INTERNATIONAL JOURNAL OF AGRICULTURE & BIOLOGY
ISSN Print: 1560–8530; ISSN Online: 1814–9596
19–1690/2020/24–3–461–472
DOI: 10.17957/IJAB/15.1460
http://www.fspublishers.org

Full Length Article

Compared to Traditional Extract, *Moringa oleifera* Leaf Extract Nanoparticles Effectively Boost the Performances and Antioxidant Defense Systems in Cadmium-Stressed *Phaseolus vulgaris* Plants

Saad M. Howladar
University of Jeddah, College of Science, Department of Biology, Jeddah, Saudi Arabia
*For correspondence: smhowladar@uj.edu.sa
Received 04 November 2019; Accepted 24 March 2020; Published 11 July 2020

Abstract

Phaseolus vulgaris (L.) is cadmium (Cd^{2+})-sensitive crop that needs an exogenous support to grow well under Cd^{2+} stress. Therefore, it was grown in the presence of 1 m*M* Cd^{2+} beginning from 15 days after sowing (DAS) and was foliar-sprayed with *Moringa oleifera* leaf extract nanoparticles (n-MLE) in comparison to traditional extract (t-MLE) at 20 and 30 DAS. Plants were sampled for growth, physio-biochemical, antioxidant analysis, and yield. The optimized levels of 2 and 6% of n-MLE and t-MLE, respectively from the preliminary study were used. Growth and yield attributes, levels of leaf chlorophylls and carotenoids, plant water use efficiency (WUE), photosynthesis efficiency in terms of PI and Fv/Fm, cellular relative water content (RWC) and cellular membrane stability (MSI) were significantly decreased. In contrast, Cd^{2+} content in different plant parts, ion leakage (EL), peroxidation of lipids (MDA content), content of hydrogen peroxide (H_2O_2), free proline, soluble sugars, α-tocopherol (αTOC), glutathione (GSH), ascorbic acid (AsA), and activity of glutathione peroxidase (GPX), ascorbate peroxidase (APX), catalase (CAT), and superoxide dismutase (SOD) were markedly elevated with exposing plants to Cd^{2+} stress compared with the corresponding control. However, foliar spray with n-MLE or t-MLE considerably detoxified the Cd^{2+} stress effects and significantly improved the abovementioned parameters by significantly increasing growth and yield attributes, photosynthetic pigment levels, Fv/Fm, PI, WUE, RWC, and MSI, and further elevating the levels of αTOC, free proline, soluble sugars, AsA, GSH, and the activities of SOD, CAT, APX, GPX. In contrast, Cd^{2+}, EL, MDA and H_2O_2 contents were markedly reduced compared to the corresponding controls. In comparison to t-MLE, n-MLE was more effective treatment that is recommended to use for ameliorating the harmful effects of Cd^{2+} stress up to the level of 1 m*M*.

Keywords: Plant extracts in nanotechnology; Snap bean; Heavy metal stress; Growth and yield; Antioxidants

Introduction

Fabaceae vegetable crops, including *Phaseolus vulgaris* (L.), are important legume food. *Phaseolus vulgaris* plant produces significant seed amount rich in protein for human nutrition (Rady *et al.* 2013). It is considered as a cadmium (Cd^{2+})-sensitive crop, which suffers from yield loss of approximately 42–54% when grown under Cd^{2+} concentration of 0.5–1.0 m*M*, respectively (Rady 2011; Semida *et al*. 2015).

Increasing the pollution of plant environment with heavy metals is causing mainly by several reasons such as the industrial and urban activities, and the excessive soil and plant applications of pesticides, chemical fertilizers, animal manures, and sewage sludge, generating a serious global problem for agricultural sector that negatively reflect in food production (Daş *et al*. 2016; Zhu *et al*. 2017, 2018). Cadmium (Cd^{2+}) is the leading toxic metal for human health and plant growth and productivity because of its high mobility and availability (Semida *et al*. 2015; Zhu *et al*. 2018). Exposure of plant to Cd^{2+} inhibits its growth and productivity through the impairing effects on photosynthetic pigments, photosynthetic efficiency, water relations, cell membranes, and the enzymatic and non-enzymatic antioxidant (the defense system components), and induction of oxidative stress and metabolic imbalances due to the stimulation of reactive oxygen species (ROS) overproduction (Liu *et al*. 2015; Semida *et al*. 2015; Zhu *et al*. 2018). Nonetheless, plants have adopted and/or developed a number of adaptive mechanisms against Cd^{2+} stress such as cellular exclusion, sequestration and chelation of Cd^{2+}, as well as osmotic modification and metabolic use, development, and production of antioxidant system, etc. (Zhang *et al.* 2015; Kushwaha *et al*. 2016; Rahman *et al*. 2017; Zhu *et al*. 2018). These adaptive mechanisms have improved to a certain extent, but not to prevent the harmful effects of cadmium on plant performance.

To cite this paper: Howladar SM (2020). Compared to traditional extract, *Moringa oleifera* leaf extract nanoparticles effectively boost the performances and antioxidant defense systems in cadmium-stressed *Phaseolus vulgaris* plants. *Intl J Agric Biol* 24:461–472

To improve cadmium tolerance in *Phaseolus vulgaris* plants, different antioxidants (Rady 2011; Rady and Hemida 2015; Alzahrany *et al*. 2018; Semida *et al.* 2015, 2018) and/or natural plant extracts (biostimulants) rich in antioxidants, nutrients, and phytohormones such as moringa (*Moringa oleifera*) and licorice (*Glycyrrhiza glabra*) extracts (Howladar 2014; Desoky *et al*. 2019) can be tested to support plant defense mechanisms. To our knowledge, the Moringa leaf extract (MLE) has not been used in nanoparticles before. Besides, the traditional MLE is rarely reported to increase plant (*Phaseolus vulgaris*) tolerance to Cd^{2+} stress through the improvement of water status of plant tissues, photosynthetic chlorophylls and carotenoids, cell membrane stability, different (enzymatic and non-enzymatic) antioxidants, and the reduction in ion leakage, lipid peroxidation, and Cd^{2+} ion content in different plant parts, positively reflecting in plant growth and yield (Howladar 2014; Bulgari *et al*. 2019; Desoky *et al*. 2019). The positive effects of MLE against Cd^{2+} stress are attributed to that moringa leaf is a substantial source of osmoprotectants, antioxidants, nutrients, and phytohormones (Howladar 2014). Besides its antioxidant properties (Siddhuraju and Becker 2003), MLE is rich in zeatin-type cytokinin (Makkar *et al.* 2007), which is the major part of hormones found in MLE (Rady *et al*. 2013).

In contrast to chemical fertilizers, growth stimulants derived from living resources such as seaweeds are biodegradable, nonpolluting, nontoxic, and non-hazardous substances to soil ecosystem, humans and animals (Dhargalkar and Pereira 2005; Ambika and Sujatha 2017). As such, we have followed this method with moringa extract.

To our knowledge, there are infrequent works that discussed the role of MLE in increasing the tolerance to Cd^{2+} stress in plants such as *Phaseolus vulgaris* (Howladar 2014). Also, no reports are conducted using MLE in nano-sized particles to examine its potentiality to increase plant tolerance to Cd^{2+} stress. Therefore, the present study aims mainly at assessing the potential impacts of exogenous treatment of nano-size MLE (n-MLE) in comparison with traditional MLE (t-MLE) on potential changes in growth and production, physiological and biochemical traits, and antioxidant defense system components of *Phaseolus vulgaris* plants exposed to 1 m*M* Cd^{2+} stress. The hypothesis examined, herein, is that exogenous treatment of n-MLE will exceeded t-MLE in promoting plant performances and activities of enzymatic and non-enzymatic antioxidant components, which play pivotal roles in ameliorating Cd^{2+} stress.

Materials and Methods

Material and growing conditions

Plastic pots (35 cm depth and top diameter) were randomly arranged in a growth chamber set at a temperature of 30/24°C and a relative humidity of 85/60% for day/night in addition to a light intensity of approx. 3500 lx for a period of 12 h a day. Pots were evenly filled with pure sand (acid-washed and moistened with deionized water). Each pot was received 3 uniform sterilized seeds of *Phaseolus vulgaris* (L.), cv. Bronco. Seed sterilization was performed using 0.1% $HgCl_2$ for 1 min. On the 15th day of seeding, seedlings were supplemented with 1 m*M* Cd^{2+} along with a half-strength Hoagland nutritious solution (Hoagland and Arnon 1938). On the 20th and 30th day of seeding, seedlings were sprayed two times with distilled water (DW), traditional moringa leaf extract at a level of 6% (t-$MLE_{6\%}$), and moringa leaf extract in nano-sized particles at a level of 2% (n-$MLE_{2\%}$). The 3 spray application type (DW, t-$MLE_{6\%}$, and n-$MLE_{2\%}$) were supplemented for common bean plants under both normal (0 m*M* Cd^{2+}) and stress (1 m*M* Cd^{2+}) conditions to comprise 6 treatments of this study. Selection of the levels of applied Cd^{2+}, t-$MLE_{6\%}$, and n-$MLE_{2\%}$ was based on a preliminary study (Table 2). The pH of the plant growing medium was adjusted back to 6.2–6.5 by using diluted H_2SO_4. To preserve the concentration (1 m*M*) of Cd^{2+} in the growing medium, an inductively coupled plasma atomic emission spectrometry (ICP-AES, IRIS-Advan type, Thermo, U.S.A.) was used. All pots of all treatments were arranged in a completely randomized factorial as the applied experimental design for two factors; Cd^{2+} in two levels (0 and 1.0 m*M*) and three levels of MLE (0, 6% t-MLE, and 2% n-MLE) under each level of Cd^{2+}. The experiments were repeated three times to assure the results.

Plant samples were collected on the 40th day of seeding, at which the supplementation of Cd^{2+} was stopped, to assess the growth traits, physiological-biochemical parameters, and antioxidant defense system components. At green pod harvest (on the 56th – 66th day of seeding), yield attributes, pod protein and pod Cd^{2+} contents and WUE were assessed.

Preparation of nano and traditional moringa leaf extracts

Leaves of *Moringa oleifera* trees were collected from the upper half of the tree at evening (at this time the leaf will be restored higher photosynthates obtained from the photosynthesis process throughout the day). After excluding the midribs, fresh leaves were extracted using acetone and methanol. A volume of 500 mL at a ratio of 1: 1, acetone and methanol was added to 100 g fresh moringa leaves for wet blending using an electric blender. The mixture was put on a shaker for 5 h and then the extract was purified through filtering 2 times using Whatman (No. 1) filter papers. Then, by using a Rotary Evaporator, the extract was evaporated to completely exclude all acetone and methanol residues to obtain the paste of traditional moringa leaf extract (t-MLE). By using the t-MLE paste, the level of 3, 6, or 9% were prepared by dissolving 30, 60, or 90 g in 1 L distilled water for both the preliminary and main studies.

The paste was then freeze-dried, ground, and then ball-milled for 5 h by using Planetary Ball Mill (model PULVERISETTE with double drive power for premium

line version) to obtain nano-sized particles of moringa leaf extract (n-MLE; near 10 nm, Fig. 1) (Ambika and Sujatha 2017). The levels of n-MLE were prepared by dissolving 10, 20, or 30 g in 1 L distilled water to obtain n-$MLE_{1\%}$, n-$MLE_{2\%}$, or n-$MLE_{3\%}$ for both the preliminary and main studies. The two types of moringa leaf extracts (t-$MLE_{6\%}$ and n-$MLE_{2\%}$) were analyzed and their chemical constituents are presented in Table 1.

Assessment of growth and yield attributes, cadmium content (Cd^{2+}) and water use efficiency (WUE)

At 40 DAS, plants were removed from pots and cleaned gently using distilled water. Leaf area per plant was taken with a Portable digital-leaf area meter (LI-3000, LI-COR Lincoln, NE, USA). Shoot and root lengths were assessed using a meter scale. Using an electric oven at 70°C, drying of plants for 48 h were performed to record dry mass. At the end of the experiment, pods were collected for counting and weighing.

The dried powdered plant parts (*e.g*., pods, leaves, and roots) were used to assess the content of Cd^{2+}. By using an acid mixture; 3 HNO_3: 1 $HClO_4$ (v/v), samples were digested and measurements were performed on a Flame-Atomic spectrometry (Shi *et al*. 2009).

Protein was determined in all enzyme preparations and the bovine serum albumin (BSA, Sigma) was utilized as a standard (Bradford 1976).

At harvest, WUE was calculated as g pods per liter of water applied. Calculations were performed for all treatments based on the equation:

$$WUE = \text{Pod yield (g pot}^{-1}) / \text{Water applied (L pot}^{-1})$$

Determination of leaf pigments and photosynthetic efficiency

At the same time, chlorophyll fluorescence was assessed in 2 sunny days ("as a photosynthetic efficiency"). A Handy portable PEA fluorometer (Hansatech Instruments Ltd., Kings Lynn, U.K.) was utilized for determinations on the 4th leaf of plant top. The *F*v/*F*m ("maximum quantum yield of PS II") was assessed (Maxwell and Johnson 2000) and photosynthetic performance index (PI_{ABS}) was calculated based on the similar absorption (Clark *et al.* 2000).

Chlorophyll and carotenoid levels in fresh leaves were assessed according to Arnon (1949) using acetone (80%, v/v) and leaf disks for extraction. Pure extracts were obtained with centrifugation (15,000 ×*g* for 10 min) process. Reads were taken (663, 645 and 470 nm) with a Visible Recording Spectrometer (UV-160A, Shimadzu, Japan).

Assessments of plant water status, stability index of cell membrane (MSI), and ion leakage (EL)

After excluding leaf midrib, 2 cm-diameter discs were taken for relative water content (RWC) determination (Osman and Rady 2014). To record fresh mass (FM), discs were weighed and immediately immersed in deionized water for 24 h in dark. Water-saturated discs were blotted dry from adhering water drops for recording the turgid mass (TM). At 70°C, discs were dried for 48 h to record dry mass (DM). The following formula was applied: "RWC (%) = (FM – DM) / (TM – DM) × 100"

After excluding leaf midrib, 0.2 g leaf disks were taken twice and putted in test tubes each with 10 mL of deionized water to determine leaf MSI (Rady 2011). At 40°C, a sample was heated for 30 min in a water-bath and solution electrical conductivity (C_1) was measured. At 100°C, another sample was boiled for 10 min and solution conductivity (C_2) was measured also. The following formula was applied:

$$\text{"MSI (\%)} = 1 - (C_1 / C_2) \times 100\text{"}$$

The ions totally leaked from leaves (EL) were assessed with Sullivan and Ross (1979) procedure. Twenty discs were immersed in 10 mL of deionized water in a boiling tube and solution electrical conductivity (C_1) was recorded. By using a water-bath, tube content was then heated to 45 – 55°C for 30 min. Electrical conductivity (C_2) of solution was scored. At 100°C, tube content was boiled for 10 min and electrical conductivity (C_3) was measured also. The following formula was applied:

$$\text{"EL (\%)} = [(EC2 - EC1) / EC3] \times 100\text{"}$$

Assessment of contents of α-tocopherol (αTOC), H_2O_2 and lipid peroxidation (MDA)

To determine αTOC content, butylated hydroxytoluene was used to dissolve an extraction solvent. Standard and stock solutions were prepared using R-TOC. Preparation and saponification were performed (Konings *et al*. 1996). Sliced dried leaf samples were homogenized and suspended in 1 L conical flask containing water. A weight of 21 g KOH was added after dissolving using ethanol. Ascorbic acid was added for saponification. Extraction was performed for three times and after evaporation to dryness the residues were dissolved again with HPLC grade n-hexane. HPLC system was used to assess αTOC content as described in Ching and Mohamed (2001) method.

The H_2O_2 content (in μmol g^{-1} FW) was determined by homogenizing the samples by using the TCA (5%) and centrifuging (at 12,000 × g for 15 min) the homogenates. Supernatants were added to a reaction medium (K-phosphate buffer; 10 m*M*, pH 7.0 + KI; 1000 m*M*) and the absorbance readings were measured on 390 nm using H_2O_2 as a standard (Velikova *et al*. 2000).

Peroxidation of membrane lipids was assessed as μmol of malondialdehyde (MDA) per 1 g of fresh tissue of leaf. Assessment was performed by using the previous extract that used to assess the H_2O_2 (Heath and Packer 1968).

Assessment of sugars, proline, ascorbate (AsA), and glutathione (GSH) contents

Many methods were utilized to determine the contents of total soluble sugars, free proline, AsA, GSH such as Irigoyen *et al.* (1992), Bates *et al.* (1973), Mukherjee and Choudhari (1983), and Griffth (1980) using the extraction solutions of ethanol (96%, v/v), 3% (v/v) $C_7H_6O_6S$; sulphosalicylic acid, TCA (6%, w/v), and metaphosphoric acid (2%, v/v), respectively. For all these determinations, the 4th fresh upper fully-expanded leaves were used.

Assaying of activities of enzymatic antioxidants

The freeze-dried powdered leaf samples (200 mg) were used for enzyme extraction. By using a cold mortar (4°C), sample homogenization was performed by utilizing K-phosphate buffer (2 mL, 100 m*M*, pH 7.0), containing 0.1 m*M* EDTA. To assay the activity of ascorbate peroxidase (APX), the extraction buffer was received 2 m*M* ascorbic acid (AsA). A nylon cloth was used for filtering the homogenate. The obtained filtrate was centrifuged (12,000 ×*g*) for 15 min. The extract was used, immediately, or it may be stored at − 25°C until utilization.

The methods outlined in Beauchamp and Fridovich (1971), Nakano and Asada (1981), Havir and McHale (1987) and Martinez *et al.* (2018) were used to assay different activities of catalase (CAT; μmol H_2O_2 min^{-1} g^{-1} protein), superoxide dismutase (SOD; U mg^{-1} protein), ascorbate peroxidase (APX; μmol H_2O_2 min^{-1} g^{-1} protein), and glutathione peroxidase (GPX; μmol H_2O_2 min^{-1} g^{-1} protein), respectively. The diminishing in the absorbance reading (at 240 nm) due to the breakdown of H_2O_2 was measured to record the CAT activity. The inhibition capability of the photochemical reduction of NBT was utilized to measure the SOD activity. The oxidation of AsA that monitored as a reduction in the absorbance reading (290 nm) was recorded as the APX activity. In addition, by utilizing a GPX assay kit (Abcam, Ref. ab102530, Cambridge, U.K.), the decrease measured for the NADPH (at 340 nm) was recorded as the GPX activity.

Statistical data analysis

For the preliminary experiment, a simple ANOVA test was utilized for data analysis, and at 95% probability level the differences between means were compared using the Fisher's LSD test. The $P \leq 0.05$ means that there are significant levels between treatments. For analyzing data of the main study, a 2-way ANOVA test was used with "cadmium stress; Cd^{2+}" and "moringa extract; MLE type" as main 2 fixed factors. *t*-test ($P \leq 0.05$) was utilized for comparing the differences between the treatments of Cd^{2+} under the same MLE type treatment. The PASW Statistics 18.0 program was used for statistical analysis. Means of values ± standard error (SE) of 3–9 replicates are presented.

Results

The preliminary study

Data presented in Table 2 display that all levels of traditional moringa leaf extract (t-MLE; 3, 6, and 9%) or nano-size particles of moringa leaf extract (n-MLE; 1, 2, and 3%) significantly improved plant dry mass, chlorophylls content, photosynthetic performance index; PI, and cellular membrane stability index; MSI of *Phaseolus vulgaris* plants as compared to the corresponding control (1 or 2 m*M* of Cd^{2+}). The level of 2 m*M* Cd^{2+} led to plant death, while the level of 1 m*M* Cd^{2+} led to considerable reductions in the abovementioned parameters, therefore, it was selected for the main study. In addition, the most effective levels of t-MLE and n-MLE in this regard, which selected for the main study were 6 and 2%, respectively due to they were exceeded other levels in increasing the tested parameters in Cd^{2+}-stressed common bean plants.

The main study

Response of common bean performance and water use efficiency (WUE) to t-MLE or n-MLE under Cd^{2+} stress: Under no stress (Cd^{2+}) condition, growth and yield parameters (shoot and root lengths, area of plant leaves, plant dry mass, and pods number and yield), as well as pod protein content and WUE of *Phaseolus vulgaris* plants were significantly ($P \leq 0.05$) increased with n-MLE treatment compared to those obtained from the treatment with t-MLE, which in turn considerably elevated these attributes as compared to the control (Table 3 and 4). Under 25 days of 1 m*M* Cd^{2+} stress, shoot length, root length, leaves area, dry mass, pods number, pods yield, pod protein content, and WUE of plants were considerably ($P \leq 0.05$) diminished as compared with the control. However, t-MLE or n-MLE significantly improved all of these Cd^{2+}-stressed attributes compared to those of non-treated plants with MLE. Treatment with n-MLE under Cd^{2+} stress conferred results corresponded with the non-stressed control and significantly ($P \leq 0.05$) exceeded the treatment with t-MLE. Interactions between Cd^{2+} stress and MLE treatments were significant for all above attributes, except for pod protein content (not significant).

Response of common bean part contents of Cd^{2+} to t-MLE or n-MLE under Cd^{2+} stress: Under normal condition, Cd^{2+} content in all parts (*i.e.*, pods, leaves, and roots) of common bean plants were not detected in all treatments, except the root Cd^{2+} content, which recorded trace amounts under the control and the t-MLE treatments (Table 5). Under Cd^{2+} stress condition, Cd^{2+} content in plant roots, leaves, and pods were significantly ($P \leq 0.01$) elevated as compared to the control. Nonetheless, t-MLE or n-MLE significantly reduced all roots, leaves, and pods Cd^{2+} contents compared to those of non-treated plants with MLE. Treatment with n-MLE under Cd^{2+} stress

Table 1: Major chemical components of traditional (t-MLE) and nano moringa leaf extracts (n-MLE) used in the current study (on dry weight basis)

The component	The unit	Values		Method of analysis
		t-MLE	n-MLE	
1. Antioxidants and osmoprotectants				
Free proline	g kg^{-1} DW	31.2	38.4	Bates *et al*. (1973)
Soluble sugars		172	214	Irigoyen *et al*. (1992)
Ascorbic acid	mg kg^{-1} DW	33.1	44.3	Mukherjee and Choudhari (1983)
Glutathione		20.6	28.6	Griffth (1980)
α-Tocopherol		34.1	43.2	Konings *et al*. (1996); Ching and Mohamed (2001)
DPPH-radical scavenging	%	82.2	86.0	Lee *et al*. (2003)
2. Phytohormones:				
Total auxins	mg kg^{-1} DW	3.6	2.8	Lavrich and Hays (2007)
Total gibberellins		3.2	3.0	
Total cytokinins		3.7	3.2	
3. Mineral nutrients:				
Potassium (K^+)	g kg^{-1} DW	24.2	2.8	Williams and Twine (1960)
Calcium (Ca^{2+})		8.8	3.0	
Iron (Fe)		1.4	3.2	Chapman and Pratt (1961)
Manganese (Mn)		1.2	2.8	
Zinc (Zn)		1.0	3.0	

Table 2: A preliminary experiment shows the optimum foliar spray levels of traditional moringa leaf extract (t-MLE) and nano moringa leaf extract (n-MLE), as well as the non-lethal maximum toxic level of cadmium (Cd^{+2} in $CdCl_2$) for the main study using *P. vulgaris* plants

Cd^{2+} treatments + MLE type	Assessed parameters			
	Dry mass $plant^{-1}$ (g)	Chlorophylls (mg g^{-1} FW)	PI (%)	MSI (%)
1.0 m*M* Cd^{2+} (Cd^{2+}-1)	3.72 ± 0.38d	0.69 ± 0.02c	5.14 ± 0.13cd	34.3 ± 0.9de
Cd^{2+}-1 + t-MLE (3%)	4.41 ± 0.35c	0.78 ± 0.02c	5.42 ± 0.15c	37.1 ± 1.1cd
Cd^{2+}-1 + t-MLE (6%)	4.74 ± 0.39b	0.86 ± 0.03b	5.94 ± 0.15b	41.2 ± 1.3b
Cd^{2+}-1 + t-MLE (9%)	4.75 ± 0.35b	0.86 ± 0.02b	5.90 ± 0.13b	41.3 ± 1.5b
Cd^{2+}-1 + n-MLE (1%)	4.78 ± 0.40b	0.88 ± 0.03b	5.94 ± 0.18b	41.2 ± 1.2b
Cd^{2+}-1 + n-MLE (2%)	5.62 ± 0.43a	0.97 ± 0.03a	6.50 ± 0.22a	45.4 ± 1.3a
Cd^{2+}-1 + n-MLE (3%)	5.64 ± 0.49a	0.96 ± 0.03a	6.45 ± 0.20a	45.1 ± 1.5a
2.0 m*M* Cd^{2+} (Cd^{2+}-2)	$DP^{\#}$	$DP^{\#}$	$DP^{\#}$	$DP^{\#}$
Cd^{2+}-2 + t-MLE (3%)	2.79 ± 0.22f	0.49 ± 0.01f	5.08 ± 0.10d	31.0 ± 1.1f
Cd^{2+}-2 + t-MLE (6%)	2.98 ± 0.24ef	0.55 ± 0.02e	5.39 ± 0.12c	34.2 ± 1.0de
Cd^{2+}-2 + t-MLE (9%)	2.97 ± 0.31ef	0.55 ± 0.02e	5.38 ± 0.15c	34.1 ± 1.2e
Cd^{2+}-2 + n-MLE (1%)	3.01 ± 0.28ef	0.57 ± 0.02de	5.42 ± 0.14c	34.5 ± 1.4de
Cd^{2+}-2 + n-MLE (2%)	3.24 ± 0.32e	0.64 ± 0.03d	5.89 ± 0.18b	38.2 ± 1.4c
Cd^{2+}-2 + n-MLE (3%)	3.26 ± 0.33e	0.63 ± 0.02d	5.87 ± 0.20b	37.9 ± 1.5c

Values are means ± SE (n = 9). Differences among means were compared by Fisher's LSD test ($P \leq 0.05$). Mean pairs followed by different letters are significantly different. $DP^{\#}$ means dead plants

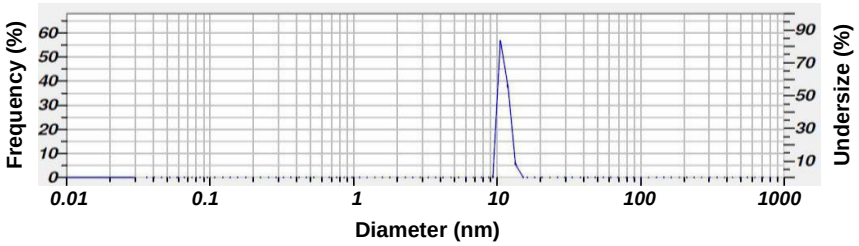

Fig. 1: Size of ball-milled MLE powder (n-MLE)

significantly decreased Cd^{2+} content of all plant parts compared to t-MLE treatment. Interactions between Cd^{2+} stress and MLE treatments were highly significant for all above attributes.

Response of common bean photosynthetic pigments and efficiency, and cell health to t-MLE or n-MLE under Cd^{2+} stress: Under no addition of Cd^{2+} to growing medium, total chlorophylls, total carotenoids, chlorophyll fluorescence (Fv/Fm), performance index (PI), tissue relative water content (RWC), and cellular membrane stability index (MSI) of common bean plants were significantly ($P \leq 0.05$) increased with n-MLE treatment compared to those obtained from the treatment with t-MLE, which in turn markedly raised these parameters as compared with the control (Table 6 and 7). The electrolyte leakage (EL) was recorded the reverse trend of the above attributes.

Table 3: Response of growth characteristics of cadmium (Cd^{2+})-stressed *P. vulgaris* plants to foliar spray with traditional moringa leaf extract (t-MLE; 5%) or MLE nano-particles (n-MLE; 2%)

Cd^{2+} treatments	MLE type	Assessed parameters			
		Shoot length (cm)	Root length (cm)	Leaves area plant^{-1} (dm^2)	Dry mass plant^{-1} (g)
0 m*M*	DW	30.2 ± 2.4c	25.6 ± 2.2c	15.1 ± 1.3c	9.8 ± 0.7c
	t-$MLE_{6\%}$	33.3 ± 2.7b	27.9 ± 2.6b	16.8 ± 1.3b	10.7 ± 0.9b
	n-$MLE_{2\%}$	37.1 ± 2.8a	31.2 ± 2.8a	18.4 ± 1.5a	11.9 ± 0.9a
1.0 m*M*	DW	16.4 ± 1.4e	12.9 ± 1.2e	8.8 ± 0.7e	4.3 ± 0.3e
	t-$MLE_{6\%}$	21.9 ± 1.8d	17.2 ± 1.5d	10.1 ± 0.9d	6.4 ± 0.6d
	n-$MLE_{2\%}$	29.4 ± 2.5c	25.1 ± 1.9c	14.9 ± 1.3c	9.6 ± 0.8c
Significance:					
Cd^{2+} level		*	*	*	*
MLE type		*	*	*	*
Cd^{2+} × MLE		*	*	*	*

Values show the means ± SE. T-test was implemented to compare differences among MLE type treatments under the same Cd^{2+} treatment. Different letters following the values indicate significant difference between each two treatments at $P \leq 0.05$. Two-way ANOVA outputs: (ns) means not significant; (*) means significant at $P \leq 0.05$, and (**) means significant at $P \leq 0.01$. DW means distilled water

Table 4: Response of yield components and water use efficiency (WUE; g pods per liter applied water) of cadmium (Cd^{2+})-stressed *P. vulgaris* plants to foliar spray with traditional moringa leaf extract (t-MLE; 5%) or MLE nano-particles (n-MLE; 2%)

Cd^{2+} treatments	MLE type	Assessed parameters			
		Pods No. pot^{-1}	Pods yield pot^{-1} (g)	Pod protein (%)	WUE
0 m*M*	DW	12.4 ± 1.1c	47.9 ± 3.5c	15.8 ± 0.4c	0.68 ± 0.05c
	t-$MLE_{6\%}$	14.0 ± 1.2b	51.2 ± 3.9b	17.1 ± 0.6b	0.73 ± 0.06b
	n-$MLE_{2\%}$	16.1 ± 1.4a	56.1 ± 4.1a	18.9 ± 0.6a	0.80 ± 0.06a
1.0 m*M*	DW	5.8 ± 0.5e	22.4 ± 2.3e	11.6 ± 0.3e	0.32 ± 0.03e
	t-$MLE_{6\%}$	9.8 ± 0.8d	32.4 ± 2.8d	13.4 ± 0.3d	0.46 ± 0.04d
	n-$MLE_{2\%}$	12.2 ± 0.9c	47.0 ± 3.8c	15.4 ± 0.3c	0.67 ± 0.05c
Significance:					
Cd^{2+} level		*	*	*	*
MLE type		*	*	*	*
Cd^{2+} × MLE		*	*	ns	*

Values show the means ± SE. T-test was implemented to compare differences among MLE type treatments under the same Cd^{2+} treatment. Different letters following the values indicate significant difference between each two treatments at $P \leq 0.05$. Two-way ANOVA outputs: (ns) means not significant; (*) means significant at $P \leq 0.05$, and (**) means significant at $P \leq 0.01$. DW means distilled water

Table 5: Response of cadmium (Cd^{2+}) contents (mg kg^{-1} DW) of Cd^{2+}-stressed *P. vulgaris* plant parts (roots, leaves, and pods) to foliar spray with traditional moringa leaf extract (t-MLE; 5%) or MLE nano-particles (n-MLE; 2%)

Cd^{2+} treatments	MLE type	Assessed parameters		
		Root Cd^{2+} content	Leaf Cd^{2+} content	Pod Cd^{2+} content
0 m*M*	DW	Trace	ND	ND
	t-$MLE_{6\%}$	Trace	ND	ND
	n-$MLE_{2\%}$	ND	ND	ND
1.0 m*M*	DW	78.4 ± 2.1a	38.61 ± 1.14a	21.82 ± 0.44a
	t-$MLE_{6\%}$	44.2 ± 1.1b	18.11 ± 0.49b	5.21 ± 0.18b
	n-$MLE_{2\%}$	22.3 ± 0.7c	8.42 ± 0.20c	0.26 ± 0.01c
Significance:				
Cd^{2+} level		**	**	**
MLE type		**	**	**
Cd^{2+} × MLE		**	**	**

Values show the means ± SE. T-test was implemented to compare differences among MLE type treatments under the same Cd^{2+} treatment. Different letters following the values indicate significant difference between each two treatments at $P \leq 0.05$. Two-way ANOVA outputs: (ns) means not significant; (*) means significant at $P \leq 0.05$, and (**) means significant at $P \leq 0.01$: Trace means amount less than 1 mg kg^{-1} DW, and ND means not detected. DW means distilled water

Under Cd^{2+} stress condition, total chlorophylls and carotenoids, Fv/Fm, PI, RWC, and MSI of plants were significantly reduced, while EL was significantly increased compared with those of the non-stressed control. However, n-MLE application considerably surpassed t-MLE application and both treatments significantly improved all of these Cd^{2+}-stressed attributes compared to those of non-treated plants with MLE. Interactions between Cd^{2+} stress and MLE treatments were significant for all abovementioned attributes, except for Fv/Fm (not significant).

Response of common bean leaf peroxidation of lipids (MDA) and hydrogen peroxide (H_2O_2) to t-MLE or n-MLE under Cd^{2+} stress: Under no stress (Cd^{2+}) condition, leaf MDA and H_2O_2 contents of *Phaseolus vulgaris* plants were significantly ($P \leq 0.05$) decreased with n-MLE or t-MLE (with a significant preference for n-MLE regarding H_2O_2 content) treatment compared with those obtained from the untreated control (Table 8). Under 25 days of Cd^{2+} (1 m*M*) stress, leaf contents of MDA and H_2O_2 in common bean plants were markedly increased compared with those of the non-stressed control. However, t-MLE or

Table 6: Response of leaf pigments contents (mg g^{-1} FW) and photosynthetic efficiency (Fv/Fm and PI) of cadmium (Cd^{2+})-stressed *P. vulgaris* plants to foliar spray with traditional moringa leaf extract (t-MLE; 5%) or MLE nano-particles (n-MLE; 2%)

Cd^{2+} treatments	MLE type	Assessed parameters			
		Total chlorophylls	Total carotenoids	Fv/Fm	PI (%)
0 m*M*	DW	1.62 ± 0.04c	0.54 ± 0.02c	0.80 ± 0.02bc	9.42 ± 0.22c
	t-$MLE_{6\%}$	1.84 ± 0.04b	0.60 ± 0.02b	0.82 ± 0.02ab	11.12 ± 0.26b
	n-$MLE_{2\%}$	2.10 ± 0.06a	0.65 ± 0.02a	0.85 ± 0.03a	13.84 ± 0.33a
1.0 m*M*	DW	0.71 ± 0.02e	0.32 ± 0.01e	0.70 ± 0.01d	6.22 ± 0.15e
	t-$MLE_{6\%}$	1.21 ± 0.03d	0.44 ± 0.01d	0.78 ± 0.02c	7.89 ± 0.19d
	n-$MLE_{2\%}$	1.58 ± 0.04c	0.54 ± 0.02c	0.82 ± 0.02ab	9.38 ± 0.24c
Significance:					
Cd^{2+} level		*	*	*	*
MLE type		*	*	*	*
Cd^{2+} × MLE		*	*	ns	*

Values show the means ± SE. T-test was implemented to compare differences among MLE type treatments under the same Cd^{2+} treatment. Different letters following the values indicate significant difference between each two treatments at $P \leq 0.05$. Two-way ANOVA outputs: (ns) means not significant; (*) means significant at $P \leq 0.05$, and (**) means significant at $P \leq 0.01$. DW means distilled water

Table 7: Response of relative water content (RWC), membrane stability index (MSI), and electrolyte leakage (EL) of cadmium (Cd^{2+})-stressed *P. vulgaris* plants to foliar spray with traditional moringa leaf extract (t-MLE; 5%) or MLE nano-particles (n-MLE; 2%)

Cd^{2+} treatments	MLE type	Assessed parameters		
		RWC (%)	MSI (%)	EL (%)
0 m*M*	DW	78.8 ± 2.4b	62.4 ± 1.6b	9.21 ± 0.19c
	t-$MLE_{6\%}$	84.6 ± 2.8a	67.4 ± 1.8a	8.44 ± 0.17c
	n-$MLE_{2\%}$	88.2 ± 3.0a	69.8 ± 2.0a	6.54 ± 0.12d
1.0 m*M*	DW	54.4 ± 1.7d	42.3 ± 1.2d	18.8 ± 0.36a
	t-$MLE_{6\%}$	62.8 ± 2.2c	50.5 ± 1.4c	14.6 ± 0.24b
	n-$MLE_{2\%}$	76.9 ± 2.3b	62.0 ± 1.5b	9.18 ± 0.20c
Significance:				
Cd^{2+} level		*	*	*
MLE type		*	*	*
Cd^{2+} × MLE		*	*	*

Values show the means ± SE. T-test was implemented to compare differences among MLE type treatments under the same Cd^{2+} treatment. Different letters following the values indicate significant difference between each two treatments at $P \leq 0.05$. Two-way ANOVA outputs: (ns) means not significant; (*) means significant at $P \leq 0.05$, and (**) means significant at $P \leq 0.01$. DW means distilled water

Table 8: Response of leaf contents of lipid peroxidation (in terms of malondialdehyde; MDA), hydrogen peroxide (H_2O_2), and α-tocopherol (αTOC) of cadmium (Cd^{2+})-stressed *P. vulgaris* plants to foliar spray with traditional moringa leaf extract (t-MLE; 5%) or MLE nano-particles (n-MLE; 2%)

Cd^{2+} treatments	MLE type	Assessed parameters		
		MDA (μmol g^{-1} FW)	H_2O_2 (μmol g^{-1} FW)	αTOC (μmol g^{-1} DW)
0 m*M*	DW	24.8 ± 0.3c	5.26 ± 0.07c	1.74 ± 0.02e
	t-$MLE_{6\%}$	22.6 ± 0.2d	4.74 ± 0.06d	1.98 ± 0.03d
	n-$MLE_{2\%}$	21.8 ± 0.2d	4.36 ± 0.06e	2.88 ± 0.03b
1.0 m*M*	DW	41.8 ± 0.4a	12.62 ± 0.18a	2.69 ± 0.03c
	t-$MLE_{6\%}$	34.2 ± 0.3b	9.44 ± 0.11b	2.94 ± 0.05b
	n-$MLE_{2\%}$	25.2 ± 0.2c	5.30 ± 0.06c	3.46 ± 0.06a
Significance:				
Cd^{2+} level		*	*	*
MLE type		*	*	*
Cd^{2+} × MLE		*	*	*

Values show the means ± SE. T-test was implemented to compare differences among MLE type treatments under the same Cd^{2+} treatment. Different letters following the values indicate significant difference between each two treatments at $P \leq 0.05$. Two-way ANOVA outputs: (ns) means not significant; (*) means significant at $P \leq 0.05$, and (**) means significant at $P \leq 0.01$. DW means distilled water

n-MLE significantly ($P \leq 0.05$) improved these Cd^{2+}-stressed attributes compared to those of non-treated plants with MLE. Treatment with n-MLE under Cd^{2+} stress conferred results corresponded with the non-stressed control and significantly exceeded the treatment with t-MLE. Interactions between Cd^{2+} stress and MLE treatments were significant for MDA and H_2O_2 contents.

Response of common bean antioxidant defense system components to t-MLE or n-MLE under Cd^{2+} stress: Under normal condition, all tested osmoprotectants and low molecular weight antioxidants (*e.g.*, soluble sugars, free proline, α-tocopherol, ascorbic acid, and glutathione) contents, as well as antioxidant enzymes (*e.g.*, glutathione peroxidase, ascorbate peroxidase, superoxide dismutase, and catalase) activities of *Phaseolus vulgaris* plants were markedly ($P \leq 0.05$) elevated due to the treatment of n-MLE as compared with the treatment of t-MLE, which in turn significantly ($P \leq 0.05$) increased these attributes as compared to the control (Table 8–10). Under the stress of 1 m*M* Cd^{2+}, osmoprotectant contents, low molecular weight antioxidant contents, and antioxidant enzyme activities were markedly raised as compared with the control. However, t-

Table 9: Response of leaf contents of total soluble sugars, free proline, ascorbate (AsA), and glutathione (GSH) of cadmium (Cd^{2+})-stressed *P. vulgaris* plants to foliar spray with traditional moringa leaf extract (t-MLE; 5%) or MLE nano-particles (n-MLE; 2%)

Cd^{2+} treatments	MLE type	Assessed parameters			
		Soluble sugars (mg g^{-1} DW)	Free proline (µmol g^{-1} DW)	AsA (µmol g^{-1} FW)	GSH (µmol g^{-1} FW)
0 m*M*	DW	12.4 ± 0.21e	3.27 ± 0.05e	1.32 ± 0.00e	0.91 ± 0.00e
	t-$MLE_{6\%}$	14.8 ± 0.24d	3.69 ± 0.06d	1.43 ± 0.01d	0.98 ± 0.00d
	n-$MLE_{2\%}$	18.2 ± 0.32c	3.98 ± 0.06c	1.67 ± 0.01c	1.24 ± 0.00c
1.0 m*M*	DW	18.0 ± 0.27c	3.90 ± 0.06c	1.72 ± 0.01c	1.22 ± 0.01c
	t-$MLE_{6\%}$	20.4 ± 0.37b	4.24 ± 0.08b	1.94 ± 0.02b	1.52 ± 0.01b
	n-$MLE_{2\%}$	25.1 ± 0.42a	4.86 ± 0.08a	2.33 ± 0.02a	1.84 ± 0.01a
Significance:					
Cd^{2+} level		*	*	*	*
MLE type		*	*	*	*
Cd^{2+} × MLE		*	*	*	*

Values show the means ± SE. T-test was implemented to compare differences among MLE type treatments under the same Cd^{2+} treatment. Different letters following the values indicate significant difference between each two treatments at $P \leq 0.05$. Two-way ANOVA outputs: (ns) means not significant; (*) means significant at $P \leq 0.05$, and (**) means significant at $P \leq 0.01$. DW means distilled water

Table 10: Response of leaf activities of superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), and glutathione peroxidase (GPX) of cadmium (Cd^{2+})-stressed *P. vulgaris* plants to foliar spray with traditional moringa leaf extract (t-MLE; 5%) or MLE nano-particles (n-MLE; 2%)

Cd^{2+} treatments	MLE type	Assessed parameters			
		SOD (U mg^{-1} protein)	CAT	APX	GPX
			(µmol H_2O_2 min^{-1} mg^{-1} protein)		
0 m*M*	DW	2636 ± 32e	162 ± 2e	13.2 ± 0.2e	20.4 ± 0.3e
	t-$MLE_{6\%}$	2842 ± 30d	174 ± 2d	15.0 ± 0.2d	22.4 ± 0.3d
	n-$MLE_{2\%}$	3106 ± 32c	191 ± 3c	16.9 ± 0.2c	24.8 ± 0.4c
1.0 m*M*	DW	3148 ± 33c	194 ± 3c	16.7 ± 0.2c	25.0 ± 0.4c
	t-$MLE_{6\%}$	3452 ± 36b	212 ± 3b	19.0 ± 0.3b	28.2 ± 0.4b
	n-$MLE_{2\%}$	3850 ± 38a	241 ± 4a	22.2 ± 0.3a	33.4 ± 0.5a
Significance:					
Cd^{2+} level		*	*	*	*
MLE type		*	*	*	*
Cd^{2+} × MLE		*	*	*	*

Values show the means ± SE. T-test was implemented to compare differences among MLE type treatments under the same Cd^{2+} treatment. Different letters following the values indicate significant difference between each two treatments at $P \leq 0.05$. Two-way ANOVA outputs: (ns) means not significant; (*) means significant at $P \leq 0.05$, and (**) means significant at $P \leq 0.01$. DW means distilled water

MLE or n-MLE significantly/further increased all of these Cd^{2+}-stressed attributes compared to those of non-treated plants with MLE. Treatment with n-MLE in the presence of 1 mM Cd^{2+} notably exceeded the treatment with t-MLE for the above attributes. Interactions between Cd^{2+} stress and MLE treatments were significant for all the tested attributes.

Discussion

Cadmium (Cd^{2+}) stress causes an excessive output of reactive oxygen species (ROS); hydroxyl anion (OH^-), superoxide ($O_2^{\bullet-}$), singlet oxygen ($^1O_2$), and hydrogen peroxide (H_2O_2) radicals. To maintain healthy metabolic functions as a result of avoiding oxidative injuries under Cd^{2+} stress conditions, ROS generation and ROS degradation are required to be in equilibrium (Howladar 2014; Rady and Hemida 2015; Semida *et al*. 2015). Using most of its resources under stress, plant improves its defense mechanisms to maintain its development and growth (Kolbert *et al*. 2012). Antioxidant system components in plants include two major types; non-enzymatic antioxidants (*e.g*., glutathione, ascorbic acid, proline, α-tocopherol, carotenoids, *etc*.) and enzymatic antioxidants (*e.g*., ascorbate peroxidase, glutathione peroxidase, superoxide dismutase, catalase, *etc*.), which were extensively explained to dominate levels of ROS in plants (Howladar 2014; Semida and Rady 2014; Rady and Hemida 2015; Semida *et al*. 2015; Desoky *et al*. 2019). Nonetheless, the control of ROS by plant antioxidant systems is limited; therefore, it is necessary to use external support for stressful plant.

Foliar application of traditional leaf extract of moringa (t-MLE) to common bean plants in presence or in absence of Cd^{2+} stress significantly elevated the activities of all tested enzymatic antioxidants and low molecular weight antioxidants (Table 8–10). Nonetheless, the interesting thing obtained in the current study is that nano-sized moringa extract (n-MLE) has significantly exceeded t-MLE in elevating the activities of antioxidant defense system components. This may be attributed to the easy penetration of n-MLE particles into leaf cells more than t-MLE particles. As shown in Table 1, these n-MLE and t-MLE are rich in osmoprotectants and antioxidants (*i.e*., soluble sugars, free proline, α-tocopherol, glutathione, and ascorbic acid), nutrient elements (*i.e*., K^+, Ca^{2+}, Fe, Mn, and Zn), and plant hormones (*i.e*., cytokinins, gibberellins, and auxins). As an important event, foliar spray of n-MLE exceeded t-

MLE and caused considerable improvements in the activities of different antioxidants (non-enzymatic and enzymatic; Table 8–10). The elevation in antioxidant enzyme building and activities, based on physiological, molecular and genetic approaches, is proved to be the outcome of improved expression of *DET2* gene that is improved the resistance to oxidative stress in *Arabidopsis* (Cao *et al*. 2005). Many reports have reported previously that foliar spray with MLE considerably increased enzyme activities under stress conditions (Rady *et al*. 2013; Howladar 2014; Rady and Mohamed 2015; Latif and Mohamed 2016; Rady *et al*. 2019). Results of this study displayed that foliar spray with n-MLE or t-MLE for common bean plants caused considerable elevations in activities of superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), and glutathione peroxidase (GPX) (Table 10), and contents of α-tocopherol (αTOC; Table 8), ascorbic acid (AsA) and glutathione (GSH) (Table 9) along with increase of membrane stability index (MSI; Table 7) in contrast to ion leakage (EL; Table 7), lipid peroxidation of cell membranes (MDA), and hydrogen peroxide (H_2O_2) (Table 8) that were considerably reduced. The first defense line in different tissues of plants is SOD that converts $O_2^{\bullet-}$ to H_2O_2, which is then converted to H_2O in the presence of peroxidase enzyme (Alscher *et al.* 2002) by use of several reductants; AsA, and phenols including guaiacol (Apel and Hirt 2004). In addition, like APX, CAT reduces H_2O_2 to H_2O and O_2. Moreover, α-tocopherol (αTOC), non-enzymatic lipophilic antioxidant, which was significantly increased with MLE application (Table 8), is capable of scavenging many ROS and free radicals (Bano *et al*. 2014). αTOC deactivates the photosynthesis-derived ROS, especially $^1O_2$ and OH–, and prevents the excess peroxidation of lipids (MDA) by eliminating the lipid peroxyl radicals in the thylakoid membranes of chloroplasts (Semida *et al*. 2014). Thus, it contributes to decrease MDA levels and EL, and increase MSI (Table 7 and 8).

Like αTOC, other antioxidants such as free proline, AsA and GSH display crucial antioxidative protective activities as preventive roles against the oxidative stress (Valero *et al*. 2016) and lipid peroxidation induced by Cd^{2+} stress. Along with sufficient APX activity, AsA/GSH pool should be modified strictly to enhance the capacity of antioxidants in plant cells, avoiding oxidative damages (Foyer and Noctor 2011). AsA is an extremely powerful eliminator of ROS due to its ability to donate electrons in various enzymatic and non-enzymatic reactions. It protects cellular membranes by the directly eliminating of OH^- and $O_2^{\bullet-}$ by the regeneration of αTOC from the tocopheroxyl radical. In chloroplasts, it also functions as a cofactor of violaxanthin de-epoxidase to maintain the dissipation of excess excitation energy (Smirnoff 2000; Semida and Rady 2014). In addition, AsA plays a pivotal role to maintain enzyme activities due to those enzymes contain transitional metal ions (Noctor and Foyer 1998). The AsA redox system consists of three AsA forms; L-AsA, dehydroascorbate (DHAR), and monodehydroascorbate (MDHAR). Both DHAR and MDHAR (the oxidized AsA forms) are relatively unstable in aquatic environments, while DHAR can be reduced chemically by GSH to AsA (Foyer and Halliwell 1976). The two main antioxidants GSH and AsA are the main components of AsA-GSH cycle. They function to control the levels of H_2O_2 in the plant cells. Mainly, GR, DHAR, and MDHAR are responsible for substrates supplying for APX *via* AsA and GSH formation (Zhou *et al*. 2017). Under the stress of 1 mM Cd^{2+}, AsA content was elevated (Table 9). Additionally, the elevated content of GSH was noted under Cd^{2+} stress compared with the control (Table 9). The combined application of MLE and Cd^{2+} increased considerably AsA and GSH contents as compared with the control or Cd^{2+} treatment (Table 9). Results, herein, showed that n-MLE treatment was more effective than t-MLE, providing plant with more AsA and GSH contents for defense against Cd^{2+} stress.

Free proline accumulates in plant tissues under stress conditions. It reaches approximately 5% of the pool of amino acids under normal conditions. However, it rises by up to 20–80% under the conditions of stress due to the elevated synthesis of proline and its reduced degradation in plant species (Kishor *et al*. 2005). It decreases the damage of ROS, enhancing plant tolerance through some mechanisms. Among them, it reduces Cd^{2+} stress influences by ROS detoxification. It can also physically quench the $^1O_2$ or directly react with OH^- radicals. Therefore, it conferred lower MDA levels in this study. Siripornadulsil *et al.* (2002) reported also that the high level of GSH facilitates the phytochelatin synthesis and the sequestration of heavy metal-phytochelatin conjugates in vacuoles of plant cells. This raised Cd^{2+}-phytochelatin complexes sequestration in cellular vacuoles accounts for the transiently elevated Cd^{2+} content of P5CS-expressing cells. In addition, free proline, as it is highly water-soluble, is a compatible osmolyte/osmoprotectant, which is not charged at a neutral pH. Proline can drive the influx of water or reduce its efflux. This mechanism provides turgor of cells that necessary for the cellular expansions. Proline possesses various distinctive roles under the conditions of osmotic stress like stabilizing cellular proteins, maintaining cell membrane integrity and subcellular structures, as well as it protects cellular functions *via* eliminating different ROS (Kishor *et al*. 2005). This leads to increased cellular relative water content (RWC) and MSI with reducing the EL (Table 7). In the current study, increased activities of the low molecular weight antioxidants and antioxidant enzymes, and proline pool led to an elevation of the tolerance capacity to Cd^{2+} stress because of the application of proline enriching-MLE, especially n-MLE.

The increased tolerance to the Cd^{2+} stress due to the raised activities of different enzymatic and non-enzymatic antioxidants was emerged in terms of considerable reductions in the *Phaseolus vulgaris* roots, leaves, and pods contents of Cd^{2+} that considerably reflect in improvements

of Cd^{2+}-stressed plant growth and output, and leaf pigments (Table 3–6). The increased levels of photosynthetic pigments is attributed to the limiting activity of the chlorophyllase (chlorophyll-degrading enzyme) under stress conditions through growth stimulators application such as the bioactive components detected in MLE (Table 1). These MLE bioactive components, especially for n-MLE, at least in part, alleviate the decrease in chlorophylls and carotenoids contents under Cd^{2+} stress (Table 6). Foliar spray of MLE, especially n-MLE prevented the premature leaf senescence (data not shown) and conferred more area of plant leaves with higher photosynthetic pigments and strong photosynthetic apparatus efficiency (Fv/Fm and PI) (Howladar 2014; Rady *et al*. 2019). This positive result may be occurred mainly due to cytokinins (CKs) found in MLE as the highest phytohormones content (Table 1). The CKs are considered, in general, as antagonists of ABA in several developmental processes. It has been reported that zeatin-type cytokinin (CK) functions as a direct ROS eliminator and/or it may implicate in the antioxidative mechanism (Chakrabarti and Mukherji 2003).

As a highly toxic pollutant, Cd^{2+} affects different metabolic processes in plant cells (Li *et al*. 2008) and causes loss in the photosynthesis rate in the current study (Table 6). However, MLE, especially n-MLE improved water relations such as the increase in water use efficiency (WUE), RWC, and the rate of photosynthesis due to the raised contents of photosynthetic pigments (Table 6 and 7). Additionally, MLE, especially n-MLE modified positively the structure/stability of Cd^{2+}-stressed membranes, therefore, application of plants with n-MLE, particularly, either in presence or in absence of stress had higher MSI and lower MDA content (peroxidation of membrane lipids) (Table 7 and 8). All of these improved attributes with the improved antioxidant (non-enzymatic and enzymatic) defense systems led to a healthy metabolism status of stressful plants that treated with MLE, especially n-MLE and led to a healthy plant growth (Table 3), and consequently an increased pods yield and quality (Table 4).

Different MLE were previously used as growth enhancers for a variety of plants grown under normal or different stress conditions (Rady *et al*. 2013; Howladar 2014; Rady and Mohamed 2015; Hanafy 2017; Rehman *et al*. 2017; Desoky *et al*. 2019). The MLE-sprayed *Phaseolus vulgaris* plants showed healthy growth (in terms of plant dry mass; Table 3) with larger leaves (in terms of leaves area; Table 3) and dark green (in terms of chlorophylls content; Table 6), early and excessive flowering and pods bearing (in terms of pods number and pods yield of plants; Table 4) as compared to the MLE-unsprayed plants. The increase in common bean plant growth by MLE might be due to that it causes its nutrients to be readily available and would assist for efficient absorption and subsequent transport, improving growth parameters. MLE also concentrates triggers early flowering and pod set, conferring more yields associating with the number of flowers (data not shown), which are initiated from robust plant growth under Cd^{2+} stress. It is believed that higher yields of MLE-treated plants are associated with plant hormones presented in MLE, especially CKs, which are linked with nutrient partitioning in plants and may be associated with nutrient mobilization (Ambika and Sujatha 2017).

All of the obtained results of this study that effectively improved by spraying plants with MLE are attributed to that MLE is distinctive source of osmoprotectants, antioxidants, mineral nutrients, and phytohormones (auxins, GAs, and CKs). Additionally, MLE has a high DPPH-radical scavenging (82–86%) activity, granting MLE the higher power to enable common bean plants to effectively tolerate the Cd^{2+} stress by many mechanisms. Among them, scavenging of ROS and strong decreasing the contents of Cd^{2+} in plant roots, leaves, and pods (Table 5).

Tolerance of Cd^{2+} stress in common bean plants, in this study, was efficiently enhanced with the raised activities of various antioxidant system components through the application of mineral nutrients, AsA, GSH, osmoprotectants (free proline and soluble sugars), CKs, auxins and gibberellins containing-MLE. Where, MLE is a rich source of zeatin-type CK, minerals and other phytohormones and antioxidants, and consequently the MLE, especially n-MLE effectiveness in alleviating Cd^{2+} stress effects by better chlorophyll and antioxidants contents, as well as healthy plant growth may be due to CKs-mediated stay green effect. Herein, applying n-MLE at a level of 2% was more effective in alleviating Cd^{2+} stress effects than t-MLE at a level of 6%. This may be attributed to that nano-compounds in MLE are rapidly absorbed by plant leaves and speedily supplied the plants with required nutrients and other useful substances. Generally, smaller (nano) particles are biologically more active than bigger particles (Shishatskaya *et al*. 2018). In addition, bigger particles of t-MLE can temporarily clog the stomata of leaves in contrast to the nanoparticles of n-MLE that easily penetrate the stomata into active leaf cells.

Conclusion

The nutritional rich MLE, especially n-MLE being a distinctive source of sugars, free proline, antioxidants, mineral nutrients, and phytohormones considerably improved the levels of antioxidant system components (ascorbate peroxidase, glutathione peroxidase, catalase, and superoxide dismutase, as well as free proline, glutathione, ascorbic acid, and carotenoids), both under Cd^{2+} stress and normal conditions. The influence of n-MLE applied as foliar spray, on different components of the antioxidant defense system was clearer under stress conditions, suggesting that the raised levels of different components of the antioxidant defense system increased the tolerance to Cd^{2+} stress in *Phaseolus vulgaris* plants and protected the machinery of photosynthesis to maintain plant growth in healthy state.

References

Alscher PG, N Erturk, LS Heath (2002). Role of superoxide dismutases (SODs) in controlling oxidative stress in plant. *J Exp Bot* 53:1331–1341

Alzahrany Y, A Kuşvuran, HF Alharby, S Kuşvuran, MM Rady (2018). The defensive role of silicon in wheat against stress conditions induced by drought, salinity or cadmium. *Ecotoxicol Environ Saf* 154:187–196

Ambika S, K Sujatha (2017). Organic seaweed nano powder effect on growth and yield attributes of pigeon pea. *Legum Res* 40:731–734

Apel K, H Hirt (2004). Reactive oxygen species: metabolism oxidatives and signal transduction. *Annu Rev Plant Physiol Plant Mol Biol* 55:373–399

Arnon DI (1949). Copper enzymes in isolated chloroplasts. Polyphenoloxidase in *Beta vulgaris* L. *Plant Physiol* 24:1–5

Bano S, M Ashraf, NA Akram (2014). Salt stress regulates enzymatic and nonenzymatic antioxidative defense system in the edible part of carrot (*Daucus carota* L.). *J Plant Interact* 9:324–329

Bates LS, RP Waldren, ID Teare (1973). Rapid determination of free proline for water stress studies. *Plant Soil* 39:205–207

Beauchamp C, I Fridovich (1971). Superoxide dismutase: Improved assays and an assay applicable to acrylamide gels. *Anal Biochem* 44:276–287

Bradford MM (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 72:248–254

Bulgari R, G Franzoni, A Ferrante (2019). Biostimulants application in horticultural crops under abiotic stress conditions – A review. *Agronomy* 9; Article 9060306

Cao S, Q Xu, Y Cao, K Qian, K An, Y Zhu, H Binzeng, H Zhao, B Kuai (2005). Loss-of-function mutations in *DET2* gene lead to an enhanced resistance to oxidative stress in *Arabidopsis*. *Physiol Plantarum* 123:57–66

Chakrabarti N, S Mukherji (2003). Alleviation of NaCl stress by pretreatment with phytohormones in *Vigna radiata*. *Biol Plantarum* 46:589–594

Chapman HD, PF Pratt (1961). Methods of analysis for soils, plants and water. *PhD Thesis*, Univ. California, Berkeley, California, USA

Ching LS, S Mohamed (2001). Alpha-tocopherol content in 62 edible tropical plants. *J Agric Food Chem* 49:3101–3105

Clark AJ, W Landolt, JB Bucher, RJ Strasser (2000). Beech (*Fagus sylvatica*) response to ozone exposure assessed with a chlorophyll a fluorescence performance index. *Environ Pollut* 109:501–507

Daş ZA, G Dimlioğlu, M Bor, F Özdemir (2016). Zinc induced activation of GABA-shunt in tobacco (*Nicotiana tabaccum* L.). *Environ Exp Bot* 122:78–84

Desoky EM, AS Elrys, MM Rady (2019). Integrative Moringa and Licorice extracts application improves performance and reduces fruit contamination content of pepper plants grown on heavy metals-contaminated saline soil. *Ecotoxicol Environ Saf* 169:50–60

Dhargalkar VK, N Pereira (2005). Seaweed: Promising plant of the millennium. *Sci Cult* 71:60–66

Foyer CH, B Halliwell (1976). The presence of glutathione and glutathione reductase in chloroplasts: A proposed role in ascorbic acid metabolism. *Planta* 133:21–25

Foyer CH, G Noctor (2011). Ascorbate and glutathione: The heart of the redox hub. *Plant Physiol* 155:2–18

Griffth OW (1980). Determination of glutathione and glutathione disulfide using glutathione reductase and 2 vinyl pyridine. *Anal Biochem* 106:207–212

Hanafy RS (2017). Using *Moringa olifera* leaf extract as a bio-fertilizer for drought stress mitigation of *Glycine max* L. plants. *Egypt J Bot* 57:281–292

Havir EA, NA McHale (1987). Biochemical and developmental characterization of multiple forms of catalase in tobacco leaves. *Plant Physiol* 84:450–455

Heath RL, L Packer (1968). Photoperoxidation in isolated chloroplasts: I. Kinetics and stoichiometry of fatty acid peroxidation. *Arch Biochem Biophys* 125:189–198

Hoagland DR, DI Arnon (1938). *The water-culture method for growing plants without soil*. Circular 347, University of California College of Agriculture, Berkeley, California, USA

Howladar SM (2014). A novel *Moringa oleifera* leaf extract can mitigate the stress effect of salinity and cadmium in bean (*Phaseolus vulgaris* L.) plants. *Ecotoxicol Environ Saf* 100:69–75

Irigoyen JJ, DW Emerich, M Sanchez-Diaz (1992). Water stress induced changesin the concentrations of proline and total soluble sugars in nodulated alfalfa (*Medicago sativa*) plants. *Physiol Plantarum* 84:455–460

Kishor PBK, S Sangam, RN Amrutha, PS Laxmi, KR Naidu, KRSS Rao, S Rao, KJ Reddy, P Theriappan, N Sreenivasulu (2005). Review: Regulation of proline biosynthesis, degradation, uptake and transport in higher plants: Its implications in plant growth and abiotic stress tolerance. *Curr Sci* 88:424–438

Kolbert Z, A Peto, N Lehotai, G Feigl, L Erdei (2012). Long-term copper (Cu^{2+}) exposure impacts on auxin, nitric oxide (NO) metabolism and morphology of *Arabidopsis thaliana* L. *Plant Growth Regul* 68:151–159

Konings EJ, HH Roomans, PR Beljaars (1996). Liquid chromatographic determination of tocopherols and tocotrienols in margarine, infant foods, and vegetables. *J AOAC Intl* 79:902–906

Kushwaha A, R Rani, S Kumar, A Gautam (2016). Heavy metal detoxification and tolerance mechanisms in plants: Implications for phytoremediation. *Environ Rev* 24:39–51

Latif HH, HI Mohamed (2016). Exogenous applications of moringa leaf extract effect on retrotransposon, ultrastructural and biochemical contents of common bean plants under environmental stresses. *South Afr J Bot* 106:221–231

Lavrich RJ, MD Hays (2007). Validation studies of thermal extraction-GC/MS applied to source emissions aerosols. 1. Semivolatile analyte-nonvolatile matrix interactions. *Anal Chem* 79:3635–3645

Lee SC, JH Kim, SM Jeong, DR Kim, JU Ha, KC Nam (2003). Effect of far-infrared radiation on the antioxidant activity of rice hulls. *J Agric Food Chem* 51:4400–4403

Li M, LJ Zhang, L Tao, W Li (2008). Ecophysiological responses of *Jussiaea rapens* to cadmium exposure. *Aquat Bot* 88:347–352

Liu Z, W Chen, X He (2015). Influence of Cd^{2+} on growth and chlorophyll fluorescence in a hyperaccumulator: *Lonicera japonica* Thunb. *J Plant Growth Regul* 34:672–676

Makkar HPS, G Francis, K Becker (2007). Bioactivity of phytochemicals in some lesser-known plants and their effects and potential applications in livestock and aquaculture production systems. *Animal* 1:1371–1391

Martinez V, M Nieves-Cordones, M Lopez-Delacalle, R Rodenas, TC Mestre, F Garcia-Sanchez, F Rubio, PA Nortes, R Mittler, RM Rivero (2018). Tolerance to stress combination in tomato plants: New insights in the protective role of melatonin. *Molecules* 23:535–555

Maxwell K, GN Johnson (2000). Chlorophyll fluorescence - a practical guide. *J Exp Bot* 51:659–668

Mukherjee SP, MA Choudhari (1983). Implications of water stress induced changes in the levels of endogenous ascorbic acid and hydrogen peroxide in *Vigna* seedlings. *Physiol Plantarum* 58:116–170

Nakano Y, K Asada (1981). Hydrogen peroxide is scavenged by ascorbate-specific peroxidase in spinach chloroplasts. *Plant Cell Physiol* 22:867–880

Noctor G, CH Foyer (1998). Ascorbate and glutathione: Keeping active oxygen under control. *Annu Rev Plant Physiol Plant Mol Biol* 49:249–279

Osman AS, MM Rady (2014). Effect of humic acid as an additive to growing media to enhance the production of eggplant and tomato transplants. *J Hortic Sci Biotechnol* 89:237–244

Rady MM (2011). Effect of 24-epibrassinolide on growth, yield, antioxidant system and cadmium content of bean (*Phaseolus vulgaris* L.) plants under salinity and cadmium stress. *Sci Hortic* 129:232–237

Rady MM, GF Mohamed (2015). Modulation of salt stress effects on the growth, physio-chemical attributes and yields of *Phaseolus vulgaris* L. plants by the combined application of salicylic acid and *Moringa oleifera* leaf extract. *Sci Hortic* 193:105–113

Rady MM, KA Hemida (2015). Modulation of cadmium toxicity and enhancing cadmium-tolerance in wheat seedlings by exogenous application of polyamines. *Ecotoxicol Environ Saf* 119:178–185

Rady MM, A Kuşvuran, HF Alharby, Y Alzahrani, S Kuşvuran (2019). Pretreatment with proline or an organic bio-stimulant induces salt tolerance in wheat plants by improving antioxidant redox state and enzymatic activities and reducing the oxidative stress. *J Plant Growth Regul* 38:449–462

Rady MM, CB Varma, SM Howladar (2013). Common bean (*Phaseolus vulgaris* L.) seedlings overcome NaCl stress as a result of presoaking in *Moringa oleifera* leaf extract. *Sci Hortic* 162:63–70

Rehman HU, SMA Basra, MM Rady, AM Ghoneim, Q Wang (2017). Moringa leaf extract improves wheat growth and productivity by delaying senescence and source-sink relationship. *Intl J Agric Biol* 19:479–484

Semida WM, MM Rady (2014). Presoaking application of propolis and maize grain extracts alleviates salinity stress in common bean (*Phaseolus vulgaris* L.). *Sci Hortic* 68:210–217

Semida WM, KA Hemida, MM Rady (2018). Sequenced ascorbate-proline-glutathione seed treatment elevates cadmium tolerance in cucumber transplants. *Ecotoxicol Environ Saf* 154:171–179

Semida WM, MM Rady, TAA El-Mageed, SM Howladar, MT Abdelhamid (2015). Alleviation of cadmium toxicity in common bean (*Phaseolus vulgaris* L.) plants by the exogenous application of salicylic acid. *J Hortic Sci Biotechnol* 90:83–91

Semida WM, RS Taha, MT Abdelhamid, MM Rady (2014). Foliar-applied α-tocopherol enhances salt-tolerance in *Vicia faba* L. plants grown under saline conditions. *S Afr J Bot* 95:24–31

Shi GR, QS Cai, QQ Liu, L Wu (2009). Salicylic acid-mediated alleviation of cadmium toxicity in hemp plants in relation to cadmium uptake, photosynthesis, and antioxidant enzymes. *Acta Physiol Plantarum* 31:969–977

Shishatskaya E, N Menzyanova, S Pyatina, A Shabanov, A Shershneva (2018). Biological activity of humic acid preparations in the form of nanoscale particles in the hydroponic culture of *Triticum aestivum* seedlings. *MOJ Curr Res Rev* 1:180–188

Siddhuraju P, K Becker (2003). Antioxidant properties of various solvent extracts of total phenolic constituents from three different agroclimatic origins of drum-stick tree (*Moringa oleifera* Lam.) leaves. *J Agric Food Chem* 51:2144–2155

Siripornadulsil S, S Traina, DS Verma, RT Sayre (2002). Molecular mechanisms of proline-mediated tolerance to toxic heavy metals in transgenic microalgae. *Plant Cell* 14:2837–2847

Smirnoff N (2000). Ascorbic acid: Metabolism and functions of a multifaceted molecule. *Curr Opin Plant Biol* 3:229–235

Sullivan CY, WM Ross (1979). Selecting the drought and heat resistance in grain sorghum. *In*: *Stress Physiology in Crop Plants*, pp:263–281. Mussel H, RC Staples (Eds.). John Wiley & Sons, New York, USA

Valero E, H Marcià, IMDL Fuente, JA Henandez, MI Gonzàles-Sànchez, F Garcia-Carmona (2016). Modeling the ascorbate-glutathione cycle in chloroplasts under light/dark conditions. *BMC Syst Biol* 10; Article 11

Velikova V, I Yordanov, A Edreva (2000). Oxidative stress and some antioxidant systems in acid rain-treated bean plants. *Plant Sci* 151:59–66

Williams V, S Twine (1960). Flame photometric method for sodium, potassium and calcium. *In: Modern Methods of Plant Analysis*, Vol. 5. Springer International, New York, USA

Zhang HZ, QJ Guo, JX Yang, JX Shen, TB Chen, GX Zhu, H Chen, CY Shao (2015). Subcellular cadmium distribution and antioxidant enzymatic activities in the leaves of two castor (*Ricinus communis* L.) cultivars exhibit differences in Cd accumulation. *Ecotoxicol Environ Saf* 120:184–192

Zhou Y, Z Wen, J Zhang, X Chen, J Cui, W Xu, HY Liu (2017). Exogenous glutathione alleviates salt-induced oxidative stress in tomato seedlings by regulating glutathione metabolism, redox status and the antioxidant system. *Sci Hortic* 220:90–101

Zhu G, H Xiao, Q Guo, Z Zhang, J Zhao, D Yang (2018). Effects of cadmium stress on growth and amino acid metabolism in two Compositae plants. *Ecotoxicol Environ Saf* 158:300–308

Zhu GX, QJ Guo, HY Xiao, TB Chen, J Yang (2017). Multivariate statistical and lead isotopic analyses approach to identify heavy metal sources in topsoil from the industrial zone of Beijing capital iron and steel factory. *Environ Sci Pollut Res* 24:14877–14888