.73	7.78	dS m^{-1}
Soluble cations and anions**:			
Ca^{2+}	20.6	21.4	mmolc L^{-1}
Mg^{2+}	15.8	15.1	
Na^{+}	30.4	30.9	
K^{+}	5.26	5.62	
$CO3^{2-}$	-	-	
HCO^{3-}	8.91	6.86	
Cl^{-}	21.3	20.4	
$SO4^{2-}$	48.5	51.9	
Available nutrient:			
N	33.4	31.9	mg kg^{-1} soil
P	8.72	7.87	
K	104	103	
Heavy metals:			
Cu	106	109	mg kg^{-1} soil
Cd	18.1	18.7	
Ni	259	253	
Pb	253	248	

*Soil paste; **Soil paste extract.

Growth and yield attributes

Ten pepper plants were selected randomly and cut off from the two outer rows of every experimental plot at60DAT to estimate shoot dry weight ($tonha^{-1}$). At the merchantable stage, fruits from 50 plants on each plot were reaped to estimate number of fruits $plant^{-1}$, and fruit yield ($tonsha^{-1}$).

Table 2 Chemical analysis of the *Moringa oleifera* seed extract (MSE) (on dry weight basis)

Component	Values	Unit
1. Antioxidants and osmoprotectants:		
Free proline	32	g kg^{-1} DW
Soluble sugars	176	
Glutathione (GSH)	22.4	mg kg^{-1} DW
Ascorbic acid (AsA; Vit. C)	34.8	
Selenium (Se)	0.8	
DPPH-radical scavenging	81.3	%
2. Phytohormones:		
Total auxins	3.3	mg kg^{-1} DW
Total gibberellins	2.9	
Zeatin-type cytokinin	3.2	
3. Mineral nutrients:		
Nitrogen(N)	30.8	g kg^{-1} DW
Phosphorus(P)	15.8	
Potassium(K)	21.7	
Calcium(Ca)	9.6	
Magnesium(Mg)	4.5	
Sulfur(S)	2.7	
Iron(Fe)	1.5	
Manganese(Mn)	0.9	
Zinc(Zn)	0.4	
Cupper(Cu)	0.2	

Determination of physio-biochemical constituents

Total chlorophylls and total carotenoids were extracted from fresh leaf using pure acetone and determined (as mg g^{-1} fresh mass) according to Fadeels,[30] the method of Bates et al.[31] was used to determine proline accumulation in pepper leaves, and total soluble sugars content was estimated according to Irigoyen et al.,[32] For nutrients and heavy metal determinations, a weight of 0.2 g of dried leaves was digested with H2SO4 in the presence of H2O2[33] and then, N content was determined using a micro Kjeldahl method according to Chapman and Pratt,[34] P content was determined calorimetrically using ascorbic acid method of Watanabe and Olsen,[35] and Na+ and K+ contents were measured directly using Flame photometer.[36] The powdery dried plant samples (upper fully-expanded leaves and yielded fruits) were ashed at 500°C for 12 h to determine heavy metal ions concentrations. The ashed samples were dissolved in 3.3% HNO3 (v/v). The concentrations of Cd, Cu, Pb, and Ni were measured by inductively coupled plasma optical emission spectroscopy (ICP-OES, Varian, and Australia). Measurements of the all tested heavy metals in plants were checked against certificated Cd, Cu, Pb, and Ni values in different reference plant materials obtained from the National Institute of Standards and Technology (Gaithersburg, USA).

Determination of antioxidants activities

Enzymes were extracted according to Vitoria et al.,[37] The activity of catalase (CAT) enzyme was assessed spectrophotochemically according to Chance & Maehly.[38] Peroxidase (POX) activity was estimated according to Thomas et al.,[39] Ascorbate peroxidase (APX) activity was determined spectrophotochemically according to Fielding & Hall.[40] Activity of superoxide dismutase (SOD) was determined by recording the drop in absorbance of superoxide-nitro blue tetrazolium complex by the enzyme.[41] Glutathione reductase (GR) activity was measured after monitoring the oxidation of NADPH for three absorbance times taken at 340 nm.[42]

Statistical analysis

Statistically significant variations between means were compared at P≤0.05 by Duncan's Multiple Range Test. The statistical analysis was done by COSTAT computer software (CoHort Software version 6.303, Berkeley, CA, USA).

Results

Growth, yield, photosynthetic pigments, antioxidants, and osmoprotectants

Compared to the control (both drip irrigation water and foliar sprays were free from MSE), the single (i.e., MSE applied as Rhizosphere supplementation with the drip irrigation water; SA or as foliar spray; FS) or integrative (i.e., MSE-SA+ MSE-FS) treatments significantly increased plant growth (in terms of shoot dry weight), yield parameters (i.e., No. of fruits per plant, and fruits yield per hectare), and the contents of total chlorophylls, total carotenoids, free proline, and total soluble sugars of pepper plants grown on a heavy metals-contaminated saline soil (Table 3) & (Table 4). In addition, the integrative MSE-SA+MSE-FS treatment significantly exceeded the single treatments conferring the best results. It increased shoot dry weight by69 and 70%, No. of fruits on plant by 111 and 142%, fruits yield by 138 and 146%, chlorophylls content by 66 and 64%, carotenoids content by 45 and 36%, proline content by 80 and 78%, and soluble sugars by 88 and 94% in both 2016 and 2017 growing seasons, respectively. These results show similar trends over both growing seasons.

Nutrients contents and K+/Na+ ratio

The single (i.e., MSE-SA or MSE-FS) or integrative (i.e., MSE-SA+MSE-FS) treatments significantly increased the contents of N, P, and K^+, and the ratio of K^+/Na^+, while significantly reduced the content of Na^+ of pepper plants grown on a heavy metals-contaminated saline soil compared to the control (SA and FS were applied with tap water) (Table 5). Further, the integrative MSE-SA + MSE-FS treatment significantly exceeded the single treatments recording the best results. It increased N content by73 and 69%, P content by 67 and 61%, K^+ content by 44 and 50%, and K^+/Na^+ ratio by121 and 141%, and reducing Na^+ content by 35 and 37% in both 2016 and 2017 growing seasons, respectively. These results represent identical trends in both growing seasons.

Antioxidant enzymes

The results in Table 6 show analogous trends in 2016 and 2017 growing seasons regarding the activity of antioxidant enzymes in pepper plants grown on heavy metals-contaminated saline soil. The single (i.e., MSE-SA or MSE-FS) or integrative (i.e., MSE-SA+MSE-FS) treatments significantly increased the activities of catalase (CAT), peroxidase (POX), ascorbate peroxidase (APX), superoxide dismutase (SOD) and glutathione reductase (GR) compared to those of the control (SA and FS were applied with tap water). Moreover, the integrative MSE-SA + MSE-FS treatment significantly exceeded both MSE single treatments and collected the best results. It increased CAT activity by 55 and 55%, POX activity by 135 and 138%, APX activity by 99 and 98%, SOD activity by 54 and 56%, and GR activity by 114 and 112% in both 2016 and 2017 growing seasons, respectively.

Table 3 Effect of *Moringa oleifera* seed extract (MSE) applications on growth (in terms of dry weight; DW) and yield attributes of pepper plants grown on a heavy metals-contaminated saline soil

Treatments	Shoot DWha^{-1}(tons)	Fruits No.plant^{-1}	Fruits yield ha^{-1} (tons)
2016			
Control	9.8±0.9d	1.9±0.6d	17.0±0.5d
MSE-SA	11.3±0.9c	2.3±0.4c	21.6±0.6c
MSE-FS	13.1±0.8b	3.2±0.4b	29.7±1.3b
MSE-SA + MSE-FS	16.6±0.9a	4.0±0.3a	40.5±1.2a
2017			
Control	10.3±0.9d	1.9±0.4d	17.7±0.6d
MSE-SA	12.3±0.8c	2.3±0.5c	24.5±0.6c
MSE-FS	14.0±0.7b	3.5±0.8b	32.4±1.1b
MSE-SA + MSE-FS	17.5±0.9a	4.6±0.7a	43.5±2.3a

Data are means (n = 9 for growth traits) ± SE. The same letters in each column indicate not significant differences according to the LSD test (P≤ 0.05).
Control; no extracts were added in irrigation water + foliar spray was done with tap water,
MSE-SA; moringa seed extract was added in irrigation water + foliar spray was done with tap water,
MSE-FS; no extracts were added in irrigation water + foliar spray was done with moringa seed extract, and
MSE-SA + MSE-FS; moringa seed extract was added in irrigation water + foliar spray was done with moringa seed extract.

Table 4 Effect of *Moringa oleifera* seed extract (MSE) applications on leaf contents of photosynthetic pigments, proline and total soluble sugars of pepper plants grown on a heavy metals-contaminated saline soil

Treatments	Total chlorophylls (mg g^{-1} DW)	Total carotenoids	Proline	Soluble Sugars
		(mg g–^{1} DW)	(mg g^{-1} DW)	(mg g^{-1} DW)
2016				
Control	1.01±0.02d	0.20±0.004c	17.2±0.7d	8.40±1.0d
MSE-SA	1.27±0.04c	0.25±0.003b	19.1±0.2c	10.2±0.2c
MSE-FS	1.40±0.32b	0.26±0.001b	24.6±0.7b	12.7±0.9b
MSE-SA + MSE-FS	1.68±0.03a	0.29±0.001a	30.9±0.2a	15.8±0.2a
2017				
Control	1.07±0.02c	0.22±0.004c	18.1±0.7d	8.14±0.9d
MSE-SA	1.35±0.04b	0.26±0.003b	20.2±0.2c	9.30±0.3c
MSE-FS	1.46±0.04b	0.27±0.002b	28.8±0.5b	12.9±1.2b
MSE-SA + MSE-FS	1.76±0.03a	0.30±0.001a	32.2±0.2a	15.8±0.2a

Data are means (n = 9 for growth traits) ± SE. The same letters in each column indicate not significant differences according to the LSD test (P≤ 0.05).
Control; no extracts were added in irrigation water + foliar spray was done with tap water,
MSE-SA; moringa seed extract was added in irrigation water + foliar spray was done with tap water,
MSE-FS; no extracts were added in irrigation water + foliar spray was done with moringa seed extract, and
MSE-SA + MSE-FS; moringa seed extract was added in irrigation water + foliar spray was done with moringa seed extract.

Heavy metals contents in pepper leaves and fruits

The data in Tables 7 and 8 represent similar trends in both 2016 and 2017 growing seasons regarding the contents of the heavy metals; cadmium (Cd), copper (Cu), lead (Pb) and nickel (Ni) in leaves and fruits of pepper plants grown on a heavy metals-contaminated saline soil. The single (i.e., MSE-SA or MSE-FS) or integrative (i.e., MSE-SA+MSE-FS) treatments significantly decreased the leaf and fruit contents of Cd, Cu, Pb and Ni compared to those of the controls (SA and FS were applied with tap water). Additionally, the integrative MSE-SA+MSE-FS treatment significantly exceeded both MSE single treatments collecting the best results. It decreased Cd content by 81 and 79% in leaves and 82 and 79% in fruits, Cu content by 51 and 53% in leaves and 52 and 54% in fruits, Pb content by 50 and 52% in leaves and 58 and 58% in fruits, and Ni content by 71 and 69% in leaves and 75 and 67% in fruits in both 2016 and 2017 growing seasons, respectively.

Table 5 Effect of *Moringa oleifera* seed extract (MSE) applications on leaf contents of nutrients and K^+/Na^+ ratio of pepper plants grown on heavy metals-contaminated saline soil

Treatments	N%	P%	K+%	Na+%	K+/Na+ratio
2016					
Control	1.21±0.02d	0.18±0.004c	1.96±0.09d	2.33±0.06a	0.84±0.05d
MSE-SA	1.28±0.03c	0.22±0.003b	2.39±0.02c	1.81±0.03b	1.32±0.03c
MSE-FS	1.66±0.03b	0.23±0.003b	2.61±0.08b	1.62±0.02c	1.61±0.04b
MSE-SA + MSE-FS	2.09±0.03a	0.30±0.010a	2.83±0.04a	1.52±0.03d	1.86±0.01a
2017					
Control	1.24±0.12d	0.18±0.010c	1.90±0.01d	2.39±0.05a	0.79±0.02d
MSE-SA	1.30±0.04c	0.23±0.007b	2.26±0.12c	1.84±0.10b	1.23±0.01c
MSE-FS	1.68±0.07b	0.23±0.004b	2.66±0.10b	1.72±0.12b	1.55±0.10b
MSE-SA + MSE-FS	2.10±0.09a	0.29±0.003a	2.85±0.05a	1.50±0.07c	1.90±0.08a

Data are means (n = 9) ± SE. The same letters in each column indicate not significant differences according to the LSD test ($P \leq 0.05$).
Control; no extracts were added in irrigation water + foliar spray was done with tap water,
MSE-SA; moringa seed extract was added in irrigation water + foliar spray was done with tap water,
MSE-FS; no extracts were added in irrigation water + foliar spray was done with moringa seed extract, and
MSE-SA + MSE-FS; moringa seed extract was added in irrigation water + foliar spray was done with moringa seed extract.

Table 6 Effect of *Moringa oleifera* seed extract (MSE) applications on leaf activity of antioxidant enzymes in pepper plants grown on heavy metals-contaminated saline soil

Treatments	CAT	POX	APX	SOD	GR
	(mM H2O2 g^{-1} FW)				
2016					
Control	40.7±0.8d	0.81±0.01d	36.8±1.1d	4.28±0.12d	20.4±1.0d
MSE-SA	45.7±1.2c	1.03±0.02c	42.0±1.7c	4.46±0.08c	24.6±1.0c
MSE-FS	51.8±0.9b	1.49±0.12b	58.1±1.7b	5.38±0.08b	31.2±1.3b
MSE-SA + MSE-FS	63.0±1.2a	1.90±0.04a	73.3±1.6a	6.59±0.13a	43.7±0.7a
2017					
Control	41.1±1.0d	0.82±0.02d	37.3±2.1d	4.31±0.04d	20.8±1.5d
MSE-SA	46.1±2.3c	1.04±0.01c	42.3±2.0c	4.79±0.07c	23.9±1.0c
MSE-FS	52.2±1.0b	1.52±0.02b	58.6±2.2b	5.42±0.07b	31.7±1.7b
MSE-SA + MSE-FS	63.5±1.0a	1.95±0.24a	73.7±1.9a	6.74±0.22a	44.1±2.5a

Data are means (n = 3) ± SE. The same letters in each column indicate not significant differences according to the LSD test ($P \leq 0.05$).
Control; no extracts were added in irrigation water + foliar spray was done with tap water,
MSE-SA; moringa seed extract was added in irrigation water + foliar spray was done with tap water,
MSE-FS; no extracts were added in irrigation water + foliar spray was done with moringa seed extract, and
MSE-SA+MSE-FS; moringa seed extract was added in irrigation water + foliar spray was done with moringa seed extract.

Discussion

The soil tested in the current study has salinity (EC = 7.73–7.78 dS m^{-1}) and heavy metals (i.e., Cd, Cu, Pb, and Ni at a concentration of 18.1–18.7, 106–109, 248–253, and 253–259 mg kg^{-1} soil, respectively) contamination that cause a severe (doubled) stress to plants (Table 1). This soil caused a reduction in leaf chlorophylls (Table 4) and mineral nutrients (Table 5) and an increase in heavy metals contents (Table 7) & (Table 8), which reflected in a severe decrease in growth and fruit yield of pepper plants (Tables 3). These deleterious effects ascribe to the long persistence of heavy metals in the soil in addition to the osmotic pressure "physiological drought" caused by salt stress, all of which cause extreme toxic effects both on production of crops and on human health due to the human consumption of these crops;[43,44] It is well known that the toxicity of heavy metals in the agricultural environment causes extreme toxic effects on plant processes including leaf chlorosis induction,[45] root and shoot growth reductions,[46] undesirable enzyme activation and inhibition.[47] Further, salt stress disorders the metabolic processes, including meristematic activity and cell elongation reductions, connecting with high respiration rate due to high energy requirements, all of which negatively affect plant growth and production.[48] The stress causes excess generation of ROS that induces oxidative stress in plants.[49] These increased ROS injure chlorophylls, DNA, membrane functions and protein. To repair and alleviate the harms caused by ROS, plants develop their antioxidant defense systems,[50] comprising of many enzymatic (i.e., CAT, POD, SOD, APX, GR, etc.) and non-enzymatic low molecular weight (i.e., proline, AsA, GSH, tocopherols, carotenoids, etc.) antioxidants[16,21] These developed antioxidant defense systems are not enough, in most cases, to assist plants to cope with the stresses under study, therefore plants need to exogenous support to increase the efficiency of their antioxidant defense systems such as antioxidants- and growth promoting-containing plant extracts, which are proved to stimulate plant defenses effectiveness in this regard.[21–24] This study showed that a beneficial use of moringa seed extract (MSE), especially the integrative MSE-SA + MSE-FS (applied as Rhizosphere supplementation with drip irrigation water; SA and as foliar spray; FS) treatment. This integrative MSE-SA+MSE-FS treatment significantly increased plant growth and production and leaf physio-biochemical attributes (Tables 3–5), and leaf antioxidant enzyme activities (Table 6), and significantly reduced leaf and fruit accumulation of heavy metals (Table 7) & (Table 8) of salt (EC=7.73–7.78 dS m^{-1})- and heavy metal (Cd, Cu, Pb, and Ni)-stressed pepper plants compared to those of the other MSE single treatments and the control (free from the extract). These improvements conferred by the integrative MSE-SA+MSE-FS treatment for pepper plants grown on saline soil contaminated with heavy metal may be attributed to the supplementations of antioxidants, osmoprotectants, phytohormones, and nutrients (Table 2) found in abundant amounts in MSE to pepper plants through their roots and leaves to support the plant antioxidant defense systems to cope with the doubled stress (salinity and heavy metals) under this study. The analysis of MSE as shown in Table 2 indicates that this extract considers as effective bio stimulant for stressed plants. The enhanced pepper growth and yield due to the application of MSE under the doubled stress may be attributed to the improved mobilization of germination and growth-linked metabolites dissolved substances such as mineral nutrients, soluble sugars, antioxidants, and amino acids of MSE that participate in early seed activities and strong plumule growth, positively reflecting in seedling growth[51,52] under stress due to the improvements in the activity of antioxidant defense systems (Tables 4–6). These positive MSE effects, coming from SA, are supported by FS of MSE, conferring positive effects of extract active components, especially GAs and Se to further improve the doubled stressed plant growth, and consequently its production.[53,54] By looking at the most important active components of MSE, it has been documented salt or heavy metal stress alleviation by exogenous GAs addition through enhancing plant growth and conferred heavy metal tolerance in microalgae (*Chlorella vulgaris*) exposed to Cd and Pb.[55] They attributed this result to the positive effect of GA_3 on the growth, metal bioaccumulation, and biochemical composition of *C. vulgaris* under stress due to the great effect of GA_3 on the basic developmental plant processes. GAs may be increased in plant roots by MSE-SA and in leaves by MSE-FS, perhaps due to enhanced activities of GAs biosynthesis enzymes such as GA20ox and GA3ox,[56] contributing to stronger growth to face salt and metal stress conditions. Application of MSE may be maintained an appropriate level of endogenous GAs for stimulated growth in different stages through activation of cell division and elongation, leading to an increase in leaf area along with stay-green effect by cytokinins found in MSE that stimulate plant photosynthetic rate.

Mineral nutrients, as an essential part of MSE (Table 2), are very important for plant growth and development under both normal and stress conditions.[9,51] It has been reported that mineral nutrients applications increased metal tolerance capacity of plants and alleviating the metal toxicity by maintain photosynthetic machinery through affecting, positively, the PSII reaction centers and regeneration of ribulose-1,5-bisphosphate, and by decreasing the level of free radical production and lipid peroxidation through stimulating the antioxidant defense systems[57-59] These nutrients maintain leaves number on plants to maximize photosynthesis, elevating the sink capacity fulfilled during supply of photo-assimilates from stressed leaves.[60] Application of nutrients-containing MSE maximized the number of photosynthetic active leaves and leaves area with staying green (observed; data not shown) to longer time, maintaining chlorophylls in higher contents (Table 4). Presence of GAs and nutrients in MSE inhibits premature leaf senescence and maintains higher leaf area, increasing the efficiency of photosynthetic machinery. In addition, Fe found in MSE may be available in plants after treatment to activate many enzymes involved in pathways of chlorophyll biosynthesis and some antioxidant enzymes such as APOX and GR that scavenge the ROS and protect chlorophyll from degradation.[61] K^+, as an important nutrient component in MSE, is a major osmo protectant to preserve higher tissue water content and to regulate the stomatal opening/closure controlling photosynthesis rate of plant grown under stress conditions.[62,63] Stomatal regulation depends on K^+ supplying in the guard cell and leaf apoplast.[64] Our results showed that the increase of chlorophyll content by MSE positively reflected in growth and yield characteristics that might be attributed to more assimilations correlated with nutrient elements, GAs, and Se.[51,65] that found in the applied MSE to support the antioxidant defense systems of the doubled stressed pepper plants under study.

The undesirable results obtained under the saline soil contaminated with heavy metals were repaired by antioxidants-containing MSE together with stimulating the endogenous antioxidant defense systems against ROS that generate by stress.[14] Leaf stomata of stressed plants close, leading to a decrease in CO_2 fixation, while electrons transfer and light reaction continue normally. Under such conditions, accepting the electrons by NADP will be limited and so oxygen can act as electrons acceptor, leading to produce more ROS such as $^1O_2$, $O_2^{\bullet-}$, H_2O_2 and OH^- radicals that lead to peroxidation of cell membranes and increasing ion leakage and lipid peroxidation.[65] Photosynthesis is coupled with transpiration rate of plants, and the transpiration inhibition is a credible and prompt measure of toxic effects of stress.[66] Application of osmoprotectants (sugars and proline)-containing MSE helps modify the water imbalance in the plant.[44] Therefore; these materials may be included in doubled stressed pepper growth and yield improvements. Proline is also altered in salt + heavy metal-stressed plants by the MSE application. It, as a main component of MSE, contributes to cell osmotic adjustment under stress conditions, with which plants usually accumulate more levels of proline[10] and is further accumulated by MSE application (Table 4). Proline accumulation (from 5% of the amino acid pool up to 20–80%) in stressed plants attributes to acclimation to recompense the energy for growth and survival, helping plants to tolerate the stress.[67] The mechanisms by which proline reduces the ROS damages and enhances plant tolerance are that proline declines stress by detoxification of ROS overproduced by stress poisoning, in addition to that it may physically quenches $^1O_2$ or reacts directly with OH^- radicals.[68] The increase in proline and other MSE components promoted the antioxidant defense systems of pepper plants (Table 4) & (Table 6) to avoid damages caused by stress.[44] Another osmo protectant that was further increased in stressed pepper plants by MSE application, soluble sugars (Table 4) are participated to plant osmotic adjustment.[69] and can directly or indirectly modify genes expressions involved in metabolic processes, storage functions, and defense systems.[70] Accumulation of soluble sugars might have a physiologically important role in energy supply, osmotic adjustment to maintain leaf water potential and plant water content, and it can reduce cell osmotic potential and increase stress tolerance.[71] In addition, Se was found as an important component of MSE (Table 2). It is a constituent of seleno-proteins, and it has many important functions, including energy metabolism and antioxidant protection.[72] Supplementation of Se to plants (through applied MSE) may be contributed to improvements of pepper performance (growth and yield) and antioxidant systems activity,[73] and the ability to reduce ROS by increasing the antioxidant systems activity.[14]

Table 7 Effect of *Moringa oleifera* seed extract (MSE) applications on heavy metals accumulation in leaves of pepper plants grown on heavy metals-contaminated saline soil

Treatments	**Cd(mg kg^{-1})**	**Cu(mg kg^{-1})**	**Pb(mg kg^{-1})**	**Ni(mg kg^{-1})**
2016				
Control	11.8±0.24a	66.4±1.9a	36.6±1.0a	17.2±0.31a
MSE(SA)	4.85±0.10c	43.6±1.6c	26.3±0.9c	12.6±0.26c
MSE(FS)	6.80±0.14b	52.7±1.7b	30.4±0.8b	14.9±0.40b
MSE(SA)+MSE(FS)	2.29±0.07d	32.5±1.0d	18.2±1.0d	4.92±0.77d
2017				
Control	11.6±0.31a	65.4±0.9a	38.0±0.8a	17.6±0.47a
MSE(SA)	4.79±0.13c	42.3±0.7c	25.3±0.5c	13.2±0.46c
MSE(FS)	6.02±0.22b	50.1±1.3b	30.0±1.0b	15.2±0.75b
MSE(SA)+MSE(FS)	2.43±0.18d	30.8±1.1d	18.1±0.8d	5.39±0.30d

Data are means (n = 3) ± SE. The same letters in each column indicate not significant differences according to the LSD test (P≤0.05).
Control; no extracts were added in irrigation water + foliar spray was done with tap water,
MSE-SA; moringa seed extract was added in irrigation water + foliar spray was done with tap water,
MSE-FS; no extracts were added in irrigation water + foliar spray was done with moringa seed extract, and
MSE-SA + MSE-FS; moringa seed extract was added in irrigation water + foliar spray was done with moringa seed extract.

Table 8 Effect of *Moringa oleifera* seed extract (MSE) applications on heavy metals accumulation in fruits of pepper plants grown on heavy metals-contaminated saline soil

Treatments	**Cd(mg kg^{-1})**	**Cu (mg kg^{-1})**	**Pb (mg kg^{-1})**	**Ni (mg kg^{-1})**
2016				
Control	8.45±0.21a	47.4±1.0a	26.1±0.5a	12.3±0.21a
MSE(SA)	2.75±0.12c	29.0±0.6b	18.8±0.7b	8.98±0.12b
MSE(FS)	4.14±0.14b	30.5±0.7b	20.3±0.8b	9.19±0.22b
MSE(SA)+MSE(FS)	1.55±0.10d	22.8±0.3c	11.0±0.3c	3.09±0.17c
2017				
Control	8.27±0.11a	46.7±0.9a	26.4±0.2a	12.6±0.37a
MSE(SA)	2.65±0.13c	29.5±0.5c	18.1±0.2c	9.25±0.16b
MSE(FS)	4.30±0.12b	34.8±0.8b	20.3±0.9b	9.49±0.25b
MSE(SA)+MSE(FS)	1.75±0.08d	21.6±0.4d	11.2±0.2d	4.19±0.18c

Data are means (n = 3) ± SE. The same letters in each column indicate not significant differences according to the LSD test (P≤0.05).
Control; no extracts were added in irrigation water + foliar spray was done with tap water,
MSE-SA; moringa seed extract was added in irrigation water + foliar spray was done with tap water,
MSE-FS; no extracts were added in irrigation water + foliar spray was done with moringa seed extract, and
MSE-SA + MSE-FS; moringa seed extract was added in irrigation water + foliar spray was done with moringa seed extract.

The increased accumulations of Na^+ and heavy metals (i.e., Cd, Cu, Pb, and Ni) ions together with the reductions in the N, P, and K^+ contents, and K^+/Na^+ ratio in the doubled stressed plants were positively modified by the MSE application (Table 5) & (Table 7). This positive nutrient status of pepper plants mainly due to the nutrient-containing MSE (Table 2) and may be attributed to the antagonistic effects of extract nutrients with heavy metals maintaining membrane health.[74,75] Plant nutrient homeostasis depends primarily on the degree of the membrane vector activity, which is involved in the transfer of ions from soil to plant, then regulates its distribution within and between plant cells.[76] Membranes can lead to chemical disturbances in stressed-plant cells, reflecting deficiency symptoms of some essential nutrients on plants due to the antagonistic effect of Na^+ ion against nutrients (i.e. N, K^+, P, and Ca^{2+}).[77] In the present study, K^+ imbalance in cells cytosols under stress was positively modified by MSE application, therefore, maintaining high concentrations of K^+ within the cytosols and maintaining an appropriate K^+/ Na^+ ratio as a basic mechanism to help plant to tolerate stress.[78] The increase in nutrients contents (N, P, and K^+) by MSE application may be attributed to that MSE is rich source in mineral nutrients and hormones like GAs, auxins, and zeatin-type cytokinin that increase the metabolic processes including nutrient absorption to increase the nutrients contents in plant tissues.[79] at the expense of Na^+ and heavy metal ions.

Phytoremediation (i.e., the use of plants) sequester and/or detoxify contaminants and has been reported to be an effective, nonintrusive, inexpensive, aesthetically pleasing and socially accepted technology to remediate contaminated soils. Plants for phytoextraction, i.e. metal removal from soil, should be tolerant to heavy metals with profuse root system, rapid growth rate, and producing reasonably high biomass.[80] Plant extract, herein (i.e., MSE), may be considered as an effective tool for Phytoremediation of heavy metals, especially they significantly decreased the accumulation of Cd, Cu, Pb, and Ni in pepper plants (leaves and fruits) when applied through drip irrigation water and foliar spray (Table 7). This reduction in heavy metals accumulation by these extracts may be attributed to the increased efficiency of the antioxidant defense systems (enzymatic and non-enzymatic; (Table 4) & (Table 6) and/or to the increased nutrients (Table 5) that may antagonize heavy metal entry into plants.[74,75] Plant generally faces oxidative damages when exposed to heavy metal stress.[81] The phyto-potential of plant can be assessed with tolerance mechanism for toxic metal-induced ROS.[82] The major ROS scavenging pathways of plants include SOD found in almost all cellular compartments, the ascorbate-glutathione cycle in chloroplasts, the water–water cycle in chloroplasts, and cytosol, GR and CAT found in peroxisomes, mitochondria, apoplast, and peroxisomes.[82] In addition, glutathione (GSH), is an important component of MSE, may be involved in heavy metal resistance. It helps to reduce the effect of secondary oxidative stress resulting from the production of ROS,[83] and it also constitutes the precursor of phytochelatins, which are small peptides binding to metal accumulating in vacuoles.[84] MSE used in this study contains many other antioxidants and vitamins that may have an important role in reducing the heavy metals accumulation in pepper plants. Another mechanism, by which pepper plants had tolerated the doubled stress under study, is antioxidant defense system activity that was increased significantly by MSE to overcome the ROS damages. Antioxidants found in MSE may be translocated into plants through roots (by drip irrigation) and leaves (through foliar spray) to contribute to increase the efficiency of the antioxidant defense systems including non-enzymatic (e.g., proline, AsA, GSH, tocopherols, carotenoids, etc.) and enzymatic (e.g., GR, SOD, CAT, POD, APX, etc.) antioxidants. Among the group of antioxidant enzymes, SOD is considered as the first line of defense against ROS and converts $O_2^{\bullet-}$ to H_2O_2.[85] H_2O_2 is then further scavenged by CAT and APX into H_2O and O_2.[86] In the AsA-GSH cycle, APX reduces H_2O_2 by using AsA as an electron donor, and the oxidized AsA is then reduced by GSH generated from GSSG that is catalyzed by GR at the expense of NADPH. Therefore, levels of antioxidant enzymes increase when plants are exposed to oxidative stress including salinity.[18,20] The regenerating enzymes GR and DHAR 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 reported.[87] The present study reported significant increases in the antioxidant enzymes activity (i.e. CAT, POX, APX, SOD, GR; Table 6) by the integrative MSE-SA+MSE-FS application compared to the controls without MSE. These increases in the activity of antioxidant enzymes as an effective defense system supported stressed plants to tolerate stress and to reduce, significantly, the ROS damages.

Economically**,** we had used 18kg moringa seeds per hectare for the best treatment; MSE-SA+MSE-FS (5g $L^{-1}\times$ 600 L MSE for each addition through drip irrigation+600 L MSE for each spray per hectare×3 sprays=18kg moringa seeds), this amount of moringa seeds equals approx. 150 USD (18 kg×8.33 USD) as local prices. The best treatment conferred average pepper fruit yield of approx.42 tons per hectare as an average of the two seasons. As local prices of pepper fruits, the 42 tons of fruits equal approx. 12,000 USD. By subtraction the value of the control production (17.3 tons per hectare) from the best treatment production; 12,000–4,800, therefore, we will obtain equal approx. 7,200 USD (minus 150 USD for applied materials) = approx. 7,050 USD earnings per hectare. This with the easy preparation of the MSE for farmers/producers due to that the extraction method requires only alcohol as described in the material and methods section.

Conclusion

Stress tolerance in pepper plants grown on a heavy metal (Cd, Cu, Pb and Ni)-contaminated saline (EC=7.73–7.78 dS m^{-1}) soil was effectively improved by the integrative application of MSE-SA + MSE-FS that provided plants with excess desirable materials (i.e., phytohormones, proline, sugars, ascorbic acid, glutathione, vitamins, selenium and mineral nutrients) for rapid and strong growth to strongly face the doubled stress. The leverage of MSE in alleviating the doubled stress in plants reflecting better growth and yield is found to be due to the improved antioxidant defense systems; non-enzymatic and enzymatic antioxidants (i.e., free proline, TSS, carotenoids, CAT, POD, SOD, APX, and GR) to decline the ROS damages by the addition of minerals-, osmoprotectants-, phytohormones-, Se-, and vitamins-containing MSE applied, especially as integrative additions through drip irrigation and foliar spray, which reported herein as the best integrative treatment. The leverage of MSE is also reported in our study as "stay-green effect" due to MSE active components (i.e., mineral nutrients, phytohormones, soluble sugars, and proline), supporting the anti oxidative defense systems in pepper plants under severe stress.

Acknowledgments

None.

Conflicts of interest

The author declares there is no conflicts of interest.

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