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Licorice Root Extract Boosts *Capsicum annuum* L. Production and Reduces Fruit Contamination on a Heavy Metals-Contaminated Saline Soil

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Abstract. Natural supplementations are used in agriculture nowadays not only for improving plant performance but also for reducing the contamination of plant edible parts. Two field trials were conducted to study the potential effects of licorice root extract (LRE; 0.5%) on performance, physio-biochemical components, antioxidant defense system, and contaminants concentrations of *Capsicum annuum* L. plants grown on a saline soil contaminated with heavy metals. LRE was applied in single (i.e., as rhizosphere application with drip irrigation water; -RA or as foliar spray; -FA) or in integration (i.e., LRE-RA + LRE-FA) treatment. The results showed that both single or integrative treatments significantly increased plant growth and yield, leaf concentrations of 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 concentrations in plant leaves and fruits on heavy metals-contaminated saline soil compared to the control (without LRE). Additionally, the integrative LRE-RA + LRE-FA treatment significantly exceeded both single treatments in this concern, which had been recommended for maximizing pepper plant performances with minimizing heavy metals in fruits on contaminated saline soils.

Introduction

Pepper (*Capsicum annuum* L.) is an economically important vegetable crop due to its nutritional value, antioxidant compounds, bioactive products, and natural colours for human health [39]. Pepper is considered as salt sensitive [19] or moderately salt sensitive [50] based on the growth stage. Peppers like most of the vegetables cultivated in Egypt are sometimes grown in heavy metals-contaminated soils close to factories and industrial cities, in addition to soil irrigation with wastewater nowadays to overcome the problem of water deficit. These soils are exposed to heavy metals contamination by which soils are contaminated up to the unpermitted levels [72], negatively reflecting in plant growth and productivity, 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 [43]. In order to cope with heavy metal stress plants adopt 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 [51].

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 [8]. High salinity causes ion disequilibrium, osmotic (physiological drought) and oxidative stresses in plant tissues, which inhibit the synthesis of photosynthetic pigments and photosynthetic process, inducing an over-reduction of the reaction centers in photosystem II that may destruct the photosynthetic machinery if the plant is unable to consume excess energy [7]. 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 [44].

The common consequence of most abiotic stresses, including salinity and heavy metals [32] are an overproduction of reactive oxygen species (ROS; $^1O_2$, $O_2^{\bullet-}$, and H_2O_2) that are extremely toxic to plants. They caused damages to DNA, proteins, and chlorophyll [62]. 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, tocopheroles, carotenoids, glutathione, ascorbic acid, etc.) to counteract the oxidative stress to protect plants from the oxidative injuries [5], and the extent of oxidative cellular damage in abiotic-stressed plants is controlled by the capacity of their antioxidant systems [64, 57]. In most cases, the endogenous antioxidant defense systems of plants are not enough to maintain healthy growth of plants, therefore, plants need exogenous support such as plant extracts to increase their tolerance to the stress [20, 60].

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, licorice (*Glycyrrhiza glabra*) root extract (LRE) is contributed to improving plant growth and production [23]. LRE contains a substance so-called "glycyrrhizin"; the calcium and potassium salts of glycyrrhizic acid and trihydroxy acid [53]. LRE contains plant performance enhancing capabilities due to that it is a rich source in antioxidants and osmoprotectants such as amino acids, proline, soluble sugars, salicylic acid, α-tocopherol, ascorbic acid, glutathione, some vitamins (i.e., A, E, and B-group), and selenium. It is also rich in phytohormones including significant amounts of auxins, gibberellins, and cytokinins (zeatin-type), and nutrient elements [66].

There are many different plant extracts that are used as effective natural biostimulants for supporting the plants grown under several environmental stresses [56, 1]. To our knowledge, using LRE in alleviating biotic and abiotic stresses in plants is very scarce. Therefore, the aim of this study was to assess the potential effects of the exogenous application of LRE 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 = 7.73–7.78 dS m^{-1}) soil. The hypothesis tested herein is that integrative supplementation of LRE (i.e., LRE-RA + LRE-FA) maybe promote plant growth and productivity through reducing the accumulation of heavy metals and maybe improve the levels of nutrients and osmoprotectants, 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

Two 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 studied sites before each agricultural season and analyzed according to Black [11] and Jackson [42] and the results are shown in Table 1. According to soil analyses results before planting, soil EC values were 7.73 and 7.78 dS m^{-1} for the soil of both 2016 and 2017 seasons, respectively, indicating saline soils [18]. EC analyses for the soil 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 [16].

Seeds of pepper (*Capsicum annuum* L., cv. Top Star; moderately salt sensitive) 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 m width × 3.50 m long = 10.5 m^2) on rows spaced 60 cm, and the distances among transplants were 15 – 20 cm. Before sowing, as recommended by the Egyptian Agricultural Ministry all plots of the experiments were fertilized with 100 kg potassium sulfate (50% K_2O) ha^{-1} and 200 kg calcium superphosphate (15.5% P_2O_5) ha^{-1}. Nitrogen fertilizer was added at a rate of 250 kg ammonium nitrate (33% N) ha^{-1}.

The empirical design of the study was a completely randomized blocks (CRBD) for 4 treatments each with 9 replicates. The details of the 4 treatments are as follows: 1. **Control**; no extracts were added in irrigation water + foliar spray was done with tap water, 2. **LRE-RA**; licorice root extract was added in irrigation water + foliar spray was done with tap water, 3. **LRE-FA**; no extracts were added in irrigation water + foliar spray was done with licorice root extract, and 4. **LRE-RA + LRE-FA**; licorice root extract was added in irrigation water + foliar spray was done with licorice root extract (5% of roots weight for each).

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

Soil characteristic	Value		Unit
	2016	2017	
Soil particles distribution:			
Sand	434	433	
Silt	287	286	g kg^{-1}
Clay	279	281	
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	
Mg^{2+}	15.8	15.1	
Na^{+}	30.4	30.9	
K^{+}	5.26	5.62	$mmol_c$ L^{-1}
CO_3^{2-}	-	-	
HCO_3^{-}	8.91	6.86	
Cl^{-}	21.3	20.4	
SO_4^{2-}	48.5	51.9	
Available nutrient:			
N	33.4	31.9	
P	8.72	7.87	mg kg^{-1} soil
K	104	103	
Heavy metals:			
Cu	106	109	
Cd	18.1	18.7	
Ni	259	253	mg kg^{-1} soil
Pb	253	248	

* Soil paste; ** Soil paste extract.

Extraction, analysis, and applications of LRE

For extraction of licorice root active ingredients, roots were collected at the end of licorice season and dried (the climate of the agricultural area; Egypt is characterized as dry and loamy soil). Each 100 g of dried root was soaked in 20 L of distilled water at 50 °C for 24 hours then filtered and the final volume was reached to 20 L by distilled water. The LRE should be used within 5 h from extraction process otherwise was stored at – 20 °C and only taken out when it requested to use. LRE was analyzed for main chemical compositions that are shown in Table 2. LRE (0.5%; 5 g roots per L) was applied as foliar spray applications (FA) 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, LRE was applied as a rhizosphere application (RA) by adding 200 L extract (0.5%) ha^{-1} to drip irrigation water three times with the 2^{nd}, 4^{th} and 6^{th} irrigation.

Table 2. Chemical analysis of the licorice root extract (LRE) (on dry mass; DM basis)

Component	Values	Unit
1. Antioxidants and osmoprotectants:		
Free proline	36	g kg^{-1} DM
Soluble sugars	148	
Glutathione (GSH)	30.2	
Ascorbic acid (AsA; Vit. C)	41.0	mg kg^{-1} DM
Selenium (Se)	0.90	
DPPH-radical scavenging	84.6	%
2. Phytohormones:		
Total auxins	4.2	
Total gibberellins	5.2	mg kg^{-1} DM
Zeatin-type cytokinin	4.1	
3. Mineral nutrients:		
Nitrogen (N)	20.2	
Phosphorus (P)	21.3	
Potassium (K)	47.2	
Calcium (Ca)	2.20	
Magnesium (Mg)	3.80	g kg^{-1} DM
Sulfur (S)	2.40	
Iron (Fe)	0.94	
Manganese (Mn)	0.62	
Zinc (Zn)	0.21	
Cupper (Cu)	0.02	

Growth and yield attributes

Ten pepper plants were selected randomly and cut off from the two outer rows of every experimental unit at 60 DAT to estimate shoot dry mass (ton ha^{-1}). At the merchantable stage (75 DAT), fruits from 50 plants on each plot were reaped to estimate the number of fruits $plant^{-1}$, and fruit yield (ton ha^{-1}).

Determination of physio-biochemical constituents

Total chlorophylls and total carotenoids were extracted from fresh leaf (the 4^{th} upper) using pure acetone and determined (as mg g^{-1} fresh mass) according to Fadeels [25], and values were converted based on dry mass. The method of Bates et al. [9] was used to determine proline accumulation in dried pepper leaves, and total soluble sugars concentration was estimated according to Irigoyen et al. [41]. For nutrients and heavy metal determinations, a weight of 0.2 g of dried leaves was digested with H_2SO_4 in the presence of H_2O_2 [75] and then, N concentration was determined using a micro Kjeldahl method according to Chapman and Pratt [14], P concentration was determined colorimetrically using ascorbic acid method of Watanabe and Olsen [73], and Na^+ and K^+ concentrations were measured directly using Flame photometer [48].

The powdery dried plant samples (old 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% HNO_3 (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 antioxidant enzyme activities

Enzymes were extracted from the upper full-expanded leaves according to Vitoria et al. [71] The activity of catalase (CAT) enzyme was assessed spectrophotochemically according to Chance and Maehly [12]. Peroxidase (POX) activity was estimated according to Thomas et al. [68]. Ascorbate peroxidase (APX) activity was determined spectrophotochemically according to Fielding and Hall [28]. The activity of superoxide dimutase (SOD) was determined by recording the drop in absorbance of superoxide-nitro blue tetrazolium complex by the enzyme [61]. Glutathione reductase (GR) activity was measured after monitoring the oxidation of NADPH for three absorbance times taken at 340 nm [59].

Statistical analysis

Statistically significant variations between means were compared at $P \leq 0.05$ 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 LRE), the single (i.e., LRE applied as rhizosphere supplementation with the drip irrigation water; RA or as foliar application; FA) or integrative (i.e., LRE-RA + LRE-FA) treatments significantly increased plant growth (in terms of shoot dry mass), yield parameters (i.e., No. of fruits per plant, and fruits yield per hectare), and the concentrations of total chlorophylls, total carotenoids, free proline, and total soluble sugars of pepper plants grown on a heavy metals-contaminated saline soil (Tables 3 and 4). In addition, the integrative LRE-RA + LRE-FA treatment significantly exceeded the single treatments conferring the best results. It increased shoot dry weight by 100 and 99%, No. of fruits per plant by 142 and 153%, fruits yield by 157 and 157%, chlorophylls concentration by 59 and 58%, carotenoids concentration by 50 and 36%, proline concentration by 85 and 83%, and soluble sugars by 83 and 92% in both 2016 and 2017 growing seasons, respectively. These results show similar trends over both growing seasons.

Table 3. Effect of licorice root extract (LRE) applications on shoot dry mass (DM) and yield attributes of pepper plants grown on a heavy metals-contaminated saline soil

Treatments	Shoot DM ha^{-1}	Fruits No. $plant^{-1}$	Fruits yield (ton ha^{-1})
2016			
Control	9.8±0.9d	1.9±0.6d	17.0±0.5d
LRE-RA	11.5±0.8c	2.4±0.7c	21.4±0.9c
LRE-FA	14.0±1.1b	3.5±0.5b	32.9±1.5b
LRE-RA + LRE-FA	19.6±1.3a	4.6±0.2a	43.7±1.1a
2017			
Control	10.3±0.9d	1.9±0.4d	17.7±0.6d
LRE-RA	11.7±0.8c	2.5±0.9c	21.9±0.8c
LRE-FA	14.5±0.9b	3.6±0.5b	33.4±1.4b
LRE-RA + LRE-FA	20.5±0.8a	4.8±0.4a	45.5±2.0a

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,

LRE-RA; licorice root extract was added in irrigation water + foliar spray was done with tap water,

LRE-FA; no extracts were added in irrigation water + foliar spray was done with licorice root extract, and

LRE-RA + LR-FA; licorice root extract was added in irrigation water + foliar spray was done with licorice root extract.

Table 4. Effect of licorice root extract (LRE) applications on leaf concentrations 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} DM)	Total carotenoids (mg g^{-1} DM)	Proline (mg g^{-1} DM)	Soluble Sugars (mg g^{-1} DM)
2016				
Control	1.01±0.02d	0.20±0.004c	17.2±0.7d	8.40±1.0d
LRE-RA	1.32±0.03c	0.25±0.002b	20.4±0.3c	10.5±0.3c
LRE-FA	1.46±0.04b	0.27±0.001b	27.9±0.2b	13.4±0.7b
LRE-RA + LRE-	1.61±0.02a	0.30±0.001a	31.9±0.2a	15.4±0.3a
2017				
Control	1.07±0.02d	0.22±0.004c	18.1±0.7d	8.14±0.9d
LRE-RA	1.39±0.03c	0.27±0.002b	21.5±0.3c	10.6±0.6c
LRE-FA	1.52±0.04b	0.27±0.001b	29.1±0.1b	13.3±0.5b
LRE-RA + LRE-	1.69±0.12a	0.30±0.001a	33.2±0.2a	15.6±0.5a

Data are means ± 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,
LRE-RA; licorice root extract was added in irrigation water + foliar spray was done with tap water,
LRE-FA; no extracts were added in irrigation water + foliar spray was done with licorice root extract, and
LRE-RA + LR-FA; licorice root extract was added in irrigation water + foliar spray was done with licorice root extract.

Nutrients concentrations and K^+/Na^+ ratio

The single (i.e., LRE-RA or LRE-FA) or integrative (i.e., LRE-RA + LRE-FA) treatments significantly increased the concentrations of N, P, and K^+, and the ratio of K^+/Na^+, while significantly reduced the concentration of Na^+ of pepper plants grown on a heavy metals-contaminated saline soil compared to the control (RA and FA were applied with tap water) (Table 5). Further, the integrative LRE-RA + LRE-FA treatment significantly exceeded the single treatments recording the best results. It increased N concentration by 67 and 73%, P concentration by 61 and 56%, K^+ concentration by 44 and 53%, and K^+/Na^+ ratio by 140 and 168%, and reducing Na^+ concentration by 40 and 43% in both 2016 and 2017 growing seasons, respectively. These results represent identical trends in both growing seasons.

Table 5. Effect of licorice root extract (LRE) applications on leaf concentrations 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
LRE-RA	1.47±0.04c	0.23±0.004b	2.36±0.08c	1.78±0.02b	1.33±0.04c
LRE-FA	1.69±0.03b	0.24±0.009b	2.60±0.06b	1.63±0.03c	1.60±0.07b
LRE-RA + LRE-	2.02±0.12a	0.29±0.011a	2.83±0.04a	1.40±0.01d	2.02±0.02a
2017					
Control	1.24±0.12d	0.18±0.010c	1.90±0.01d	2.39±0.05a	0.79±0.02d
LRE-RA	1.52±0.08c	0.23±0.007b	2.38±0.08c	1.80±0.05b	1.32±0.09c
LRE-FA	1.79±0.11b	0.24±0.006b	2.66±0.08b	1.60±0.06c	1.66±0.06b
LRE-RA + LRE-	2.15±0.21a	0.28±0.009a	2.91±0.05a	1.37±0.03d	2.12±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,
LRE-RA; licorice root extract was added in irrigation water + foliar spray was done with tap water,
LRE-FA; no extracts were added in irrigation water + foliar spray was done with licorice root extract, and
LRE-RA + LR-FA; licorice root extract was added in irrigation water + foliar spray was done with licorice root extract.

Table 6. Effect of licorice root extract (LRE) applications on leaf activity of antioxidant enzymes in pepper plants grown on heavy metals-contaminated saline soil

Treatments	CAT	POX	APX	SOD	GR
	(mM H_2O_2 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
LRE-RA	46.2±1.2c	1.26±0.10c	47.1±2.5c	4.79±0.13c	25.3±1.2c
LRE-FA	54.0±1.1b	1.60±0.06b	60.8±2.5b	5.75±0.06b	36.0±0.8b
LRE-RA + LRE-	63.1±1.3a	1.94±0.02a	73.3±2.2a	6.62±0.12a	43.9±1.2a
2017					
Control	41.1±1.0d	0.82±0.02d	37.3±2.1d	4.31±0.04d	20.8±1.5d
LRE-RA	46.9±1.3c	1.30±0.08c	47.2±2.8c	4.83±0.24c	25.4±1.2c
LRE-FA	54.4±0.8b	1.63±0.15b	61.3±3.1b	5.79±0.18b	36.5±1.9b
LRE-RA + LRE-	63.6±2.2a	1.98±0.16a	73.7±2.4a	6.67±0.04a	44.4±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,
LRE-RA; licorice root extract was added in irrigation water + foliar spray was done with tap water,
LRE-FA; no extracts were added in irrigation water + foliar spray was done with licorice root extract, and
LRE-RA + LR-FA; licorice root extract was added in irrigation water + foliar spray was done with licorice root extract.
CAT means catalase, **POX** means peroxidase, **APX** means ascorbate peroxidase, **SOD** means superoxide dismutase, and **GR** means glutathione reductase.

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., LRE-RA or LRE-FA) or integrative (i.e., LRE-RA + LRE-FA) 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 (RA and FA were applied with tap water). Moreover, the integrative LRE-RA + LRE-FA treatment significantly exceeded both MSE single treatments and collected the best results. It increased CAT activity by 55 and 55%, POX activity by 140 and 141%, APX activity by 99 and 98%, SOD activity by 55 and 55%, and GR activity by 115 and 113% in both 2016 and 2017 growing seasons, respectively.

Heavy metals concentrations in pepper leaves and fruits

The data in Tables 7 and 8 represent similar trends in both 2016 and 2017 growing seasons regarding the concentrations of the heavy metals; cadmium (Cd), copper (Cu), lead (Pb) and nickel (Ni) in leaves and fruits of pepper plants grown on heavy metals-contaminated saline soil. The single (i.e., LRE-RA or LRE-FA) or integrative (i.e., LRE-RA + LRE-FA) treatments significantly decreased the leaf and fruit concentrations of Cd, Cu, Pb and Ni compared to those of the controls (RA and FA were applied with tap water). Additionally, the integrative LRE-RA + LRE-FA treatment significantly exceeded both LRE single treatments collecting the best results. It decreased Cd concentration by 81 and 81% in leaves and 82 and 78% in fruits, Cu concentration by 51 and 54% in

leaves and 51 and 53% in fruits, Pb concentration by 53 and 52% in leaves and 57 and 55% in fruits, and Ni concentration by 77 and 74% in leaves and 77 and 74% in fruits in both 2016 and 2017 growing seasons, respectively.

Table 7. Effect of licorice root extract (LRE) 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
LRE-RA	4.74±0.06c	42.6±1.1c	23.2±0.6c	12.3±0.30c
LRE-FA	6.64±0.22b	50.5±1.5b	29.9±1.1b	14.4±0.35b
LRE-RA + LRE-FA	2.20±0.04d	32.5±0.8d	17.1±0.7d	4.02±0.87d
2017				
Control	11.6±0.31a	65.4±0.9a	38.0±0.8a	17.6±0.47a
LRE-RA	4.74±0.10c	41.9±0.8c	23.0±0.4c	13.0±0.51c
LRE-FA	5.66±0.11b	51.5±1.0b	30.1±0.4b	15.1±0.30b
LRE-RA + LRE-FA	2.21±0.11d	30.2±1.0d	18.1±1.0d	4.63±0.34d

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,
LRE-RA; licorice root extract was added in irrigation water + foliar spray was done with tap water,
LRE-FA; no extracts were added in irrigation water + foliar spray was done with licorice root extract, and
LRE-RA + LR-FA; licorice root extract was added in irrigation water + foliar spray was done with licorice root extract.

Table 8. Effect of licorice root extract (LRE) 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
LRE-RA	3.53±0.16c	30.0±0.5c	20.7±0.6c	10.2±0.16b
LRE-FA	5.67±0.22b	36.1±1.1b	24.2±0.7b	10.3±0.15b
LRE-RA + LRE-FA	1.54±0.06d	23.2±0.4d	11.2±0.3d	2.87±0.07c
2017				
Control	8.27±0.11a	46.7±0.9a	26.4±0.2a	12.6±0.37a
LRE-RA	3.46±0.14c	30.5±0.6c	20.6±0.3c	9.88±0.21c
LRE-FA	5.47±0.21b	36.8±1.0b	24.4±0.4b	10.8±0.30b
LRE-RA + LRE-FA	1.78±0.04d	22.0±0.6d	12.0±0.3d	3.31±0.14d

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,
LRE-RA; licorice root extract was added in irrigation water + foliar spray was done with tap water,
LRE-FA; no extracts were added in irrigation water + foliar spray was done with licorice root extract, and
LRE-RA + LR-FA; licorice root extract was added in irrigation water + foliar spray was done with licorice root 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 chlorophyll and an increase in Cd concentrations, which reflected in a severe

decrease in growth and yield of pepper plants (Table 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 [78]. 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 [74], root and shoot growth reductions [27], undesirable enzyme activation and inhibition [70]. 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 [45]. The stress causes excess generation of ROS that induces oxidative stress in plants [15]. 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 [34], 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 [63]. 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 [58, 56, 20].

This study showed a beneficial use of licorice root extract (LRE), especially the integrative LRE-RA + LRE-FA. This integrative LRE-RA + LRE-FA 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 (Tables 7 and 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 treatments including the control (free from LRE). These improvements conferred by the integrative LRE-RA + LRE-FA 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 LRE to pepper plants through their roots (LRE-RA) and leaves (LRE-FA) to support the plant antioxidant defense systems to cope with the doubled stress (salinity and heavy metals) under this study.

The analysis of LRE as shown in Table 2 indicates that this extract considers as an effective biostimulant for stressed plants. The enhanced pepper growth and yield due to the application of LRE 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 LRE that participate in strong seedling growth [22] under stress due to the improvements in the activity of antioxidant defense systems (Tables 4–6). These positive LRE effects, coming from RA, are supported by FA of LRE, conferring positive effects of extract active components, especially GAs and Se to further improve the doubled stressed plant growth, and consequently its production [55].

By looking at the most important active components of LRE, a previous study has documented salt or heavy metal stress alleviation by exogenous addition of GAs through enhancing plant growth [26], concluding that GA_3 conferred heavy metal tolerance in microalgae (*Chlorella vulgaris*) exposed to Cd and Pb. 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. Elrys and Merwad [23] have documented different important active compounds found in the LRE such as glycyrrhizin, polysaccharide, vitamins, and mevalonic acid that converts to GAs in plants. GAs may be increased in roots and leaves by LRE-RA + LRE-FA, perhaps due to enhanced activities of GAs biosynthesis enzymes such as GA20ox and GA3ox [49], contributing to stronger growth to face salt and metal stress conditions. Application of LRE maintains an appropriate level of endogenous GAs for stimulated growth in different stages. Of which GAs activate cell division and elongation, leading to an increase in leaf area along with stay-green effect by cytokinins found in LRE, leading to stimulation of photosynthetic rate.

Mineral nutrients, as an essential part of LRE, are very important for plant growth and development under both normal and stress conditions [3, 8]. It has been reported that mineral nutrients applications increased the metal tolerance capacity of plants and alleviating the metal toxicity by maintaining 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. These nutrients maintain leaves number on plants to maximize photosynthesis, elevating the sink capacity fulfilled during supply of photo-assimilates from stressed leaves [67]. Application of nutrients-containing LRE maximized the number of photosynthetic active leaves and leaves area with staying green (observed; data not shown) to longer time, maintaining chlorophylls in higher concentration (Table 4). Presence of GAs and nutrients in LRE inhibits premature leaf senescence and maintains higher leaf area, increasing the efficiency of photosynthetic machinery. In addition, Fe found in LRE 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 [79]. K^+, as an important nutrient component in LRE extract, is a major osmoprotectant to preserve higher tissue water concentration and to regulate the stomatal opening/closure controlling photosynthesis rate of plant grown under stress conditions [21]. Stomatal regulation depends on K^+ supplying in the guard cell and leaf apoplast [65]. Our results showed that the increase of chlorophyll concentration by LRE positively reflected in growth and yield that might be attributed to more assimilations correlated with nutrient elements, GAs and Se [77, 2] that found in the applied extracts 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 LRE together with stimulating the endogenous antioxidant defense systems against ROS that generate by stress [58]. Photosynthesis is coupled with transpiration rate of plants, and the transpiration inhibition is a credible and prompt measure of toxic effects of stress [69]. Application of osmoprotectants (sugars and proline)-containing LRE helps modify the water imbalance in the plant [78]. In addition, potassium-magnesium-calcium glycyrrhizin is a form in which glycyrrhizin exists in licorice roots prior to aqueous extraction [35]. 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 LRE application. It contributes to cell osmotic adjustment under stress conditions, with which plants usually accumulate more levels of proline [80] and is further accumulated by LRE 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 [13]. 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 singlet oxygen ($^1O_2$) radical or reacts directly with OH^- ions [40]. The increase in proline and other LRE components promoted the antioxidant defense systems of pepper plants (Tables 4 and 6) to avoid damages caused by salt stress [78]. Another osmoprotectant that was further increased in stressed pepper plants by LRE application, soluble sugars (Tables 4) are participated to plant osmotic adjustment [36] and can directly or indirectly modify genes expressions involved in metabolic processes, storage functions, and defense systems [37]. 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 [6]. In addition, Se was found as an important component of LRE (Table 2). It is a constituent of seleno-proteins, and it has many important functions, including energy metabolism and antioxidant protection [46]. Supplementation of Se to plants (through applied extracts) may be contributed to improvements of pepper performance (growth and yield) and antioxidant systems activity [76], and the ability to reduce ROS by increasing the antioxidant systems activity [58].

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^+ concentrations, and K^+/Na^+ ratio in the doubled stressed plants were positively modified by LRE application (Tables 5 and 7). This positive nutrient status of pepper plants mainly due to the nutrient-containing LRE (Table 2) and may be attributed to the antagonistic effects of extract nutrients with heavy metals maintaining membrane health [47]. 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 [24]. 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+}) [31]. In the present study, K^+ imbalance in cells cytosols under stress was positively modified by LRE 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 the stress [52]. The increase in nutrients concentrations (N, P, and K^+) by LRE application may be attributed to that these extracts are rich sources in mineral nutrients and hormones like GAs, auxins, and zeatin-type cytokinin that increase the metabolic processes including nutrient absorption to increase the nutrients concentrations in plant tissues [66] 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 the profuse root system, rapid growth rate, and producing reasonably high biomass [30]. Plant extract, herein (i.e., LRE), 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 (Tables 7 and 8). 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; Tables 4 and 6) and/or to the increased nutrients (Table 5) that may antagonize heavy metal entry into plants [3, 47]. Plant general faces oxidative damages when exposed to heavy metal stress [33]. The phyto-potential of plant can be assessed with the tolerance mechanism for toxic metal-induced ROS [10]. 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 [10]. In addition, glutathione (GSH), is an important component of LRE, may be involved in heavy metal resistance. It helps to reduce the effect of secondary oxidative stress resulting from the production of ROS [54], and it also constitutes the precursor of phytochelatins, which are small peptides binding to metal accumulating in vacuoles [17]. Extracts 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 LRE to overcome the ROS damages. Antioxidants found in the LRE extract may be translocated into plants through roots (by drip irrigation) and leaves (through foliar spray) to contribute to increasing 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 the superoxide ($O_2^{\bullet-}$) radical to H_2O_2 [38]. H_2O_2 is then further scavenged by CAT and APX into H_2O and O_2 [4]. 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 [57]. 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 [29]. The present study reported significant increases in the

antioxidant enzymes activity (i.e. CAT, POX, APX, SOD, GR; Table 6) by the integrative LRE-RA + LRE-FA application compared to the controls without both extracts. These increases in the activity of antioxidant enzymes as an effective defense system supported salt-stressed plants to tolerate salt stress and to reduce, significantly, the ROS damages.

Conclusion

Stress tolerance in pepper plants grown on heavy metal (Cd, Cu, Pb and Ni)-contaminated saline (EC = 7.73–7.78 dS m^{-1}) was effectively improved by the integrative application of LRE-RA + LRE-FA 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 LRE 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 LRE applied, especially as integrative addition through drip irrigation and foliar spray that reported herein as the best integrative treatment. The leverage of LRE also is reported in our study as "stay-green effect" due to the LRE active components (i.e., mineral nutrients, phytohormones, soluble sugars, and amino acids), supporting the antioxidative defense systems in plants under severe stress.

Conflict of Interest

The authors declare that there is no conflict of interest.

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