

PRIMING WITH MORINGA (*MORINGA OLEIFERA* LAM.) LEAF EXTRACT BOOSTS THE GROWTH AND PHYSIO-BIOCHEMICAL ATTRIBUTES OF LEAD-STRESSED FENUGREEK (*TRIGONELLA FOENUM-GRAECUM* L.) SEEDLINGS

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Abstract. Lead (Pb) is a potentially toxic environmental concern, affecting all forms of life on the planet. However, moringa leaf extract (MLE) attained enormous attention owing to its high phytohormones, antioxidants and essential nutrient contents. To evaluate the potential of MLE in alleviating the phytotoxicity of Pb on fenugreek seedlings, a pot experiment was conducted at the laboratory of Biological Sciences Department, Jeddah University. The uptake of Pb by fenugreek seedlings negatively affected growth rate, chlorophyll content, soluble sugars, enzymatic and non-enzymatic antioxidants, availability of the macronutrients (N, P, Ca, K), as well as K/Na ratio. Contrastingly, H₂O₂, MDA, soluble proteins and Mg content were increased. Seed priming in the aqueous MLE significantly ameliorated the ionic and osmotic deteriorations resulting from Pb uptake by fenugreek seedlings. MLE potentially retrieved seedling growth, balanced carbon and nitrogen metabolism, decreased the ROS-induced oxidative damage, in addition to sustaining ionic and osmotic homeostasis in Pb-stressed fenugreek seedlings. To sum up, the present study proved MLE as a potent eco-friendly approach to counteract Pb-phytotoxicity.

Keywords: metal pollution, *Moringa oleifera*, stress markers, antioxidant potential, ionic homeostasis

Introduction

Lead (Pb) is one of the most hazardous non-essential heavy metals in any ecosystem, it accumulates in excessive concentrations due to its non-degradable nature (Mahaffey, 1990). Lead pollution can originate from many sources like leaded gasoline, agro-fertilizers, mining processes, stoneware, boat constructing, paints, pipes manufacturing, batteries, artificial limbs, colorants, ink industry and traffic exhausts (Karri et al., 2008). Exposure of plants to lead stress results in damaging effects to their morphology, growth, biochemistry, ultrastructure, photosynthesis, productivity and quality. The deteriorations caused by lead stress are time, dose and growth-stage dependent. Several reports revealed that lead exposure had resulted in reduced germination ratio, biomass production, protein content (Hussain et al., 2013), photosynthetic activity, lamellar organization of the chloroplast (Hu et al., 2007) and yield criteria (Sobhy et al., 2019). Moreover, Pb stress triggered the increase in the level of some stress markers like membrane leakage, lipid peroxidation (MDA), H₂O₂ and OH[•] radical in wheat (*Triticum aestivum* L.) seedlings (Sobhy et al., 2019). The same authors reported that the activities of particular antioxidant enzymes (SOD, CAT and POD) were increased in wheat seedlings due to up-regulation of the decoding genes.

On the organelles level, lead toxicity had been proven through the damage of cristae, enlargement of mitochondria, distortion of endoplasmic reticulum, damaged dictyosomes and irregular dark nuclei in different plant cells (Sandalo et al., 2001).

Fenugreek (*Trigonella foenum-graecum* L.) is a self-pollinating annual forage leguminous plant (Family: *Fabaceae*) native to the Eastern Mediterranean region but is widely distributed in various parts of the world (Abdel Latef et al., 2017). The plant

possesses a long taproot, long erect cylindrical stem and trifoliate leaves with inversely ovate leaflets. After blossoming, the plant produces long pods with 10 to 20 angular-flattened seeds. Many factors, like farming practices, climatic conditions, use of herbicides and fertilizers, irrigation systems and genotypes, affect seed yield and quality of fenugreek (Pavlista and Santra, 2016). Choudhary et al. (2012) reported that number of pods/plant and number of seeds/pod in fenugreek subjected to Pb-stress were decreased in a dose-dependant manner.

Traditionally, fenugreek leaves and seeds have been used extensively in the treatment of various ailments (Basch et al., 2003). It has been used for centuries in folkloric medicine as a hypocholesterolemic, antidiabetic, antineoplastic, antitumor, antioxidative, anti-inflammatory, antiulcerogenic, antihypotriglyceridemic and antipyretic substance (Dixit et al., 2010; Xue et al., 2011). The therapeutical activity of fenugreek has been attributed to a variety of metabolites such as vitamins, amino acids, fatty acids, saponins (neogitogenin, disogenin, gitogenin, saponaretin, homorientin, neogigogenin and trigogenin) polysaccharides, fibers, fixed oils, flavonoids, and alkaloids (trigonelline and choline) (Yoshikawa et al., 1997).

The natural phyto-extracts of some algae, weeds, and plant species have been ascribed to be effective tools in ameliorating the deteriorations caused by various biotic and abiotic stressors. Saad-Allah and Nessim (2016) reported that the aqueous extract of the seaweed *Halimedaopuntia* potentially augmented cadmium toxicity in rocket plant (*Eruca sativa* Mill.) via improving the water content, photosynthesis efficiency and water-soluble osmolytes. Also, Hegazi et al. (2015) concluded that the extract of *Ascophyllum nodosum* seaweed functioned as an anti-salinity agent through the upregulation of some antioxidant enzymes level and enhancing the uptake of some essential macronutrients. In the meantime, Rady et al. (2013) affirmed that salt imposed common bean (*Phaseolus vulgaris* L.) seedlings exhibited an improved growth rate by seeds pre-soaking in the leaf extract of *Moringa oleifera*. The extract had increased the antioxidative, osmoprotective and ion uptake potentials of NaCl-stressed seedlings. Likewise, Abdel Latef et al. (2017) studied the response of salt-stressed fenugreek seedlings to the foliar application of fresh moringa leaf extract. They concluded that the interruption in the growth rate of stressed fenugreek had been resumed through the enhanced metabolic activity, minerals uptake and up-regulation of some salt tolerance genes. In the present study, we aimed at assessing the morpho-physiological, antioxidant and nutritional status responses of lead-stressed fenugreek seedlings to seed priming in the powdered moringa leaf extract.

Materials and Methods

Preparation of aqueous moringa leaf extract

The fully expanded and healthy fresh moringa (*Moringa oleifera* Lam.) leaves were collected from the streets of Jeddah city, KSA during the summer season (August 2019). The collected leaves were washed several times with tap water then once with distilled water and dried in an electrical oven at 60°C till constant weight. The dried leaves were ground using an electric mixer and sieved through a 0.2 mm sieve. The aqueous extract of moringa leaves was prepared by soaking of 50 g of the powdered leaves in 1L of the distilled water in a 2L Erlenmeyer flask. The flask was placed on a rotary shaker (180 rpm) for 24 hours then the extract was filtered through Whatman No1 filter paper to get rid of leaf tissues and the supernatant was taken and used in the

preparation of two different concentrations (2.5 and 5.0%) of the aqueous extract. The prepared extracts were placed in the fridge at 4°C until the time of use.

Growth conditions and treatments

Seeds of fenugreek (*Trigonellafoenum-graecum*L.) were obtained from the Ministry of Environment, Water and Agriculture, Mecca branch, Kingdom of Saudi Arabia (KSA) and chosen for superficial homogeneity of size and shape. The seeds were disinfected in 5% Clorox for 4 minutes with stirring then washed with distilled water. The seeds then were separated into 2 groups; the first was soaked in distilled water for 24 hours as a control and the second was primed by soaking either in 2.5 or 5.0% of aqueous moringa leaf extract (MLE) solutions for 24 hours. Thereafter, 10 seeds of every treatment were sown in plastic pots (25 cm diameter and 18 cm depth) filled with 8 kg of clay-sandy soil (2:1 w/w) and four pots were used as a replica for each treatment. Seeds were irrigated with tap water and left to grow for 5 days, before the treatment with Pb stress, under normal environmental conditions (10 hours photoperiod at 28/16°C±2 day/night and 62% relative humidity) at the laboratory of Biological Science Department, Faculty of Science, Jeddah University, KSA. The Pb stress was applied as lead acetate (100 mM Pb(CH₃COO)₂ solution), while the 5-days old seedlings were irrigated once at 70% field capacity with tap water (the sub-lethal concentration of Pb(CH₃COO)₂ (100 mM) was previously determined in a preliminary experiment). The freshly harvested 25-days old seedlings were separated into roots and shoots, washed thoroughly with tap water several times followed by deionized water, and stored at -80°C. For dry matter analysis, the plant samples were oven-dried at 60°C till constant weight. Three replicates were utilized for each treatment of all the experiments.

Growth parameters

The collected 25-days old seedlings were used to assess Pb toxicity through measuring growth criteria (lengths, fresh and dry weights of both roots and shoots).

Photosynthetic pigments

The third fully-expanded leaf in each seedling was selected to estimate chlorophyll a and b by Arnon (1949) method and carotenoids by Horvath et al. (1972) method. Concisely, 0.1 g of fresh leaves were extracted in 85% cold acetone, whereas the absorbance was measured using a UV/visible spectrophotometer and expressed as mg/g fw.

Soluble sugars and protein estimation

The total free sugars fraction in dry leaves and roots of fenugreek was estimated using the phenol-sulfuric acid method (Dubois et al., 1965) with glucose as a standard sugar. Protein fraction was assessed using Bovine serum albumin (BSA) as a standard protein by the method of Bradford (1976). The quantified sugars and proteins were expressed as mg/g dw.

Hydrogen peroxide and lipid peroxidation quantification

Hydrogen peroxide (H₂O₂) content of fresh fenugreek leaves was estimated by the method given by Velikova et al. (2000). The H₂O₂ content of leaves was calculated using the extinction coefficient (0.28 μM⁻¹cm⁻¹) and expressed as μmol/gfw. Malondialdehyde (MDA) in fresh leaf homogenates was estimated according to the method of Heath and

Packer (1968) by the aid of thiobarbituric acid (TBA) using the extinction coefficient ($155 \text{ mM}^{-1} \text{ cm}^{-1}$) and expressed as nmol/gfw .

Enzymatic activity assay

Fresh leaves (0.5 g) were homogenized in pre-chilled 0.1 M K-phosphate buffer (pH 6.8) containing 0.1 mM EDTA. Homogenates were centrifuged at 10000 rpm for 15 min and the resultant supernatant was used for enzymes assay.

The activity of CAT (catalase) was measured by the method of Havir and McHale (1987). The decline in H_2O_2 was monitored spectrophotometrically at 240 nm and the activity was calculated using the molar extinction coefficient $36 \times 10^3 \text{ mM}^{-1} \text{ m}^{-1}$ and expressed as $\mu\text{M/g fw. min}^{-1}$.

POD (peroxidase) activity as assessed in the leaf extracts using 7.2 mM guaiacol and the reddish-brown color development was monitored using a UV/vis spectrophotometer at 470 nm. POD activity was expressed as $\mu\text{M/g fw min}^{-1}$ based on the molar extinction coefficient of $26.6 \text{ mM}^{-1} \text{ cm}^{-1}$ (Kato and Shimizu, 1987).

APX (ascorbate peroxidase) activity was determined using a mixture of 0.5 mM ascorbic acid, 0.1 mM H_2O_2 , K-phosphate buffer and 1 mM EDTA-sodium salt (Nakano and Asada, 1981). The decrease in ascorbate absorbance at 290 nm and APX activity was calculated as $\mu\text{M/g fw. min}^{-1}$ using the extinction coefficient of $2.8 \text{ mM}^{-1} \text{ cm}^{-1}$.

Polyphenol oxidase (PPO) activity of leaf homogenates was determined as described by Kumar and Khan (1982) in a mixture of 100 mM K-phosphate buffer and 100 mM catechol. Sulfuric acid (2.5 N) was used to stop the reaction and the absorbance of the developed purpurogallin was measured at 495 nm. PPO activity was expressed as $\mu\text{M/g fw min}^{-1}$ using the extinction coefficient of catechol ($22.6 \text{ mM}^{-1} \text{ cm}^{-1}$).

Radical scavenging and total antioxidant capacity

The free radicals scavenging activity of the root and shoot ethanolic extracts was evaluated using the technique adopted by Hatano et al. (1988). A 0.1 ml aliquot of each extract was well vortexed with 3 ml of $36 \times 10^{-2}\%$ 1,1-diphenyl-2-picrylhydrazyl (DPPH $^\circ$) radical in methanol and kept for 30 min in dark. The bleaching in DPPH color by the extracts was monitored at 417 nm using UV/vis. spectrophotometer and the activity was expressed as a percentage.

The total antioxidant capacity (TAC) of the extracts was estimated using 3 ml of phosphomolybdate reagent (0.6 mol. $\text{L}^{-1} \text{H}_2\text{SO}_4$, 28 mmol L^{-1} sodium phosphate and 4 mmol L^{-1} ammonium molybdate) and 300 μL of the extracts. The contents were boiled at 95°C for 90 min, cooled and the absorbance was recorded at 765 nm. The TAC was reported in $\mu\text{g/g}$ dw using ascorbic acid as a standard (Ahmed et al., 2012).

Mineral ions content

The dry plant root and shoot samples were wetly digested by a mixture of 70% HNO_3 and 30% H_2O_2 (5:2 v/v). The concentrations of Ca, Mg, K, Na and P in the digested samples were quantified using an inductively coupled plasma-optical spectrophotometer (Polyscan 61E, Thermo Jarrell-Ash Corp., Franklin, MA, USA) at the Central Lab of Jeddah University. Nitrogen and phosphorus were calorimetrically estimated in the samples digest by the assay of Allen et al. (1974) using Rochelle reagent for N and molybdenum blue method for P against their standard

calibration curves. The mineral content of root and shoot samples was expressed as mg/g dw.

Statistical analysis

The experimental results were expressed as a mean of three replicates \pm the standard error (SE). The obtained data were statistically analyzed (ANOVA test) using SPSS software (V20) and the variations between means were evaluated using the LSD test at 5% level. All the experimental values were compared to the control treatment.

Results

Growth parameters

In the current study, fenugreek seedlings treated with 100 mM lead acetate exhibited evident toxicity symptoms reflected as a reduction in growth parameters as shown in Fig. 1. Compared with the control, Pb stress resulted in a significant reduction in the fresh and dry weights of both shoot and root. The presoaking of fenugreek seeds in aqueous moringa leaf extracts (MLEs) was considerably effective in increasing the growth parameters of non-stressed fenugreek seedlings, but non-significantly alleviated the growth reduction mediated by Pb; whereas the degree of alleviation was dose-dependent. Compared to Pb treatment, priming in 5% MLE was the most valuable and slightly increased the growth parameters of fenugreek seedlings.

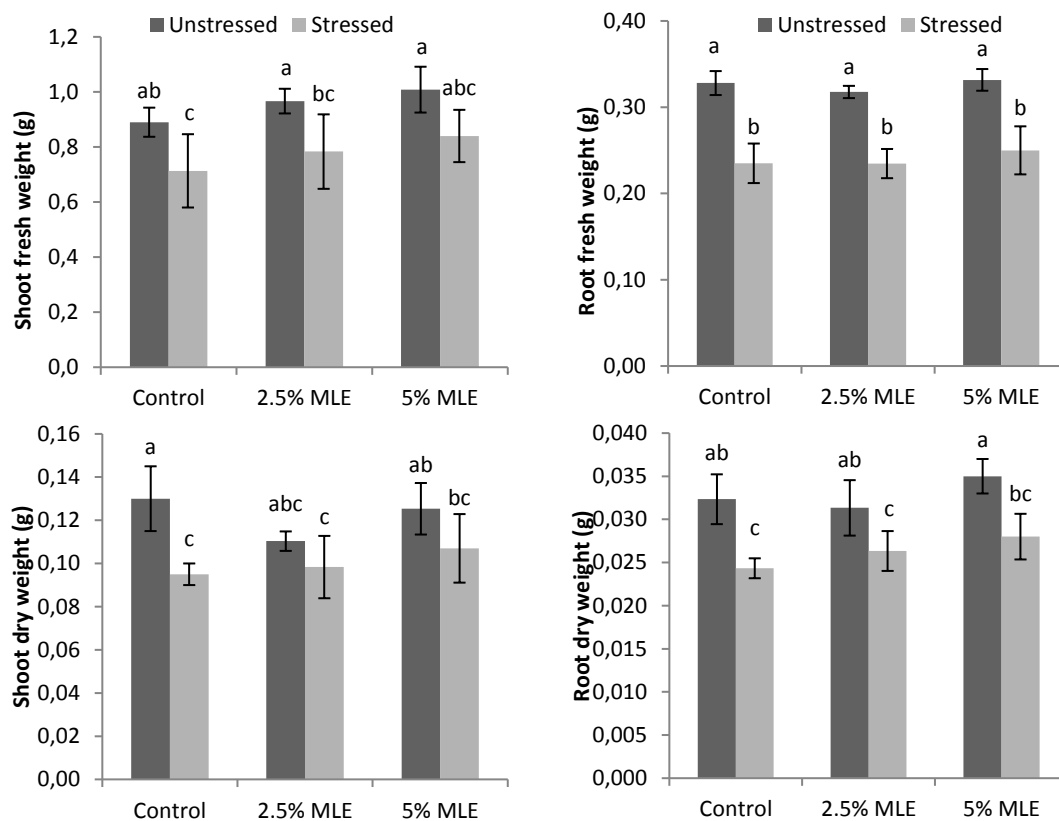


Figure 1. Growth parameters of Pb-stressed and pre-soaked in MLE (0.0, 2.5 and 5%) fenugreek seedlings. Different letters indicate significant differences at 5% level and the error bars represent SD

Photosynthetic pigments

The application of Pb was extremely destructive concerning photosynthetic pigments as observed in Fig. 2. The leaves content of Chla and Chl b were significantly declined with percentages of 26.47 and 49.50%, respectively compared to the control. Meanwhile, carotenoids content was significantly increased following lead treatment, the percentage of increase was 27.11% as compared to the control. The priming with MLE triggered an enhanced response and ameliorated Pb-stress by raising the level of chlorophyll content and lowering that of carotenoids to a significant level as compared to Pb treatment. Compared to Pb stress, 5% MLE had a more pronounced impact by increasing chlorophyll a and b contents and lowering carotenoids content.

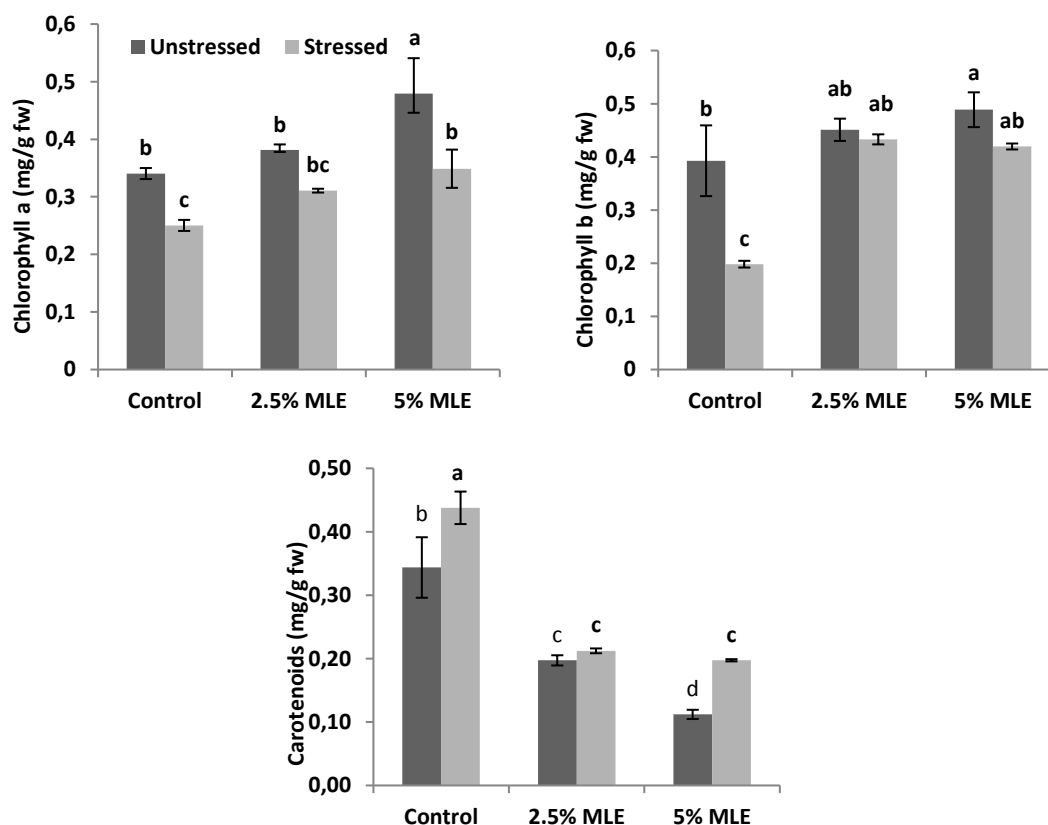


Figure 2. Photosynthetic pigments of Pb-stressed and pre-soaked in MLE (0.0, 2.5 and 5%) fenugreek seedlings. Different letters indicate significant differences at 5% level and the error bars represent SD

Soluble sugars

The total soluble sugars content of the fenugreek seedlings root and shoot was significantly affected by Pb-stress as represented in Fig. 3. The exposure to 100 mM lead acetate had lowered fenugreek root and shoot sugar content, but the effect was more pronounced on the root, as the reduction was with percentages of 43.12 and 11.90% in root and shoot, respectively, compared with the control. However, priming in MLE had declined the sugar content in fenugreek root and shoot. The most evident decline was observed at 5% MLE in the root and 2.5% MLE in the shoot. The combined

interactions of Pb and MLE non-significantly affected soluble sugar contents of both root and shoot, comparable to Pb-stress.

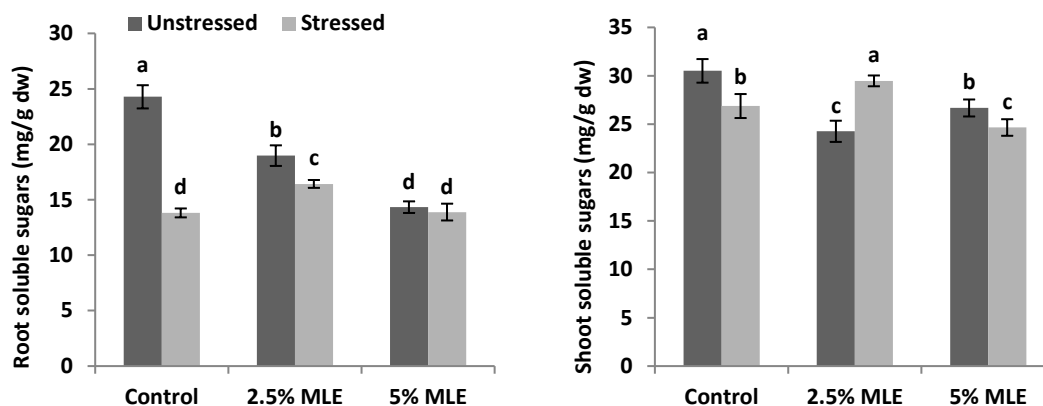


Figure 3. Soluble sugars content in roots and shoots of Pb-stressed and pre-soaked in MLE (0.0, 2.5 and 5%) fenugreek seedlings. Different letters indicate significant differences at 5% level and the error bars represent SD

Soluble proteins

Contrasting to soluble sugars, the soluble protein content of fenugreek seedlings was significantly increased as affected by lead treatment (Fig. 4). The achieved increment in protein level was 46.24 and 113.75% in root and shoot, respectively, relative to the control's content. Likewise, MLE treatments have increased the content of proteins in fenugreek seedlings compared with the control, especially 2.5% dose in roots and 5% in shoots. Meanwhile, the priming in MLEs was effective in decreasing the soluble protein content of Pb-stressed fenugreek seedlings, but the level still close to or slightly higher than that of the control.

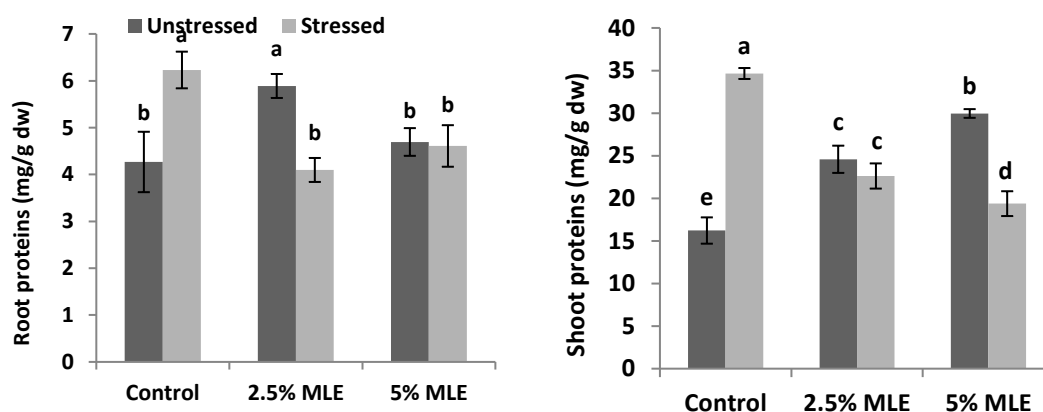


Figure 4. Soluble proteins content in roots and shoots of Pb-stressed and pre-soaked in MLE (0.0, 2.5 and 5%) fenugreek seedlings. Different letters indicate significant differences at 5% level and the error bars represent SD

Stress indices

Hydrogen peroxide (H_2O_2) and malondialdehyde (MDA) contents in the leaves of fenugreek seedlings were significantly affected by the applied dose of lead stress (Fig. 5). Irrigating fenugreek seedlings with 100 mMPb-acetate triggered a reasonable increment in the oxidative stress markers; H_2O_2 and MDA, in the leaf tissues, compared to the control. The applied dose of Pb acetate showed 43.24 and 47.90% increase in the level of H_2O_2 and MDA, respectively, relative to the control levels. Nevertheless, seed pre-soaking in MLE effectively lowered the leaf content of both stress markers; H_2O_2 and MDA, in fenugreek leaves, particularly 2.5% MLE. Likewise, the pre-treatment with MLE efficiently ameliorated the deleterious impact of Pb-stress on fenugreek seedlings.

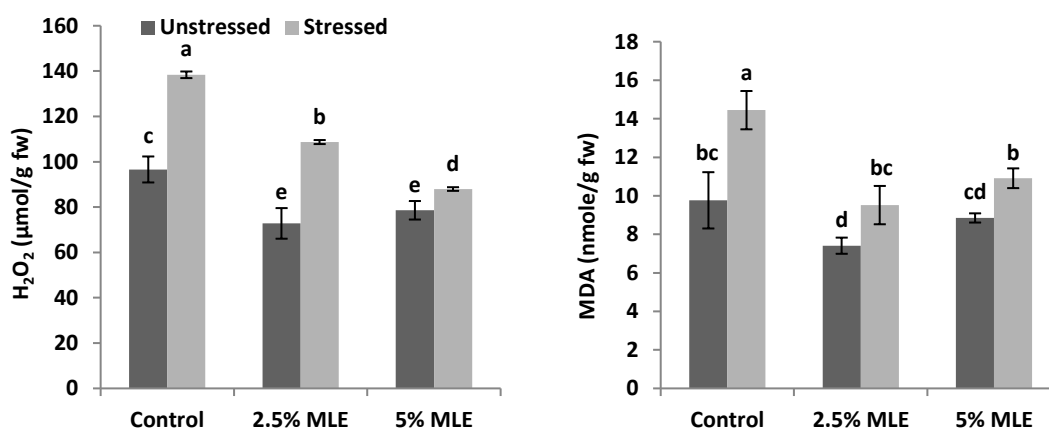


Figure 5. Stress markers (H_2O_2 and MDA) of Pb-stressed and pre-soaked in MLE (0.0, 2.5 and 5%) fenugreek seedlings. Different letters indicate significant differences at 5% level and the error bars represent SD

Enzymatic activity

The activities of catalase (CAT), peroxidase (POD), ascorbate peroxidase (APX) and polyphenol oxidase (PPO) in fenugreek leaves were significantly affected by lead and aqueous moringa leaf extract (MLE) treatments (Fig. 6). As for CAT, the exposure of fenugreek seedlings to 100 mMPb resulted in 47.32% decline in CAT activity, compared with the control. Additionally, both MLE treatments caused a sharp decline in CAT activity (53.36 and 53.68% with 2.5 and 5% MLE, respectively). The combined interactions between Pb and MLE showed the least CAT activity throughout the experiment.

Nonetheless, POD activity showed a 20.84% rise following Pb exposure, but non-significantly affected by both MLE treatments, either as single treatments or combined with Pb stress, compared with the untreated control. Likewise, Pb stress significantly affected APX activity, as it caused a 31.59% increase compared to the control. Both, 2.5 and 5.0% MLE treatments non-significantly diminished APX activity relative to the control activity, 5.95 and 13.69%, respectively. Combined interaction of Pb and MLE was concentration dependant. The low dose (2.5%) slightly declined APX activity, but the higher dose (5.0%) reasonably increased its activity, compared to their stress counterparts.

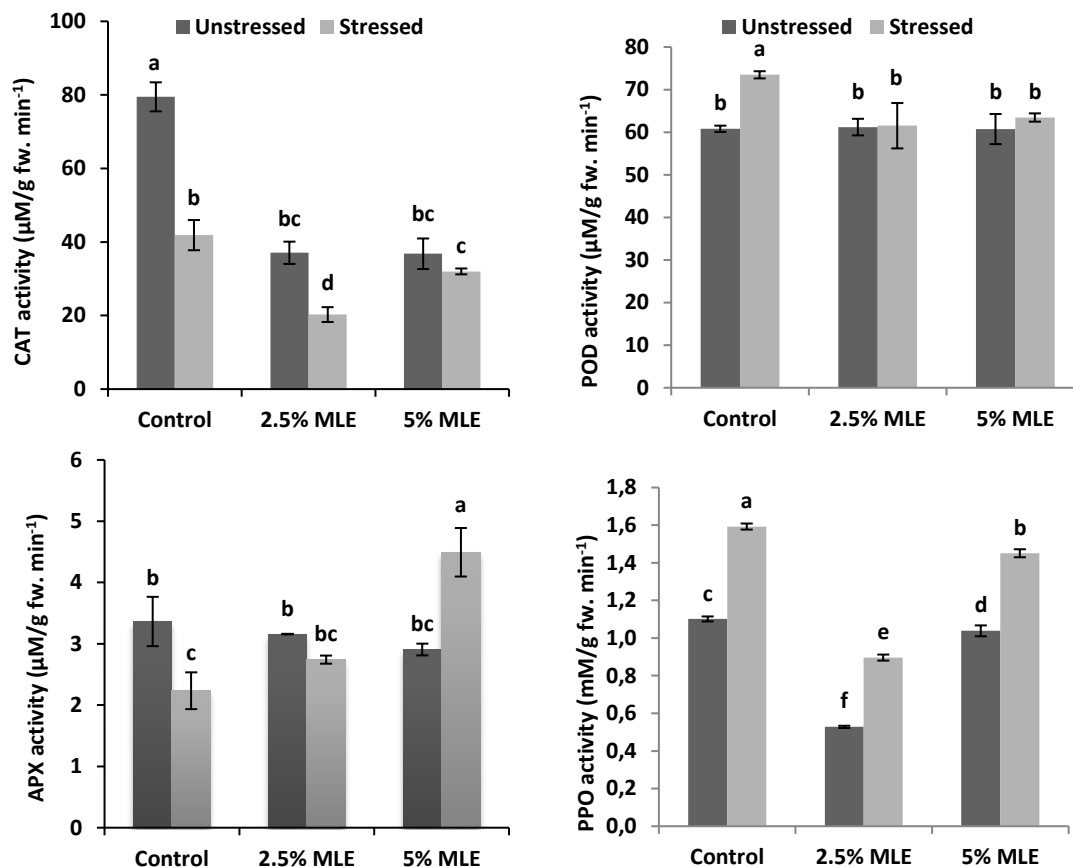


Figure 6. Enzymatic activities (CAT, POD, APX and PPO) of Pb-stressed and pre-soaked in MLE (0.0, 2.5 and 5%) fenugreek seedlings. Different letters indicate significant differences at 5% level and the error bars represent SD

Like POD, PPO activity was markedly induced (44.54%) by Pb treatment. However, both MLE treatments; 2.5 and 5.0%, showed a pronounced decrease in PPO activity in comparison with the untreated control, particularly the low dose (49.09 and 5.45%, respectively). Combined treatments of Pb and MLE significantly enhanced PPO activity, compared to single MLE treatments, but the activity still lower than that of Pb sole treatment.

Free radical scavenging activity

The free radical (DPPH) scavenging activity of fenugreek root and shoot ethanolic extracts was significantly affected by Pb and MLE treatments (Fig. 7). Treatment of fenugreek seedlings with 100 mMPb-acetate non-significantly lowered the DPPH activity of the root and the shoot extracts (3.79 and 11.33%, respectively). However, the effect of MLE on DPPH activity was dose-dependent. Priming in 2.5% MLE significantly increased the DPPH activity in both root and shoot of fenugreek extracts (33.67 and 18.83%, respectively), but priming in 5.0% MLE resulted in a 16.15 and 19.26% decrease in the DPPH activity of root and shoot extracts, respectively.

The interactive combinations of MLE and Pb treatments decreased the scavenging activity of fenugreek ethanolic root and shoot extracts, except for the mutual treatment with both Pb-acetate and 5.0% MLE in case of the shoot, this treatment remarkably

decreased the DPPH activity of the ethanolic extract, compared to the sole Pb treatment. All in all, the DPPH scavenging activity of fenugreek shoot extracts was higher than those of the root under all treatments.

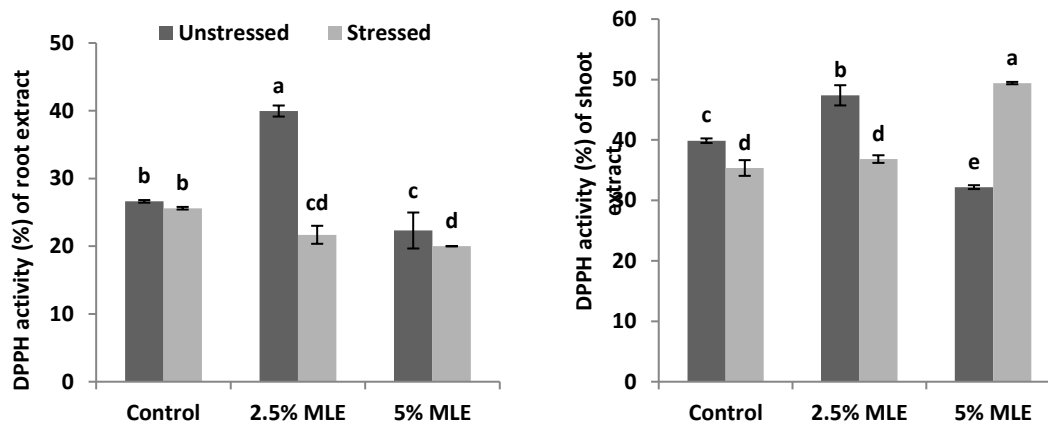


Figure 7. DPPH free radical scavenging activity of root and shoot ethanolic extracts of Pb-stressed and pre-soaked in MLE (0.0, 2.5 and 5%) fenugreek seedlings. Different letters indicate significant differences at 5% level and the error bars represent SD

Total antioxidant capacity

The phosphomolybdate assay is used as a criterion for total antioxidant capacity (TAC) in fenugreek root and shoot ethanolic extracts (Fig. 8). The TAC of root extract was not affected by Pb treatment, however, that of the shoot extract was slightly decreased, compared to the non-stressed counterparts. The TAC of the root extract was significantly raised by priming in 2.5% MLE, however priming in 5.0% MLE did not affect the TAC, compared to the control treatment. As for shoot ethanolic extract, 2.5% MLE did not affect TAC but 5.0% MLE addressed a slight decrease in TAC, comparable to the control treatment.

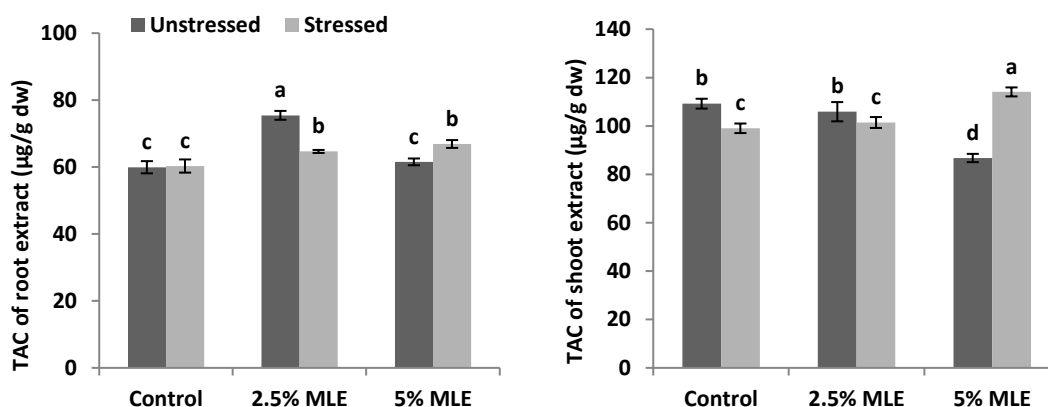


Figure 8. Total antioxidant capacity (TAC) of root and shoot ethanolic extracts of Pb-stressed and pre-soaked in MLE (0.0, 2.5 and 5%) fenugreek seedlings. Different letters indicate significant differences at 5% level and the error bars represent SD

The interactive treatments of Pb-acetate and MLE were concentration dependant. The low concentration of MLE (2.5%) decreased the TAC, but the high concentration (5.0%) increased it in the root and the shoot extracts, relative to the individual lead-exposed treatments.

Elemental content of fenugreek root

The contents of N, P, Ca, Mg, K, Na and Pb, as well as K/Na ratio in the roots of fenugreek grown under Pb-stress and pre-soaked in MLE were determined and tabulated as mg g⁻¹dw (Table 1). Treatment with Pb-acetate resulted in a pronounced accumulation of Pb ions in the roots of stressed-untreated fenugreek seedlings as compared with the control. Comparable with Pb treatment, priming with 2.5 and 5.0% MLE distinctly declined the Pb content of fenugreek. Likewise, the levels of Mg and Na were increased as affected by lead stress, but the priming with MLE lowered their accumulation to values relatively near to the control. However, N, P, and K contents, as well as the ratio of K/Na, were decreased by the exposure of fenugreek to Pb stress, but Ca content relatively remained unaffected by Pb treatment. Fenugreek priming with MLE significantly increased the root content of N, P, and K ions of Pb-stressed fenugreek, supporting improved ionic homeostasis and enhanced root growth.

Table 1. Ion concentrations (N, P, Ca, Mg, K, Na, and Pb) in the root of Pb-stressed fenugreek seedlings pre-treated with 2.5 and 5.0% MLE. Different letters in the same column represent significant variations at 0.05 level

Treatments	Mineral ions (mg g ⁻¹ dw)							
	N	P	Ca	Mg	K	Na	K/Na	Pb
Control	2.81±0.03 ^a	0.31±0.02 ^b	0.35±0.00 ^b	0.49±0.00 ^c	1.64±0.01 ^b	0.62±0.02 ^b	2.63±0.10 ^{bc}	0.00
Pb	1.71±0.06 ^c	0.22±0.01 ^c	0.34±0.00 ^c	0.66±0.00 ^c	1.16±0.06 ^d	0.71±0.02 ^a	1.64±0.12 ^e	1.25
2.5% MLE	2.78±0.05 ^a	0.31±0.01 ^b	0.35±0.00 ^b	0.71±0.01 ^a	1.49±0.02 ^c	0.60±0.00 ^b	2.48±0.03 ^c	0.00
5.0% MLE	2.73±0.03 ^a	0.34±0.06 ^{ab}	0.30±0.00 ^e	0.68±0.01 ^b	1.64±0.04 ^b	0.60±0.00 ^b	2.73±0.05 ^b	0.00
Pb+2.5% MLE	2.64±0.07 ^b	0.38±0.07 ^a	0.33±0.00 ^d	0.55±0.00 ^d	1.16±0.06 ^d	0.62±0.00 ^b	1.87±0.09 ^d	0.34
Pb+5.0% MLE	2.64±0.02 ^b	0.39±0.02 ^a	0.36±0.00 ^a	0.70±0.01 ^a	2.00±0.01 ^a	0.62±0.01 ^b	3.25±0.01 ^a	0.18
	Statistics							
F	230.47	8.60	361.64	266.16	146.31	32.70	117.93	18872.6
P	0.0000 ***	0.0012 **	0.0000 ***	0.0000 ***	0.0000 ***	0.0003 ***	0.0000 ***	0.0000 ***
LSD at 0.05	0.0847	0.0657	0.0042	0.0193	0.0921	0.0249	0.1884	0.0122

*** = very highly significant, ** = highly significant, * = significant at 0.05 level

Elemental content of fenugreek shoot

The contents of N, P, Ca, Mg, K, Na and Pb, in addition to K/Na ratio in the shoots of fenugreek grown under Pb-stress and presoaked in MLE were estimated and represented as mg g⁻¹dw (Table 2). Treatment with Pb-acetate resulted in an evident uptake of Pb ions into the fenugreek shoot system as compared with the control. Comparable with Pb-stress, priming with 2.5 and 5.0% MLE distinctly declined Pb content (57.14 and 71.42%, respectively) in fenugreek shoot. Similarly, Na level increased as affected by Pb-stress, nevertheless, pre-soaking in MLE slightly decreased Na uptake into fenugreek shoot. On the other hand, other minerals (N, P, Ca, K, and K/Na ratio) were observably decreased by fenugreek exposure to Pb-stress, but Mg content relatively increased as

affected by Pb treatment. Fenugreek priming with MLE significantly increased the shoot content of N, P, Ca, Mg, and K ions of Pb-stressed fenugreek, contributing to ionic homeostasis in the stressed fenugreek seedlings.

Table 2. Ion concentrations (N, P, Ca, Mg, K, Na, and Pb) in the shoot of Pb-stressed fenugreek seedlings pre-treated with 2.5 and 5.0% MLE. Different letters in the same column represent significant variations at 0.05 level

Treatments	Mineral ions (mg g ⁻¹ dw)							
	N	P	Ca	Mg	K	Na	K/Na	Pb
Control	2.47±0.11 ^{bc}	0.32±0.02 ^{cd}	1.05±0.00 ^b	0.34±0.01 ^d	2.05±0.08 ^b	0.56±0.02 ^c	3.63±0.26 ^b	0.00
Pb	1.68±0.01 ^e	0.24±0.02 ^d	0.92±0.01 ^d	0.42±0.00 ^c	1.67±0.03 ^c	0.63±0.00 ^a	2.65±0.05 ^c	0.28
2.5% MLE	2.61±0.12 ^{ab}	0.49±0.04 ^b	1.04±0.00 ^b	0.60±0.01 ^b	2.49±0.01 ^a	0.60±0.02 ^{ab}	4.15±0.13 ^a	0.00
5.0% MLE	2.71±0.03 ^a	0.88±0.16 ^a	1.18±0.03 ^a	0.67±0.01 ^a	2.40±0.01 ^a	0.59±0.02 ^{abc}	4.04±0.12 ^a	0.00
Pb+2.5% MLE	2.35±0.12 ^c	0.54±0.06 ^b	0.93±0.00 ^{cd}	0.41±0.01 ^c	2.01±0.01 ^b	0.62±0.01 ^a	3.27±0.04 ^b	0.12
Pb+5.0% MLE	2.08±0.06 ^d	0.42±0.04 ^{bc}	0.96±0.00 ^c	0.32±0.04 ^d	1.94±0.09 ^b	0.58±0.01 ^{bc}	3.34±0.24 ^b	0.08
	Statistics							
F	59.89	27.47	90.22	126.96	70.83	5.34	23.03	40.75
P	0.0000 ***	0.0000 ***	0.0000 ***	0.0000 ***	0.0000 ***	0.0325 *	0.0008 ***	0.0000 ***
LSD at 0.05	0.1518	0.1307	0.0355	0.0438	0.1253	0.0359	0.3974	0.0299

*** = very highly significant, * = significant at 0.05 level

Discussion

Globally, plants are endangered by the increased human, industrial and agricultural activities, particularly after the industrial revolution in the past century. One of the most challenges faced by plants is heavy metals pollution. Lead (Pb) was reported as a potential heavy metal, neither possess any role in cellular metabolism nor essential for plant growth and development (Nas and Ali, 2018), although it is simply uptaken and accumulated within various plant parts. The declined fenugreek growth by lead exposure in the current study could be ascribed to the extensive phytotoxicity represented in the distortion of cellular structures, ionic imbalance, reduced chlorophyll synthesis, induction of ROS over-accumulation and hormonal imbalance (Kumar et al., 2012). As one of the heavy metals, Pb impairs the hydrolysis and the translocation of carbohydrates leading to reduced plant growth (Kuriakose and Prasad, 2008).

On the other hand, the study results demonstrated that moringa leaf extract (MLE) pretreatment significantly improved the growth rate of Pb-stressed fenugreek seedlings. These results are in agreement with those of Latif and Mohamed (2016) in salt-stressed common bean and Abdel-Latef et al. (2017) in salt-stressed fenugreek. These results affirmed that MLE could be considered growth-promoting due to its high content of growth-regulating substances (zeatin), antioxidants (ascorbate, phenols and carotenoids) and essential nutrients (Yasmeen et al., 2013). Those authors concluded that MLE induces the endogenous hormonal level causing improved plant growth in the presence and/or absence of any other stressful factor. Additionally, Taiz and Zeiger (2010) reported that MLE is an excellent source of cytokinins which promote many physiological processes in the growing plants as chlorophyll biosynthesis, cell division and cell elongation.

The induced reduction in chlorophyll content by Pb is considered as a common consequence of ROS mediated chlorosis due to the impairment of chlorophyll biosynthesis and induction of chlorophyll degradation (Desoky et al., 2018). The study results showed a significant reduction in chlorophyll (a+b) content in Pb-stressed fenugreek leaves. Furthermore, chlorophyll b was reported to be more sensitive to lead stress than chlorophyll a. The powerful inhibition in the activity of α -amino levulinic acid dehydrogenase; a key enzyme in chlorophyll biosynthesis, by lead ions was reported to be the main cause of chlorophyll decline as a result of lead exposure (Prasad, 1996). Other reports showed that lead restrains chlorophyll biosynthesis by decreasing the uptake of some essential ions like Mg and Fe (Nas and Ali, 2018). Lead destroys the photosynthetic apparatus because it has high affinity towards protein nitrogen and sulfur ligands (Ahmed and Tajmir-Riahi, 1993). Sofy et al. (2020) reported that lead stress reduced the total chlorophyll content and disrupted the chloroplast ultrastructure of maize leaves. They attributed that to the increased levels of lipid peroxidation and membrane leakage, in addition to the interaction of lead with-SH group in chlorophyll biosynthetic enzymes.

Priming of fenugreek seeds in MLE provoked a protective mechanism for the growing seedlings against lead stress. This was apparent during this study through the improved chlorophyll content of lead-stressed fenugreek seedlings. Yasmeen et al. (2013) also previously reported that MLE improved the total chlorophyll content of wheat plants subjected to salt stress. Moreover, Latif and Mohamed (2016) reported that the foliar spray of common bean plants with MLE significantly improved the chlorophyll content of salt-stressed plants. They attributed this effect to the high content of macronutrients in MLE such as magnesium, which is a main constituent of chlorophyll. Other reports showed that the high content of cytokinins, particularly zeatin, in MLE prevent early senescence, producing large leaf area with high chlorophyll content (Elzaawely et al., 2017) through upregulating cytokinin independent isopentenyl transferase, which is a key enzyme in the biosynthetic pathway of chlorophyll.

Carotenoids, as main antioxidant molecules, were significantly boosted by lead stress. Similar results were obtained by Lim et al. (2012) in buckwheat (*Fagopyrum esculentum* Moench.) subjected to salt stress, they attributed this increase to the induction of the mevalonate pathway to induce the biosynthesis of abscisic acid (ABA) to improve the plant tolerance to salt stress. Also, Zaid et al. (2020) reported that carotenoids content highly increased by the exposure of menthol plants (*Mentha piperita* L.) to cadmium stress. The excessive accumulation of carotenoids under abiotic stress conditions was attributed to their capability to scavenge ROS, in addition to providing photoprotection against photooxidation of photosynthetic reaction centers (Gururani et al., 2015). Moreover, carotenoids were reported to offer protection to thylakoid membrane lipids, quenching singlet oxygen and chlorophyll triplet excited state (Li et al., 2012). Consequently, the increased carotenoids provide protection to the photoassimilating apparatus against Pb-stress.

Pre-treatment of fenugreek with MLE significantly reduced the level of carotenoids in the leaves of Pb-stressed fenugreek seedlings. Our results are in harmony with those obtained by Abd El-Mageed et al. (2017) in squash. The declined level of carotenoids in fenugreek subjected to Pb-stress might be due to the accompanying antioxidant properties in the aqueous MLE, as it has been reported to be a rich source of natural antioxidants such as flavonoids, ascorbic acid, carotenoids and phenolics (Siddhuraju

and Becker, 2003). These antioxidant molecules could offer protection to the stressed fenugreek seedlings, allowing it to harness the mevalonate pathway in the production of other essential molecules sustaining plant growth under stress conditions.

The results of this study revealed that Pb-stress down-regulated the accumulation of soluble sugars in the root and the shoot of fenugreek seedlings. This reduction was ascribed to the loss of chlorophyll, inhibition of photosynthetic enzymes (like RUBP carboxylase/ oxygenase) and reduced leaf area in stressed plants (Pandey et al., 2001), in addition to the increased respiration rate to adapt with the high energy demand (John et al., 2008).

The single treatment with MLE resulted in decreasing the soluble sugar content of Pb-stressed fenugreek seedlings, but the combined interaction of Pb and MLE slightly affected their content, compared to Pb treatment. Free soluble sugars function as anti-stress osmoregulators and their decrease in response to priming with MLE could be attributed to the fact that this extract contains various osmoregulators, like proline and soluble sugars (Abd El-Mageed et al., 2017), which improve the vigor, cellular turgidity, and water use efficiency of stressed plants. Accordingly, these plants direct the photosynthates into the production of other imperative compounds like structural sugars and essential secondary metabolites.

In comparison with the control, soluble proteins content of fenugreek seedlings root and shoot was significantly increased in response to Pb and MLE treatments. The increased protein accumulation in response to lead toxicity has been previously reported in wheat (Lamhamdi et al., 2011) and grass pea (*Lathyrus sativus* L.) (Brunet et al., 2009). Such accumulation of proteins may protect plants against Pb-stress through sustaining cellular redox like ascorbate, glutathione and phytochelatins make (Jiang and Liu, 2010). Lamhamdi et al. (2011) postulated that increased protein accumulation under Pb-stress could be a direct consequence of stimulating specific stress proteins. Increasing of total soluble protein content in stressed plants by using MLE has been previously approved in snap bean (*Phaseolus vulgaris* L.) (Elzaawely et al., 2017). Those authors accredited the increase in protein to the increased nitrogen concentration due to MLE treatment. Moreover, the increased protein biosynthesis could be explained by the availability of antioxidant compounds in the extract, providing protection to the metabolic machinery involved in protein biosynthetic pathway.

The current investigation revealed a prominent increment in lipid peroxidation (MDA) and H₂O₂ accumulation in the leaves of fenugreek seedlings exposed to Pb-stress, substantiating Pb-induced oxidative damage in fenugreek seedlings. Wang et al. (2012) found that eelgrass (*Vallisneria spiralis* L.) showed an increased production of MDA and H₂O₂ as a result of Pb treatment. They explained this effect by the deteriorations caused by the excessive generation of ROS through the frequent creation of fatty aldehydes and short-chained alkanes caused by Pb ions. Likewise, Ahamed and Siddiqui (2007) confirmed ROS generation due to lead toxicity, causing oxidant/antioxidant-balance disturbances, and lipid metabolism alternation. However, MLE-treated fenugreek plants counteracted oxidative damage through the decreased generation of MDA and the accumulation of H₂O₂. This could be caused by the high content of antioxidant compounds, vitamins and cytokinins in MLE, in addition to the induction of antioxidants, like ascorbate, phenols and anthocyanins, in the stressed plant tissues (Batool et al., 2016).

Lead is well-known to be a redox inert-metal, causing excessive ROS generation via disrupting cellular oxidant/antioxidant balance (Zulfiqar et al., 2019). To meet oxidative injury, plant have developed several enzymatic and non-enzymatic molecules. In the

present investigation, catalase (CAT) and ascorbate peroxidase (APX) activities decreased in fenugreek leaves by receiving 100 mM Pb-acetate. This might be a consequence of the severe oxidative damage imposed by Pb (Erdei et al., 2002). Verma and Dubey (2003) reported that the Pb-induced decline in CAT and APX activities arise from ROS-mediated inactivation of these enzymes, enzymes-synthesis decline, or enzyme subunits assembly modification. On the contrast, peroxidase (POD) and polyphenol oxidase (PPO) activities were raised following Pb-treatment. The probable explanation of the increased POD activity is the dependence of fenugreek in eliminating the rapidly accumulated H₂O₂ on this enzyme, to compensate for the diminished CAT activity. Similar results were obtained by Sobhy et al. (2019) in wheat and Hou et al. (2019) in bamboo grass (*Pogonatherum crinitum* Thunb.). PPO has been established as a key enzyme in the photosynthetic apparatus, and it can provide an evidence for heavy metals accumulation in various plant species (Lavid and Tel-Or, 2001). The exposure of plants to Pb-stress has been correlated with the increased activity of PPO, due to its crucial role in respiration, oxidation-reduction reactions, and synthesis of phenol-containing molecules like lignin (El-Beltagi et al., 2016). The declined activity of antioxidant enzymes, except for POD which remained unchanged, as consequence of pre-treating fenugreek with MLE pull the awareness to the ability of this extract, owing to its elevated antioxidative capacity, to eliminate the generated ROS, so there is no need for the upregulation of enzymatic antioxidant machinery, and the plant could sustain its normal growth based on the high content of antioxidants in MLE (Yasmeen et al., 2013).

Under normal growth conditions, plants produce specific secondary metabolites, some of which can scavenge ROS. However, the scavenging potential of these metabolites has been reported to decrease under stress conditions (Ahmad et al., 2016). In the current study, Pb-stress resulted in decreased radical scavenging (DPPH) activity and total antioxidant capacity (TAC) in fenugreek roots and shoots. Tripathi et al. (2016) attributed that the increased Pb-stress mediated accumulation of H₂O₂ and MDA is the main cause of the antioxidant potential decreasing in the stressed plants. MLE presoaked fenugreek seedlings showed a mitigating response to Pb-stress in terms of DPPH and TAC at the low concentration (2.5%), but the higher concentration (5.0%) non-significantly affected the non-enzymatic antioxidant status of fenugreek. Yasmeen et al. (2013) stated that the high content of cytokinins in MLE was behind its effectiveness in ameliorating environmental stresses via quenching ROS. Moreover, the reason may be due to the reduced absorption and transfer of lead to fenugreek tissues.

Pb-stress was demonstrated to increase the uptake of Pb and Na ions, disturbing the uptake and mobility of essential minerals (N, P, Ca and K), reducing K/Na ratio and interrupting ionic and osmotic homeostasis. Transport of Pb to the plant cells occurs through the cation channels on plasma membranes, especially Ca channels, causing other nutrients deficiency, hindering water uptake and deterioration of membrane integrity (Lamhamdi et al., 2011). Therefore, the decreased content of essential nutrients could be considered as a consequence of intensive membrane deteriorations as a direct effect of Pb ions or the generated ROS. The decreased level of essential macronutrients after Pb-exposure results in the decreased growth rate of fenugreek seedlings, which may be correlated with reduced cell division and elongation, as well as metabolic redox imbalances.

The high content of essential nutrients in MLE promoted high ionic homeostasis in tissues leading to higher levels of essential macronutrients (N, P, K and C) supporting the normal growth of Pb-stressed fenugreek. The balanced ionic status of MLE-treated plants

promoted fenugreek seedlings to sustain membrane integrity, ionic homeostasis, water use efficiency and antioxidant capacity, consequently improved growth. Several authors adjudged enhanced ionic status of stressed plants following MLE treatment (Yasmeen et al., 2013; Abdel-Latef et al., 2017).

Conclusion

Being a heavy metal, lead negatively affected growth rate, photosynthetic pigments, nitrogen metabolism, antioxidant status and ionic homeostasis in fenugreek seedlings. Seed priming with moringa leaf extract (MLE) is a cost-effective and eco-friendly approach that could be used to improve Pb-resistance in fenugreek by modulating nitrogen metabolism, antioxidative machinery, ionic homeostasis with decreased Pb and Na concentrations, thereby promoting enhanced growth rate under stress conditions. However, further studies are recommended to elucidate the phytochemical constituents of MLE and the mode of action of this extract in modulating metal-stressed plants growth.

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ELECTRONIC APPENDIX

This manuscript has 13 electronic appendices with basic data.