

## Foliar Application of Fresh Moringa Leaf Extract Overcomes Salt Stress in Fenugreek (*Trigonella foenum-graecum*) Plants

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FRESH moringa leaf extract (MLE) was applied and its impact was assessed on growth as well as some physiological and biochemical parameters of fenugreek (*Trigonella foenum-graecum*) grown under four levels (0, 50, 100 and 200 mM) of NaCl. Salinity decreased growth criteria, the contents of photosynthetic pigments, organic solutes (except proline), total phenols,  $K^+$ ,  $Ca^{2+}$ ,  $Mg^{2+}$ , the ratio of  $K^+/Na^+$  and  $Ca^{2+}/Na^+$  and peroxidase (POD) activity. On the other hand, salinity stress boosted the contents of proline,  $Na^+$ ,  $Cl^-$  malonaldehyde (MDA), the activity of catalase (CAT) and ascorbate peroxidase (APX). Interestingly, superoxide dismutase (SOD) activity remained unchanged under salinity levels. Foliar application of MLE ameliorated the negative impact of salinity to considerable extent by enhancing growth traits and all above parameters except  $Na^+$ ,  $Cl^-$  and MDA. Under saline concentrations, foliar application with MLE led to the appearance of new 12 polypeptides. The 39, 21, 19, 17 and 16 kDa protein bands that were absent under the influence of salinity occurred under the combined effect of salinity and MLE. These results proposed that foliar application of MLE could mitigate the harmful effect of salinity on fenugreek and the strategy may be employed to enhance the crop production in saline soils.

**Keywords:** Antioxidant enzymes, Moringa leaf extract, Na Cl, Osmolytes, Protein patterns, *Trigonella foenum-graecum*.

Environmental constraints like increased salinity have stern effects on plant growth and development with consequences being much evident in arid and semiarid regions of the globe where salinity has proven to be a major problem (Abdel Latef and Miransari, 2014; Ahmad *et al.*, 2015; Liu and *et al.*, 2016). Among the prime reasons that cause further aggravation in this environmental constraint is the excessive use of saline water for irrigation purposes thereby converting the arable land into asalinized wasteland. It has been reported that near about 5 to 7% of total universal land is salt affected and is expected to increase in near future (Ruiz-Lozano *et al.*, 2012). Salinity imposes both osmotic and ionic effects thereby causing reductions in normal growth through deleterious impact on the important physiological and bio-chemical processes such as photosynthesis and ion balance (Porcel *et al.*, 2012; Iqbal *et al.*, 2015; Ahmad *et al.*, 2016). Salinity triggered alterations in photosynthetic capacity is associated with the perturbations in carbon and nitrogen metabolism.

Accumulation of osmotically compatible osmolytes including proline, glycine betaine, total free amino acids, soluble protein and soluble sugar reduces noxious impact of stress by maintaining the tissue water balance (Abdel Latef and Tran, 2016; Ahmad *et al.*, 2016 and Liu *et al.*, 2016). However, more precisely, under salt stress regimes efficient and screened nutrient uptake and subsequent accumulation of ions like sodium ( $\text{Na}^+$ ), chloride ( $\text{Cl}^-$ ) and potassium ( $\text{K}^+$ ) contribute more towards osmoregulation.

Plant phenolics represent a vast group of secondary metabolites mainly comprising phenylpropanoids, flavonoids, tannins, coumarins and lignin precursors (Wilfred and Nicholson, 2006). At certain optimal concentration, phenolic compounds can protect plant metabolism through their active involvement in preventing oxidative damage through free radical scavenging and enhancing membrane stability (Arora *et al.*, 2000; Verstraeten *et al.*, 2003 and Michalak, 2006).

Presence of high salt concentrations in the soil solution initiates formation of reactive oxygen species (ROS) (including superoxide, hydroxyl and peroxide radicals) and their over accumulation result in oxidative pressure thereby affecting important macromolecules like lipids, proteins, chlorophylls and DNA (Abdel Latef and Chaoxing, 2011 and Ahmad *et al.*, 2016). In order to circumvent the stress induced deleterious effects, plants employ different mechanisms. Among these are included the up-regulation of antioxidant enzymes and the accumulation of osmotic constituents (Ahanger *et al.*, 2015). Antioxidant defense includes superoxide dismutase (SOD), catalase (CAT), peroxidase (POD) and ascorbate peroxidase (APX) which mediate the removal of ROS to provide protection to cell and the cellular functioning under stressful conditions by reducing the oxidative damage (Abdel Latef, 2010, 2011; Mostofa *et al.*, 2015 and Ahmad *et al.*, 2016).

Application of plant extracts has been reported to reinforce the growth of crop plants, however; this stimulatory effect is concentration dependent (Tomar and Agarwal, 2013; Tomar *et al.*, 2015). Chemicals released by plants show diverse variation with age and the difference in impact much depends on the plant part. Moringa (*Moringa oleifera*) leaves are well-known as vegetable from Moringaceae family and are rich in vitamins, particularly A and C, iron, calcium, carotenes and phenolics (Foidl *et al.*, 2001; Rady *et al.*, 2013; Yasmien *et al.*, 2013a,b; Rady and Mohamed, 2015). Leaf extracts of moringa show potential antioxidant properties (Siddhuraju and Becker, 2003; Nouman *et al.*, 2014a). In addition, extracts of moringa possess sufficient quantity of cytokinins (Rady *et al.*, 2013, Rady and Mohamed, 2015). Albeit knowledge about the mineral richness, presence of sugars, amino acids, increased antioxidant potential and presence of phytohormones like cytokinins in moringa extracts is available (Nouman *et al.*, 2014b), but scanty reports are available discussing the

amelioration of salt stress by moringa extracts. Application of nutrient, metabolite, vitamin and hormone rich moringa extracts to plants can be beneficial from the stress perspectives that mostly hamper the synthesis of such important metabolites.

Legumes show altered nitrogen fixation, metabolism and growth in response to abiotic stressors (Abdel Latef and Ahmad, 2015). Fenugreek (*Trigonella foenum-graecum* L.) is a self-pollinating annual forage legume within the family Fabaceae and it is used as a cover crop, green manure and forage crop in addition to its wide importance as a medicinal herb. Biochemical constituents like steroids, saponins, polysaccharides and alkaloids contribute to its medicinal and pharmaceutical importance for treatment of ailments like diabetes and hyperglycaemia (Srinivasan, 2006; Zandi *et al.*, 2015).

The aim of the present work is to study: (1) impact of NaCl on growth, photosynthetic pigments, primary and secondary metabolites, mineral uptake, oxidative stress, antioxidant machinery and protein profile of fenugreek plants, (2) possible ameliorative role of moringa leaf extract (MLE) in NaCl stressed-fenugreek plants through the modulation of the above mentioned characteristics.

### Materials and Methods

#### *Preparation of MLE*

MLE was prepared by collecting young fresh leaves harvested from moringa (*Moringa oleifera* Lam.) tree grown in Aswan botanical garden, Aswan, Egypt. Leaves were washed to remove the dust and then frozen in refrigerator at 4°C for two days (Iqbal, 2014). Leaves were pulverized in a manual juicer and the extract was filtered through muslin cloth to remove the cellular debris (Iqbal, 2014). Then, the extract was centrifuged at  $8,000 \times g$  for 15 min and the supernatant was taken and diluted 25 times (MLE25) for usage as foliar spray.

#### *Pot experiment*

Present work was carried out in the wire-house of the experimental farm of South Valley University, Qena, Egypt during growing season 2014-2015. Fenugreek seeds were sterilized in 5% sodium hypochlorite (NaOCl) solution for 5 min to avert contamination. Equal number of seeds (15 seed/pot) of fenugreek were sown in plastic pots (30 cm diameter) filled with 2 kg of dried soil and pots were arranged in completely randomized design in factorial arrangement with three replications. The climatic conditions were: mean day/night temperature cycle of 24/14°C, light 14/10 h and air humidity between 35 % and 70%. Seven days after germination, seedlings were thinned to five per pot, then the pots were irrigated with a constant amount of different salt concentrations, *i.e.*, 0, 50, 100 and 200 mM NaCl. The seedlings of both control and NaCl treated pots were

sprayed with MEL25 after 4 and 10 days of treatment with NaCl levels and the untreated control plants were sprayed with distilled water only using a hand spray pump. The MEL25 and NaCl concentrations were selected based on our preliminary experiment. All pots were supplemented once every week with Hoagland's nutrient solution (1/2 strength). Plant samples were collected after 5 weeks of sowing for further experimentation.

#### *Growth measurement*

The lengths of root and shoot were measured by manual scale. After recording fresh weights, roots and shoots were oven dried to constant weight at 80°C for their dry weight.

#### *Determination of photosynthetic pigments*

The method of Arnon (1949) was used for determination photosynthetic pigments in fresh leaves. The absorbance of the extract was measured at 663, 645 and 480 nm.

#### *Determination of osmolytes and total phenols*

Soluble sugar content of dry shoot was estimated by the anthrone sulphuric acid method as described by Badour (1959). The method of Bradford (1976) was used to estimate soluble protein content of dry shoot. Total free amino acid contents were measured in dry shoot by Lee and Takahashi (1966) method. The proline content was estimated in dry shoot according to Bates *et al.* (1973) procedure. Total phenols of dry shoot were estimated by Folin-Ciocalteu's reagent (Skerget *et al.*, 2005).

#### *Determination of mineral ions*

Na<sup>+</sup> and K<sup>+</sup> estimation of dry shoot was done by using flame photometer (Williams and Twine, 1960). Calcium (Ca<sup>2+</sup>) and magnesium (Mg<sup>2+</sup>) of dry shoot were determined titrimetrically as described by Bower and Hatcher (1962). Cl<sup>-</sup> of dry shoot was measured by titration with silver nitrate according to Cotlove (1965).

#### *Determination of malondialdehyde (MDA)*

MDA was measured in fresh leaf sample according to the method followed by Heath and Packer (1968).

#### *Assays of antioxidant enzyme activities*

Fresh leaf sample was frozen in liquid nitrogen and grinded in 10 ml of 100 mM phosphate buffer (pH 7.0) containing 0.1mM EDTA and 0.1% polyvinyl pyrrolidone. Homogenate was centrifuged at 15,000 × g at 4 °C for 10 min and used for estimation of SOD, CAT and POD (Abdel Latef and Tran, 2016). For estimation of APX, extraction medium was supplemented by 2 mM ascorbate (Ahmad *et al.*, 2016).

The activity of SOD (EC 1.15.1.1) was determined by monitoring photo inhibition of nitro blue tetrazolium (NBT) at 560 nm (Giannopolitis and Ries, 1977). Amount of enzyme required to cause 50 % inhibition of the reduction of NBT was considered as one unit. CAT (EC 1.11.1.6) activity was assayed by observation of the reduction in absorbance at 240 nm as a consequence of H<sub>2</sub>O<sub>2</sub> disappearance (Aebi, 1984). POD (EC 1.11.1.7) activity was estimated by determination of the oxidation of guaiacol in the presence of H<sub>2</sub>O<sub>2</sub> at 470 nm (Maehly and Chance, 1954 as modified by Klapheck *et al.*, 1990). APX (EC 1.11.1.11) activity was measured by the method followed by Chen and Asada (1992).

#### *Protein electrophoretic studies*

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) was performed to distinguish and fragment total soluble protein for *Trigonella foenum-graecum* leaf samples according to the methods of Lammler (1970).

#### *Statistical analysis*

All data shown are the mean values. Data were statistically analyzed by the analysis of variance (ANOVA) with SAS software (Version 9.1; SAS Institute, Cary, NC, USA) followed by Duncan's multiple range test.  $P \leq 0.05$  were considered as significant. Data represented in the Tables and Figures are means  $\pm$  standard deviation (SD) of five independent replicates of each treatment.

## **Results**

#### *Growth traits*

*Trigonella foenum-graecum* subjected to salinity stress showed considerable reduction in the growth attributes like root and shoot length, fresh weight (FW) and dry weight (DW) of root and shoot (Table 1). Relative to untreated control, 200 mM NaCl reduced root and shoot length (54.57% and 41.01%, respectively), FW of root and shoot (53.57% and 43.54%, respectively) and DW of root and shoot (70.43% and 60.31%, respectively) (Table 1). However, foliar spraying of MLE significantly enhanced these attributes and also mitigated the negative impact of salt stress (Table 1). In non-stressed plants, the percent increase in length, FW and DW by application of MLE was 25.92%, 28.57% and 12.17%, respectively in root and 40.81%, 76.01% and 42.85%, respectively in shoot as compared to untreated control (Table 1). In treatment with 200 mM NaCl + MLE, percent increase in length, FW and DW of root was 39.93%, 69.23% and 73.52%, respectively and 42 %, 46.40% and 80%, respectively in shoot, over the plants exposed to 200 mM NaCl alone (Table 1).

#### *Photosynthetic pigments*

In comparison with untreated control, the values of photosynthetic pigments gradually lessened with the rise of salinity levels (Fig. 1). The maximum

reduction in Chl a (38.47%), Chl b (41.86%) or-carotenoids (48.70%) was pronounced at 200 mM NaCl. Spraying stressed plants by MLE, *i.e.* 50 mM NaCl + MLE, 100 mM NaCl + MLE and 200 mM NaCl + MLE significantly enhanced Chla (37.06%, 20.23% and 24.59%), Chl b (36.75%, 14.83% and 42%) and carotenoids (40.14%, 30.08% and 64.55%), respectively against the salinized plants alone (Fig. 1). However, percent increase due to foliar application of MLE alone was 32.85%, 29.06% and 36.36%, respectively for Chl a, Chl b and carotenoids over the untreated control (Fig. 1).

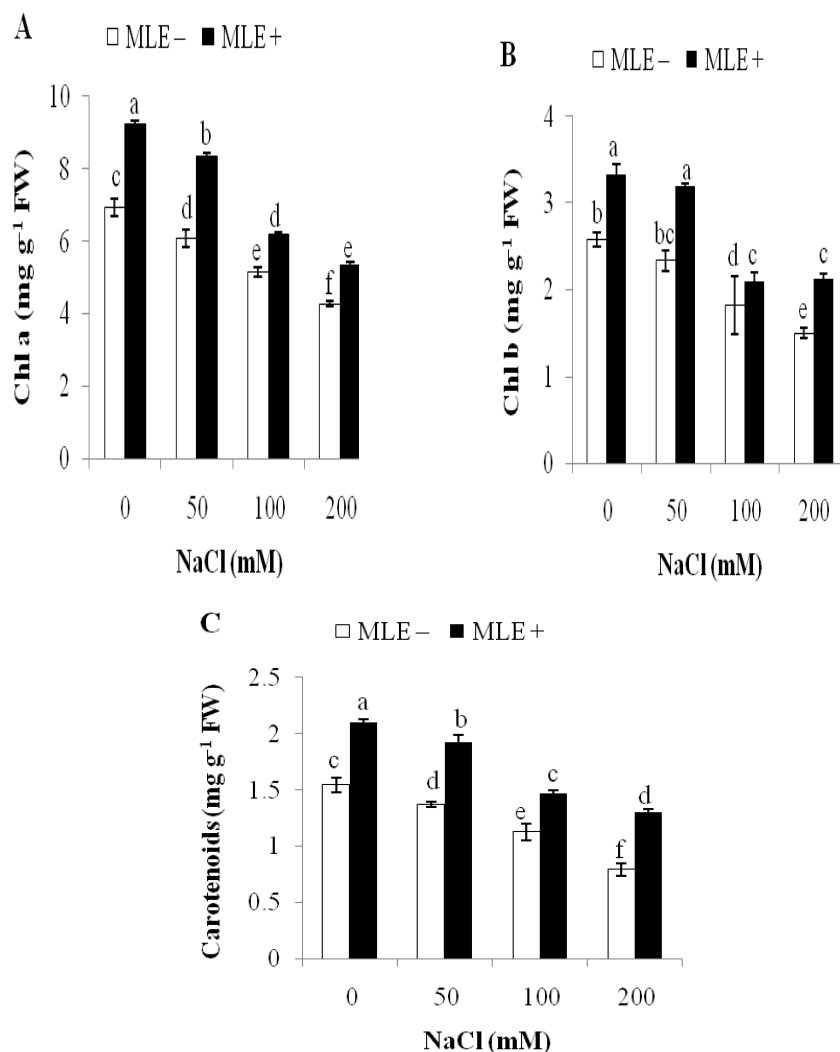
**TABLE 1. Effect of salt stress and foliar application of moringa leaf extract (MLE) on root length (cm plant<sup>-1</sup>), shoot length (cm plant<sup>-1</sup>), fresh weight (FW) of root (g plant<sup>-1</sup>), fresh weight (FW) of shoot (g plant<sup>-1</sup>), dry weight (DW) of root (g plant<sup>-1</sup>) and dry weight (DW) of shoot (g plant<sup>-1</sup>) of 5- week old fenugreek plants (*Trigonellafoenum-graecum*). Data presented are means  $\pm$  SD (n=3). Data followed by similar letters are non significantly different by Duncan's multiple range test at  $P \leq 0.05$ .**

Treatments (NaCl; mM)	MLE	Root length	Shoot length	FW Root	FW shoot	DW root	DW shoot
0	- MLE	7.33 $\pm 1.07^b$	14.53 $\pm 1.02^c$	0.28 $\pm 0.03^b$	2.71 $\pm 0.08^c$	0.115 $\pm 0.004^b$	0.63 $\pm 0.06^b$
	+ MLE	9.23 $\pm 0.21^a$	20.46 $\pm 0.40^a$	0.36 $\pm 0.03^a$	4.77 $\pm 0.52^a$	0.129 $\pm 0.004^a$	0.90 $\pm 0.05^a$
50	- MLE	5.33 $\pm 0.42^{cd}$	13.13 $\pm 0.67^{de}$	0.23 $\pm 0.02^{bc}$	2.36 $\pm 0.12^{cde}$	0.076 $\pm 0.011^d$	0.48 $\pm 0.04^c$
	+ MLE	8.60 $\pm 0.30^a$	17.89 $\pm 0.21^b$	0.33 $\pm 0.05^a$	4.13 $\pm 0.09^b$	0.120 $\pm 0.003^{ab}$	0.85 $\pm 0.03^a$
100	- MLE	4.43 $\pm 0.21^e$	11.50 $\pm 1.32^f$	0.20 $\pm 0.02^c$	2.07 $\pm 0.20^e$	0.050 $\pm 0.004^e$	0.36 $\pm 0.04^d$
	+ MLE	5.55 $\pm 0.29^c$	13.67 $\pm 0.23^{cd}$	0.25 $\pm 0.03^{bc}$	2.52 $\pm 0.18^{cd}$	0.086 $\pm 0.007^c$	0.51 $\pm 0.04^c$
200	- MLE	3.33 $\pm 0.15^f$	8.57 $\pm 0.59^g$	0.13 $\pm 0.02^d$	1.53 $\pm 0.09^f$	0.034 $\pm 0.004^f$	0.25 $\pm 0.03^e$
	+ MLE	4.66 $\pm 0.40^{de}$	12.17 $\pm 0.45^{ef}$	0.22 $\pm 0.03^c$	2.24 $\pm 0.07^{de}$	0.059 $\pm 0.005^e$	0.45 $\pm 0.04^c$

#### *Organic solutes and total phenols*

Exposure of *Trigonellafoenum-graecum* to salt stress resulted in a marked decrease in the contents of soluble sugars, soluble proteins, total free amino acids and total phenols. The concentration 200 mM NaCl recorded the highest reduction in soluble sugars (45.03%), soluble proteins (35.90%), total free amino acids (48.70%) and total phenols (38.89%), as compared to the corresponding values of the untreated control (Table 2). On the other side, an opposite pattern was noticed

in proline content, where salt stress resulted in dramatically accumulated proline content with highest accumulation (77.14%) at 200 mM NaCl, compared to control (Table 2). Application of MLE alone or in combination with salt significantly elevated the content of the above parameters over those of either the untreated control or NaCl- stressed plants (Table 2).



**Fig. 1.** Effect of salt stress and moringa leaf extract (MLE) on photosynthetic pigments content of fenugreek (*Trigonella foenum-graecum*) leaves. Data presented are the means  $\pm$  SD (n=3). Data followed by similar letters are not significantly different by Duncan's multiple range test at  $P \leq 0.05$ .

**TABLE 2.** Effect of salt stress and foliar application of moringa leaf extract (MLE) on the content of soluble sugars ( $\text{mg g}^{-1}$  DW), soluble proteins ( $\text{mg g}^{-1}$  DW), total free amino acids ( $\text{mg g}^{-1}$  DW), proline ( $\text{mg g}^{-1}$  DW) and total phenols ( $\text{mg GAE g}^{-1}$  DW) of fenugreek (*Trigonella foenum-graecum*) shoot. Data presented are the means  $\pm$  SD ( $n=3$ ). Data followed by similar letters are non significantly different by Duncan's multiple range test at  $P \leq 0.05$ . GAE, gallic acid equivalent.

Treatments (NaCl; mM)	MLE	Soluble sugars	Soluble proteins	Total free amino acids	Proline	Total phenols (GAE)
0	- MLE	54.96 $\pm 4.02^b$	73.72 $\pm 2.74^{bc}$	9.26 $\pm 0.38^c$	0.35 $\pm 0.02^f$	8.87 $\pm 0.10^c$
	+ MLE	68.36 $\pm 1.60^a$	81.03 $\pm 3.77^a$	12.62 $\pm 0.13^a$	0.45 $\pm 0.04^e$	10.85 $\pm 0.07^a$
50	- MLE	47.86 $\pm 2.54^c$	63.00 $\pm 3.02^{de}$	8.22 $\pm 0.12^d$	0.43 $\pm 0.02^e$	7.54 $\pm 0.19^e$
	+ MLE	64.35 $\pm 3.47^a$	76.28 $\pm 5.38^{ab}$	11.52 $\pm 0.41^b$	0.55 $\pm 0.03^d$	10.34 $\pm 0.08^b$
100	- MLE	41.90 $\pm 1.86^c$	58.16 $\pm 1.14^e$	6.76 $\pm 0.46^e$	0.51 $\pm 0.04^d$	6.22 $\pm 0.13^e$
	+ MLE	54.30 $\pm 3.66^b$	68.49 $\pm 3.82^{cd}$	8.82 $\pm 0.17^c$	0.71 $\pm 0.10^b$	7.83 $\pm 0.03^d$
200	- MLE	30.21 $\pm 4.93^d$	47.25 $\pm 3.24^f$	4.75 $\pm 0.31^f$	0.62 $\pm 0.05^c$	5.42 $\pm 0.10^b$
	+ MLE	44.54 $\pm 4.00^c$	58.75 $\pm 1.03^e$	7.78 $\pm 0.22^d$	0.84 $\pm 0.04^a$	7.02 $\pm 0.10^f$

#### Mineral ions

The data in Table 3 illustrate that, the contents of  $\text{Na}^+$  and  $\text{Cl}^-$  were positively affected by increasing salt stress. Reversibly, the contents of  $\text{K}^+$ ,  $\text{Ca}^{2+}$ , and  $\text{Mg}^{2+}$  were negatively affected by increasing salt stress and attained their lowest values at the highest salt level (200 mM NaCl). Accordingly,  $\text{K}^+/\text{Na}^+$  and  $\text{Ca}^{2+}/\text{Na}^+$  ratios were decreased by increasing NaCl concentration. On the other hand, foliar application of MLE reduced the levels of  $\text{Na}^+$  and  $\text{Cl}^-$ , and increased the content of  $\text{K}^+$ ,  $\text{Ca}^{2+}$ ,  $\text{Mg}^{2+}$  as well as  $\text{K}^+/\text{Na}^+$  and  $\text{Ca}^{2+}/\text{Na}^+$  ratios, compared with the corresponding MLE-untreated plants (Table 3).

#### Oxidative stress

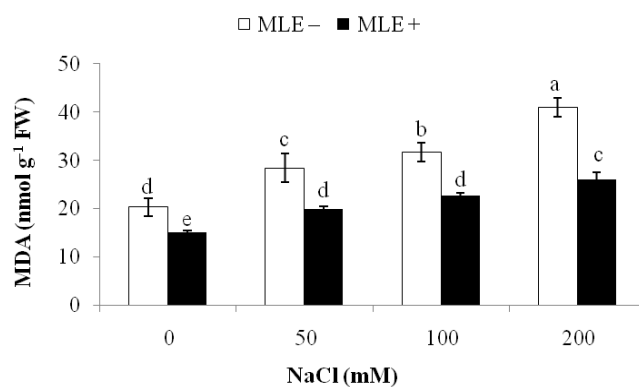
Salt stress caused increased content of MDA by 39.76%, 56% and 101.47% at 50, 100 and 200 mM NaCl, respectively over untreated control (Fig. 2). The content of MDA was reduced by 25.74%, 29.72%, 28.42% and 36.52% at 0 mM NaCl + MLE, 50 mM NaCl+ MLE, 100mM NaCl + MLE and 200 mM



NaCl + MLE, respectively, compared to 0 mM NaCl, 50 mM NaCl, 100 mM NaCl and 200 mM NaCl, respectively (Fig. 2).

**TABLE 3.** Effect of salt stress and foliar application of moringa leaf extract (MLE) on  $\text{Na}^+$ ,  $\text{Cl}^-$ ,  $\text{K}^+$ ,  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  contents each as  $\text{mg g}^{-1}$  DW as well as the ratios of  $\text{K}^+/\text{Na}^+$  and  $\text{Ca}^{2+}/\text{Na}^+$  of fenugreek (*Trigonella foenum-graecum*) shoot. Data presented are the means  $\pm$  SD (n=3). Data followed by similar letters are not significantly different by Duncan's multiple range test at  $P \leq 0.05$ .

Treatments (NaCl; mM)	MLE	$\text{Na}^+$	$\text{Cl}^-$	$\text{K}^+$	$\text{Ca}^{2+}$	$\text{Mg}^{2+}$	$\text{K}^+/\text{Na}^+$	$\text{Ca}^{2+}/\text{Na}^+$
0	- MLE	13.54 $\pm 1.23^d$	11.08 $\pm 0.55^e$	34.62 $\pm 0.12^b$	10.19 $\pm 0.17^c$	2.34 $\pm 0.10^c$	2.57 $\pm 0.23^b$	0.92 $\pm 0.06^c$
	+ MLE	10.06 $\pm 0.19^e$	8.60 $\pm 0.72^f$	36.57 $\pm 0.63^a$	13.13 $\pm 0.63^a$	4.27 $\pm 0.16^a$	3.63 $\pm 0.10^a$	1.53 $\pm 0.09^a$
50	- MLE	18.93 $\pm 1.96^c$	14.68 $\pm 0.28^c$	29.19 $\pm 0.94^d$	9.04 $\pm 0.31^{de}$	2.02 $\pm 0.07^d$	1.55 $\pm 0.19^d$	0.61 $\pm 0.03^e$
	+ MLE	13.31 $\pm 0.33^d$	10.55 $\pm 0.53^e$	35.72 $\pm 0.62^a$	17.79 $\pm 0.26^b$	3.40 $\pm 0.13^b$	2.68 $\pm 0.03^b$	1.11 $\pm 0.07^b$
100	- MLE	21.14 $\pm 1.28^b$	17.18 $\pm 0.55^b$	23.54 $\pm 0.31^f$	8.13 $\pm 0.31^f$	1.62 $\pm 0.11^f$	1.11 $\pm 0.07^e$	0.47 $\pm 0.02^f$
	+ MLE	15.33 $\pm 0.32^d$	12.53 $\pm 0.30^d$	32.40 $\pm 0.55^c$	9.30 $\pm 0.08^d$	2.05 $\pm 0.06^d$	2.14 $\pm 0.05^c$	0.74 $\pm 0.04^d$
200	- MLE	27.29 $\pm 1.28^a$	21.61 $\pm 0.55^a$	18.54 $\pm 0.87^e$	6.56 $\pm 0.31^e$	1.25 $\pm 0.12^e$	0.68 $\pm 0.06^f$	0.30 $\pm 0.01^e$
	+ MLE	17.32 $\pm 0.32^c$	14.67 $\pm 0.30^c$	25.69 $\pm 0.55^e$	8.55 $\pm 0.08^{ef}$	1.81 $\pm 0.06^e$	1.48 $\pm 0.08^d$	0.58 $\pm 0.02^e$



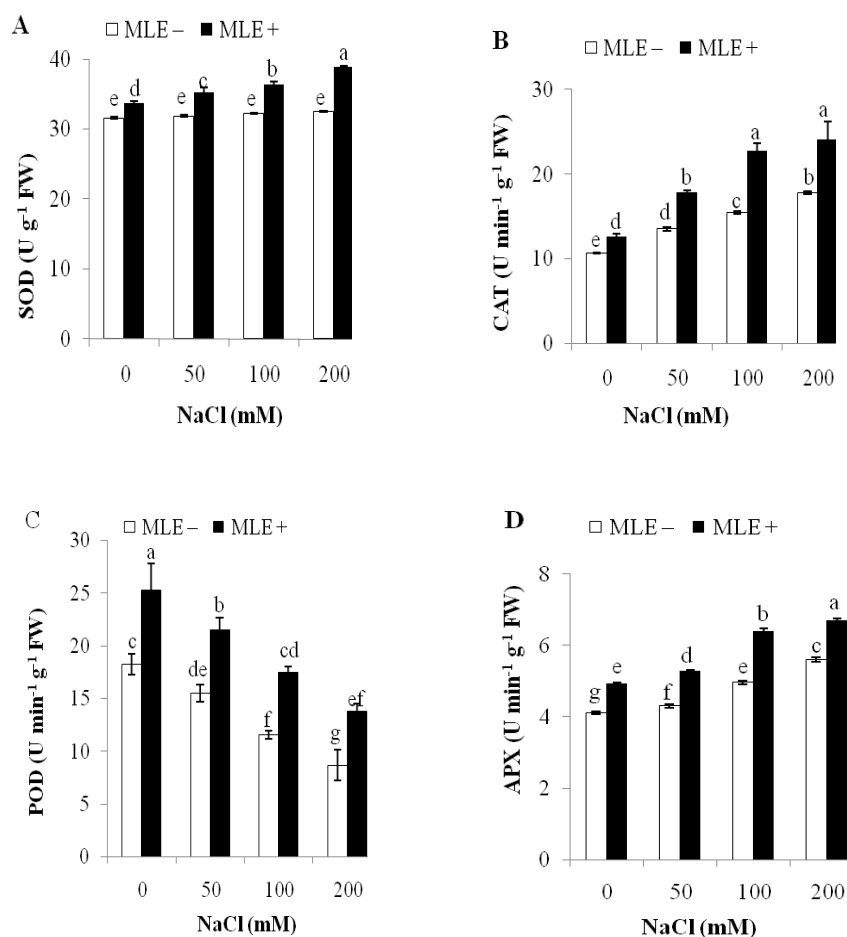
**Fig. 2.** Effect of foliar application of moringa leaf extract (MLE) on malonaldehyde (MDA) content of fenugreek (*Trigonella foenum-graecum*) leaves. Data presented are the means  $\pm$  SD (n=3). Data followed by similar letters are not significantly different by Duncan's multiple range test at  $P \leq 0.05$ .

#### *Activities of antioxidant enzymes*

Figure 3A shows that, the value of SOD activity was non significantly changed at all adopted salinity levels versus untreated control. However, foliar application of MLE induced a significant increase in SOD activity in non-salinized and salinized plants compared to non-salinized and salinized plants alone. The maximum increase (19.37%) in SOD activity was obvious at 200 mM NaCl + MLE against 200 mM NaCl alone (Fig. 3A). CAT activity was significantly increased with the rise in salinity levels and the maximum increase of 66.85% was recorded at 200 mM NaCl over the untreated control (Fig. 3B). Treatment with MLE further increased CAT activity in stressed plants relative to non-stressed and stressed plants alone. The maximum value (47.44%) of CAT activity was recorded at 100 mM NaCl + MLE versus 100 mM NaCl alone (Fig. 3B). Treatment with NaCl induced a marked and progressive decrease in POD activity, as compared with untreated control and maximum decrease of 52.35% was recorded at 200 mM NaCl, as compared to untreated control (Fig. 3C). Application of MLE modulated the POD activity in stressed plants, compared to non-stressed and stressed plants alone and the highest modulation (58.27%) was noticed at 200 mM NaCl versus 200 mM NaCl (Fig. 3C). There was a marked and progressive increment in the activity of APX with the rise of NaCl concentrations, where maximum increment (36.58%) was pronounced at 200 mM NaCl (Fig. 3D). Application of MLE further increased APX activity at all NaCl stress levels and the maximum activity (29.03%) was recorded at 100 mM+MLE, compared to 100 mM NaCl (Fig. 3D).

#### *Protein patterns*

A total of 18, 13, 12 and 20 proteins were expressed in the 0, 200 mM NaCl, 0 + MLE and 200 mM NaCl + MLE treated plants, respectively (Table 4). In the present study, two types of modifications were observed in the protein patterns of *Trigonella foenum-graecum* leaves, *i.e.* disappearance of several proteins and occurrence of new sets of proteins (Table 4). Two protein bands with molecular weights 50 and 38 kDa (Table 4) were *de novo* synthesized in *Trigonella foenum-graecum* plant grown under the influence of 200 mM NaCl. Salinity stress caused the disappearance of 7 protein bands having molecular weights 51, 39, 25, 21, 19, 17 and 16 kDa (Table 4). In 0 + MLE plants, foliar application with MLE led to the net synthesis of 5 polypeptides with molecular weights 74, 59, 52, 43 and 35 kDa and disappearance of 11 protein bands with molecular weights 66, 57, 51, 44, 42, 39, 36, 32, 18, 17 and 16 kDa (Table 4). In saline-stressed plants, it was worthy to notice that MLE application provoked the appearance of 12 polypeptides with molecular weights 83, 71, 61, 55, 48, 43, 38, 35, 33, 30, 27 and 23 kDa (Table 4).



**Fig. 3.** Effect of salt stress and foliar application of moringa leaf extract (MLE) on the activity of (A) superoxide dismutase (SOD), (B) catalase (CAT), (C) peroxidase (POD) and (D) ascorbate peroxidase (APX) activity of fenugreek (*Trigonella foenum-graecum*) leaves. Data presented are the means  $\pm$  SD ( $n=3$ ). Data followed by similar letters are not significantly different by Duncan's multiple range test at  $P \leq 0.05$ .

**TABLE 4. Comparative analysis of molecular weight (M.Wt.) and relative front (R<sub>f</sub>) of SDS-PAGE protein profile of fenugreek (*Trigonella foenum-graecum*) leaves under salt stress (200 mM NaCl) and foliar application of moringa leaf extract (MLE).**

M.Wt. (kDa)	R <sub>f</sub>	200 mM NaCl			
		0	200 mM NaCl	0 + MLE	+ MLE
83	0.107				(+)*
74	0.136			(+)*	
71	0.149				(+)*
66	0.169	+	+	-	-
61	0.194				(+)*
59	0.211			(+)*	
57	0.223	+	+	-	-
55	0.240				(+)*
52	0.273			(+)*	
51	0.281	+	-	-	-
50	0.285		(+)*		
48	0.306				(+)*
44	0.351	+	+	-	-
43	0.368			(+)*	(+)*
42	0.380	+	+	-	-
39	0.405	+	-	-	+
38	0.417		(+)*		(+)*
36	0.446	+	+	-	-
35	0.463			(+)*	(+)*
33	0.483				(+)*
32	0.500	+	+	-	-
30	0.529				(+)*
28	0.570	+	+	+	
27	0.587			+	(+)*
25	0.632	+	-	+	-
24	0.657	+	+	+	+
23	0.678				(+)*
22	0.727	+	+	+	+
21	0.798	+	-	+	+
20	0.831	+	+	+	+
19	0.868	+	-		+
18	0.897	+	+	-	-
17	0.963	+	-	-	+
16	0.988	+	-	-	+
Total numbers of bands		18	13	12	20
Number of new bands (+)*			2	5	12

### Discussion

Reduced growth rate in fenugreek plants under the influence of salinity was reflected as reduction in plant length as well as fresh and dry weights. This might be due to hampered cell division and cell elongation that is exerted by the presence of high levels of salts (Azooz *et al.*, 2004 a,b; Rady and Mohamed, 2015). Application of moringa leaf extract (MLE) not only assuaged the negative deleterious impact of salinity on growth of fenugreek plants but also increased the attributes considerably when applied alone. Such finding supported the stimulatory effects of moringa extracts as has been reported by Rady *et al.* (2013) and Rady and Mohamed (2015). Application of MLE to salt stressed *Phaseolus vulgaris* resulted in improved growth by modulating the metabolism and the water use efficiency (Howladar, 2014). Priming wheat seeds with extracts of moringa led to increased growth and biomass (Yasmeen *et al.*, 2013b) by modulating the metabolite fraction (Imran *et al.*, 2014). Aqueous fresh and dry extracts of plants show stimulatory impacts on growth through bringing up-regulation in the activities of various enzymes involved in metabolically important pathways (Tomar *et al.*, 2015).

Plants growing under salt concentrations show altered synthesis of photosynthetic pigments. Reduction in the pigments synthesis is the cumulative effect of several factors such as osmotic stress-induced reduction in water content, altered mineral uptake and down-regulation in the activities of key photosynthetic enzymes like  $\delta$ -aminolevulinic acid dehydratase and protochlorophyllide reductase (Padmaja *et al.*, 1990; Abd-Allah *et al.*, 2015). In the present study, application of MLE enhanced the chlorophyll contents under normal and NaCl stressed conditions. Foliar application of moringa leaf extract to wheat (Yasmeen *et al.*, 2013a) and bean (Howladar, 2014) showed significant positive effects on chlorophyll pigment components resulting in higher photosynthesis under normal as well as stressed conditions. Carotenoids were significantly enhanced due to MLE application which might be assumed to contribute in protection of macromolecules including proteins, DNA and RNA from the toxic effects of free radicals (Choudhary *et al.*, 2012; Ahmad *et al.*, 2015; Abdel Latef and Tran, 2016) by quenching of  $^1O_2$  generated during photosynthesis thereby mediating the optimal electron flow (Telfer *et al.*, 2003).

In this work, the reduction in growth, especially at severe salinity, was connected with diminution in the contents of soluble sugars, soluble protein and total free amino acids. Oppositely increased proline even at the low level of salinity could have been brought about by high necessities for osmoregulation and membrane adjustment (Azooz *et al.*, 2015). In the present work, when salt-stressed *Trigonella foenum-graecum* plants were sprayed with MLE, proline

content was increased, compared with stressed plants alone. This increment in proline content was linked with a marked increase of other organic solutes (soluble sugars, soluble protein, and total free amino acids), suggesting an ameliorating effect and other strategies by which MLE could adjust the osmotic potential of *Trigonella foenum-graecum* plants and hence increase their resistance to salinity stress.

Presence of phenols and other active metabolites within the MLE were assumed to prevent excessive membrane leakage and provide stability to structures suffering from lipid peroxidation and such promotion of MLE has also been observed in salt stressed *Phaseolus vulgaris* (Howladar, 2014). Our results are also in harmony with those of Yasmeen *et al.* (2013a) who reported that accumulation of phenols by MLE application enhanced salt tolerance by mediating scavenging of ROS and maintaining membrane stability.

Salt stress showed impeded uptake of essential mineral elements in *Trigonella foenum-graecum* plants. Higher concentrations of  $\text{Na}^+$  and  $\text{Cl}^-$  ions are deleterious to plant and cause disturbance in the mobility of potassium and calcium within the plant (Iqbal *et al.*, 2015). Several studies have witnessed the antagonistic relationship of  $\text{Na}^+$  with several other important ions like  $\text{K}^+$  (Tomar and Agarwal, 2013; Ahmad *et al.*, 2014; Abd\_Allah *et al.*, 2015; Ahanger *et al.*, 2015). The present study also revealed an improved uptake of  $\text{Na}^+$  posing a concomitant negative effect on the uptake of other ions like  $\text{K}^+$ ,  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$ . Our results of reduced uptake of essential mineral ions due to salt stress are in concurrence with the findings of Kohler *et al.* (2009) for lettuce, Yasmeen *et al.* (2013a) for wheat, Azooz *et al.* (2015) for okra and Iqbal *et al.* (2015) for *Brassica juncea*.

Foliar application of MLE not only allayed the negative impact of excess sodium by restricting its uptake but also caused a significant increase in the uptake of essential mineral elements including  $\text{K}^+$ ,  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$ . Improved  $\text{K}^+$  uptake has positive control over growth performances through its active participation in several metabolically important processes like enzyme activation, osmoregulation, and the selective accumulation of sodium (Tomar and Agarwal, 2013; Ahmad *et al.*, 2014 and Ahanger *et al.*, 2015). Improvement in the  $\text{Mg}^{2+}$  uptake in MLE-treated plants may contribute to improved chlorophyll pigment synthesis. Plenty of reports are available adjudging increased  $\text{K}^+/\text{Na}^+$  and  $\text{Ca}^{2+}/\text{Na}^+$  ratio as an important strategy for stress amelioration (Azooz *et al.*, 2004b, 2015; Tomar and Agarwal, 2013; Jatav *et al.*, 2014 and Ahanger *et al.*, 2015). These results suggest a positive effect of MLE on the uptake of  $\text{K}^+$  and  $\text{Ca}^{2+}$  and hampering  $\text{Na}^+$  and  $\text{Cl}^-$  uptake in fenugreek plants. Our findings suggested that salt resistance in fenugreek plants is not only correlated with boost of  $\text{K}^+$ ,  $\text{Ca}^{2+}$ ,  $\text{Mg}^{2+}$ , but also with high ratios of  $\text{K}^+/\text{Na}^+$  and  $\text{Ca}^{2+}/\text{Na}^+$  due to MLE application.

Salt stress-induced enhancement in lipid peroxidation corroborated with the findings of Rasool *et al.* (2013), Mostofa *et al.* (2015) and Ahmad *et al.* (2016). In our work, application of MLE lessened the production of MDA depicting membrane strengthening the effect of the extract and thereby protecting the biological membrane functioning from oxidative effects of toxic free radical.

In this work, the ineffective change in SOD activity under saline stress might be due to increased rate of ROS scavenging by the other antioxidant enzymes (CAT and APX). Consequently, activity of SOD seemed not to be directly in charge of safeguard against oxidative damage, where as CAT and APX might be critical to provide defense for *Trigonella foenum-graecum* plants when grown under saline stress conditions. On the other side, the decrease in POD activity in *Trigonella foenum-graecum* exposed to NaCl treatments might be due to the rising rate of other ROS scavenging enzymes (CAT and APX) suggesting that the decrease in POD activity was compensated by boosting the activity of CAT and APX. Application of MLE caused further increase in CAT and APX activity thereby providing strength to the antioxidant defense system in removal of toxic ROS. Both CAT and APX are indispensable for improving plant tolerance to stress through mediating the quick removal of H<sub>2</sub>O<sub>2</sub> thus protecting membrane functioning. Higher activities of CAT and APX in the MLE-sprayed plants was associated with improved stress tolerance to oxidative damage. Foliar spraying of MLE stimulated the activity of POD that was however reduced due to salinity consequently predicted to result in increased production of lignin's and associated protective compounds that directly or indirectly contribute for extenuating the oxidative stress induced damage. Increased activities of SOD, CAT, POD and APX by foliar application with MLE might also help in growth maintenance through quick scavenging of H<sub>2</sub>O<sub>2</sub>. In the present study it could thus be concluded that application of MLE further strengthened the antioxidant potential of *Trigonella foenum-graecum* and Similar conclusions have been reported by Yasmeen *et al.* (2013a) and Howladar (2014) in wheat and bean plants subjected to salt stress, respectively.

Considerable differential changes were observed in the expression patterns of the proteins conferred as alteration in the de novo synthesis of proteins of different molecular masses (Ahmad *et al.*, 2014). In this investigation, expression of specific proteins in response to saline stress (200 mM NaCl) might result in the protection of macromolecules like DNA damage and work for substantial repair of stress affected molecules (Oliveira *et al.*, 2015). The disappearance of certain polypeptides under saline stress may be related to suppression of their synthesis, inhibition of mRNA transcription, increase in RNAase activity, dissociation of polysomes and differential turnover (Riccardi *et al.*, 1998; Beltagi *et al.*, 2008 and Abdel Latef, 2011). It is worthy to mention that 39, 21, 19, 17 and 16 kDa protein

bands disappeared under salt stress, but were initiated again by the foliar application with MLE that might play a key role in signaling of plant adaptive responses to salinity.

Alterations in gene expression and translation under stressed condition can result in expression of stress responsive proteins and hence induce stress tolerance. Proteins expressed differentially can contribute to maintain the genome stability and integrity, thereby contributing for the enhanced genome plasticity in changing environment (Waterworth *et al.*, 2011). Proteins are ubiquitous for modulating the biosynthesis of important hormones and controlling the signaling pathways especially via transcription factors (Stone *et al.*, 2007; Lyzenga *et al.*, 2011). Increased de novo synthesis of proteins due to moringa application might contribute to stress tolerance of *Trigonellafoenum- graecum* by modulating the tolerance response. Impact of MLE on protein expression are still poorly understood and further proteomic studies can provide information on the influence of MLE on plant metabolism under salt stress.

### Conclusions

Salinity stress caused alterations in normal growth of *Trigonellafoenum-graecum* by affecting physiological and biochemical parameters under study. Foliar application of MLE improved growth by boosting the contents of photosynthetic pigments, osmolytes, total phenols,  $K^+$ ,  $Ca^{2+}$ ,  $Mg^{2+}$ , as well as the  $K^+/Na^+$  and  $Ca^{2+}/Na^+$  and the antioxidant enzyme (SOD, CAT, POD and APX) activities. In addition to lowering the accumulation of  $Na^+$  and MDA contents. Differential expression of proteins in salinity stressed plants with and without MLE application strongly justified a positive role of MLE in stress amelioration. Therefore, it could be concluded that MLE has a role in the alleviation of negative impacts of salinity stress. Thus, we recommend that foliar spray of MLE might be a useful strategy for enhancing *Trigonellafoenum-graecum* plant tolerance when exposed to salinity stress. Additionally, this technique is easy to apply and is eco-friendly and could be accepted by farmers to be used in the field to improve the growth and yield of plants.

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## الرش الورقى بمستخلص أوراق المورينجا الطازجة يتغلب على الإجهاد الملحى فى نباتات الحلبة

عرفات عبد الحميد عبداللطيف ، منى فوزى ابوالحمد و صباح أحمد حماد  
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أجرى هذا البحث لدراسة تأثير الرش الورقى بمستخلص أوراق المورينجا الطازجة على نبات الحلبة المزروع تحت أربعة مستويات من كلوريد الصوديوم (١٠٠،٥٠،٠٠ و ٢٠٠ مل مول). وتشير النتائج أن الملوحة أسفرت عن انخفاض دلالات النمو ومحتويات الأصباغ النباتية والذائبات العضوية (ما عدا البرولين) والفينولات الكلية والبوتاسيوم والكالسيوم والماغنسيوم ونسبة البوتاسيوم على الصوديوم و الكالسيوم على الصوديوم و نشاط إنزيم البيروكسيداز. على الجانب الأخر تسببت الملوحة فى زيادة محتويات البرولين والصوديوم والمالونداى الدهيد ونشاط إنزيم الكاتاليز وإنزيم الأسكوربيت بيروكسيداز. ومن المثير للإهتمام أن نشاط إنزيم السوبر أوكسيد ديسموتيز بقى دون تغيير تحت جميع مستويات التملح. الرش الورقى بمستخلص أوراق المورينجا الطازجة خفف من الأثر السلبى للملوحة وظهر ذلك جليا فى تحسن دلالات النمو والقياسات السابقة ما عدا الصوديوم و الكلور والمالونداى الدهيد. تحت الظروف الملحية ، أدى الرش الورقى بمستخلص أوراق المورينجا الطازجة إلى ظهور ١٢ من عديد الببتيدات الجديدة و التأثير المشترك لمستخلص أوراق المورينجا الطازجة والملوحة أدى الى ظهور الروابط البروتينية ٣٩ و ١٩،٢١ ، ١٦،١٧ التى لم تظهر تحت اجهاد الملوحة. نتائج هذا البحث تشير إلى أن الرش الورقى بمستخلص أوراق المورينجا الطازجة يمكن أن يخفف من الأثر الضار للملوحة على الحلبة ويمكن إستعمال هذه الأستراتيجية فى تعزيز إنتاج المحاصيل فى الأراضى المجهدة ملحياً.