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Iron oxide Nanoparticales role in micropropagation of *Moringa oleifera* L. under salinity stress

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ABSTRACT

Micropropagation is the most important tool to propagate some important economic trees such as *Moringa oleifera* L. to examine the morphological reaction of *in vitro* grown plantlets on MS culture medium supplemented with different PGR (BA, IBA, NAA and Kinetin) and investigate the *in vitro* propagation ability under NaCl different concentrations (0, 1, 2, 4 and 8g/l). Adding two levels of Iron Oxide NPs (5 and 10mg/L) was used to mitigate the effect of salt stress on micropropagation of plants. The results showed that NaCl at different concentrations significantly reduced the *in vitro* recorded shoots and roots parameters (number of shootlets, shootlet length, rooting %, number and length of roots), chlorophyll content and carotenoids. Moreover, proline content was increased with increasing NaCl concentration in the culture medium. In addition, adding Iron Oxide NPs to culture medium showed positive effects on alleviating salt stress. This study aimed to evaluate the adverse effect of salt stress on *Moringa oleifera* L. micropropagation and the possibility of using Iron oxide NPs to overcome these effects.

***Keywords*:** *Moringa oleifera* L., micropropagation, salinity, Iron oxide NPs.

Introduction

The widely cultivated *Moringa oleiferab* in tropical and subtropical regions of the world is a fast- growing deciduous tree and known for its multipurpose such as a source of food, shelter and traditional medicine especially in developing countries. Several studies have been conducted to assess the various claims of traditional medicine practitioners that Moringa tree can improve health and cure various diseases. The tree has a high nutritional profile, especially nutrient-rich leaves. Some reports also support the use of tree parts to reduce blood sugar and cholesterol levels. These attractive characteristics have led researchers to seek new uses of the Moringa tree, especially as a source of anti-cancer drugs. Researchers have tested extracts from different parts of the Moringa tree *in vitro* and *in vivo* on several types of cancers with varying success (Khor *et al*., 2018). The plant is also known as a Horse- radish tree, Drumstick tree. Each part of this plant has a valuable medical advantage. It contains a rich source of vitamin A, vitamin C and milk protein (Birendra *et al*., 2017).
Moringa oleifera is traditionally propagated by seeds. Plants obtained from seeds vary in genotype and phenotype thus leading to variations in the quality of the yield. Therefore, propagation of elite genotype can be propagated via micropropagation techniques. In general, explant type, culture conditions and plant growth regulator regimes must be defined for each genotype in order to develop the successful micropropagation protocol (Stephenson and Fahey, 2004; Marfori, 2010 and Saini *et al*., 2012)

Application of saline water in agriculture requires deep understanding of law to overcome the negative effects of salinity. Tissue culture technique supplements with artificial tools to study the effect of salinity on plants under conditions and production of the range of plant species all over the world (Gayathri *et al*., 2015).

Trace iron is an important and necessary ingredient for all living organisms. Iron is an essential element in cell metabolism and is involved in photosynthesis, respiration, etc (Rashno *et al*., 2013). The nanoparticles of iron oxide are smaller than typical iron particles and create more complexes with higher iron availability for plants (Mazaherinia *et al*., 2010).

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The aim of the present investigation was to optimum micropropagation ability of *Moringa oleifera* under saline condition and study the possibility of using Iron oxide NPs to mitigate these effects.

Materials and Methods

These investigations were conducted at Tissue Culture Technique Lab., Central laboratories, Department of Ornamental Plants and Woody Trees, National Research Centre (NRC), during years 2018-2019 to evaluate some morphological, chemical changes of *Moringa oleifera* plants cultured *in vitro* under the effect of salt stress.

Explant source and surface sterilization

Seeds of *Moringa oleifera* were collected from nursery of Timber Trees Department Horticulture Research Institute- Agriculture Research Centre, Giza, Egypt. The seeds were washed and sterilized in ethanol 70% (v/v) for 30 seconds then immersed in 15% of sodium hypochlorite (Clorox) for 7 minutes then 1% of HgC_{l2} (MC) solution (w/v) for 10 minutes and rinsed three times in sterile water.

Culture medium

After surface sterilization, seeds of *Moringa oleifera* were cultured for one month on MS free of hormones (Murashige and Skoog, 1962). The obtained seedlings were subcultured on MS medium supplemented with 0.2 ppm of 6- benzylamino-purine (BAP) and 0.1ppm indole butyric acid (IBA), 2.5% sucrose and 0.7% agar. The pH of the medium was adjusted to 5.6-5.8 then autoclaved at 121°C and 15 psi for 15 minutes. The *in vitro* obtained shootlets were used as explant source for testing the morphogenetic reaction on MS medium supplemented with three PGR: IBA (indole-3-butyric acid), BAP (benzyl amino purine), NAA (α-naphthaleneacetic acid) and Kinetin as in Table (1).

Table 1: PGR concentrations used for testing the *in vitro* morphological reaction of explants of *Moringa oleifera.*

Medium	Concentration of hormones [mg/l^{-1}]			
	IBA	BA	NAA	Kinetin
1	-	-	-	-
2	0.1	0.2	-	-
3	-	0.1	0.1	0.2

First experiment: Various concentrations (0, 1, 2, 4 and 8 g/ L) of NaCl were examined under *in vitro* conditions.

Second experiment: Studying the salt stress alleviation ability on the plant using two Iron oxide NPs (5.0 and 10 mg/l) was carried out as follows:
1-Control
2-Control+NaCl (1 g/L) +5.0 mg/L Fe NPs
3-Control+NaCl (1 g/L) +10 mg/L Fe NPs
4-Control+NaCl (2 g/L) +5.0 mg/L Fe NPs
5-Control+NaCl (2 g/L) +10 mg/L Fe NPs
6-Control+NaCl (4 g/L) +5.0 mg/L Fe NPs
7-Control+NaCl (4 g/L) +10 mg/L Fe NPs
8-Control+NaCl (8 g/L) +5.0 mg/L Fe NPs
9-Control+NaCl (8 g/L) +10 mg/L Fe NPs

Culture conditions:

Cultures were incubated in growth chamber at 24 ± 1°C under white cool florescent lamps with light intensity of 3k lux at 16 hr photoperiod.
The culture period for each experiment (first and second) took two months after start of culture then, the following data were recorded:

Shooting behavior: Survival %, number of formed shootlets per explant and shootlet length (mm).
Rooting behavior: Percentage of roots formation (%), number of roots/shootlet and root length (mm).

Extraction and chemical analysis
Photosynthetic pigments

Photosynthetic pigments (chlorophyll a and b) as well as carotenoids were determined in shootlets tissues as mg/100g F.W. using spectrophotometer, according to the procedure achieved by Saric *et al* (1967).

Proline

The proline content in leaves (mg/g F.W.) was determined using the method described by Bates *et al*. (1973).

Specification of used NPs in the experiment

Iron Oxide NPs

Specification test method

Phase	Hematite	XRD
Particle size	<50 nm	TEM
Surface area	>50m^2/gm	BET (P/Po: up to 0.35

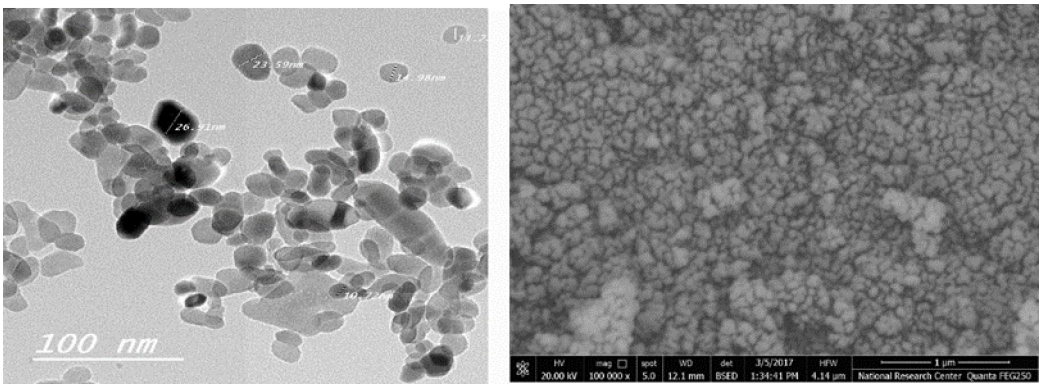

Fig. 1: Scanning electron microscopy image of Iron oxide NPs

Results and Discussion

1. Culture establishment of *Moringa oleifera* under effect of growth regulators

Data in Table (2) observed the *in vitro* shoot multiplication on MS culture media added with different PGR such as BA, IBA, NAA and Kinetin that were compared after nearly 8 weeks of culture. It can be seen that the highest number of shootlets (7.30), longest shootlets (48.33 mm), highest rooting percentage (100%), number of roots (7.8) and longest roots (51.66mm) per explants was observed for the culture media without any PGR compared to other culture media (MS contained BA 0.1mg/l and IBA 0.1 mg/l or MS containing BA 0.1mg/kinetin 0.2 mg/l and NAA 0.1 mg/l). In this study, data observations such as shoot multiplication and growth of shoot and root initiation were promoted by culture medium without PGR more than other treatments. Similar results were noticed by Nayak *et al*., (2010) on *Bambusa arundinacea* showed that the highest multiplication can be obtained in medium without Kin. In conclusion, type and concentration of growth regulators and species (genotype) are the most important factors in the plant Micropropagation. Few studies have shown the positive effect of without growth regulator on micropropagation of different plants. The current study showed the positive effect of MS without growth regulator, on micropropagation of *Moringa oleifera*. Growth regulators are significantly important for *in vitro* shoot proliferation, but some internal factors and nutrient conditions can modify their activities (Park *et al*., 2001). Moreover, endogenous levels of growth regulators in the explant showed different regeneration efficiencies (Nontaswatsri *et al*., 2002).

Table 2: *In vitro* shooting behavior of *Moringa oleifera* under effect of growth regulators.

Treatments	Characters	Number of shootlets/ explant	Shootlet length (mm)	Rooting (%)	Number of roots/ plantlet	Length of roots (mm)
MS free hormones		7.30 a	48.33a	100 a	7.8 a	51.66 a
MS + BA 0.2mg/l + IBA 0.1mg/l		4.66 ab	39.33a	88.86 a	7.8 a	20.00 b
MS + BA 0.1 mg/l + NAA 0.1mg/l + Kin 0.2 mg/l		3.5 b	26.66 b	22.2 b	2.0 c	12.33 b

2-*In vitro* shooting and rooting behaviors of *Moringa oleifera* under effect of various NaCl concentrations

Data in Fig. (2,3) showed that control explant had highest values of all *in vitro* shoot parameters (number of shootlets/ explant, shootlet length, rooting percentage, number of roots and root length).The lowest NaCl concentration in culture medium gave the best values compared to other treatments of NaCl.

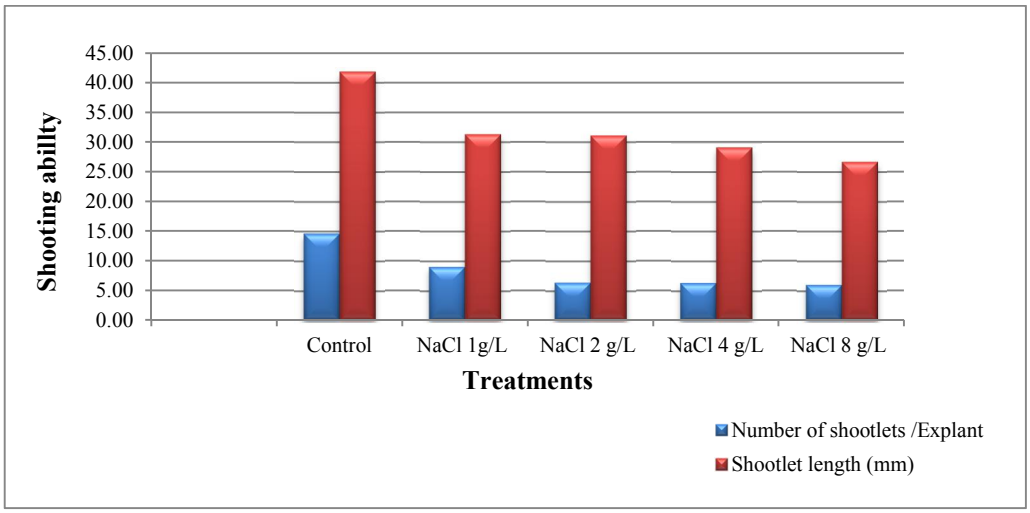

Fig. 2: *In vitro* shooting behaviors of *Moringa oleifera* under effect of various concentrations of NaCl

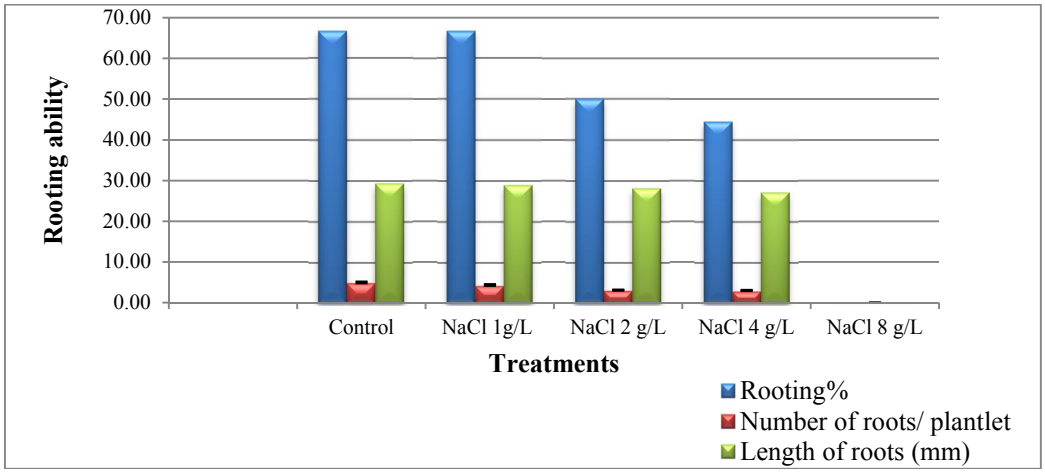

Fig. 3: *In vitro* rooting behaviors of *Moringa oleifera* under effect of various concentrations of NaCl

Similar results were found by Soliman *et al*.(2012) on *Acacia saligra* who mentioned the reduction in the number of both leaves and branches and shoot growth as a result of salt stress.

Sayed *et al*. (2014) observed that *Tagetes erecta* L. reduction of plant height, root length, no of leaves that cultured under higher concentration of NaCl.

Salem (2016) on *Moringa oleifera* observed that under salinity, retardation in shoot multiplication and growth was noticed.

Salinity negatively affects plant growth through many physiological and biochemical methods such as toxicity, osmotic pressure, nutritional deficiencies, chemical and physiological disturbances (Kao *et al*., 2003).In addition, high salinity may prevent root and shoot elongation due to slow water absorption by the plant. Over time, Na + and Cl- for concentrations will accumulate in the shoot resulting in early leaf senescence and death due to the ionic component of salt (Hairmansis *et al*., 2014).

3-Effect of NaCl on proline content

Data of proline content of leaf samples that were taken from shootlets receiving different NaCl concentrations (Fig.4) showed that, with increasing NaCl in culture medium, the proline content was generally increased. Accordingly, the highest salt concentration (4g/l) had the highest mean proline content (0.63 mg/g F.W.). On the other hand, control plants and those that were cultured on the lowest NaCl concentration had the lowest mean proline content (0.11 and 0.13 mg/g F.W., respectively).

These results agreed with Soliman *et al*., (2015) who mentioned the increase of proline content in leaves under different levels of salt stress on Moringa plant.

Proline serves as storage, sink for carbon and nitrogen and it is a free-radical. It also stabilizes subcellular structures (membranes and proteins), and buffers cellular redox potential. Hence, these organic osmolytes are known as osmoprotectants, these organic solutes may contribute to osmotic adjustment, protecting cell structure and function, and/or may serve as a metabolic or an energetic reserve (Chen and Murata, 2000).

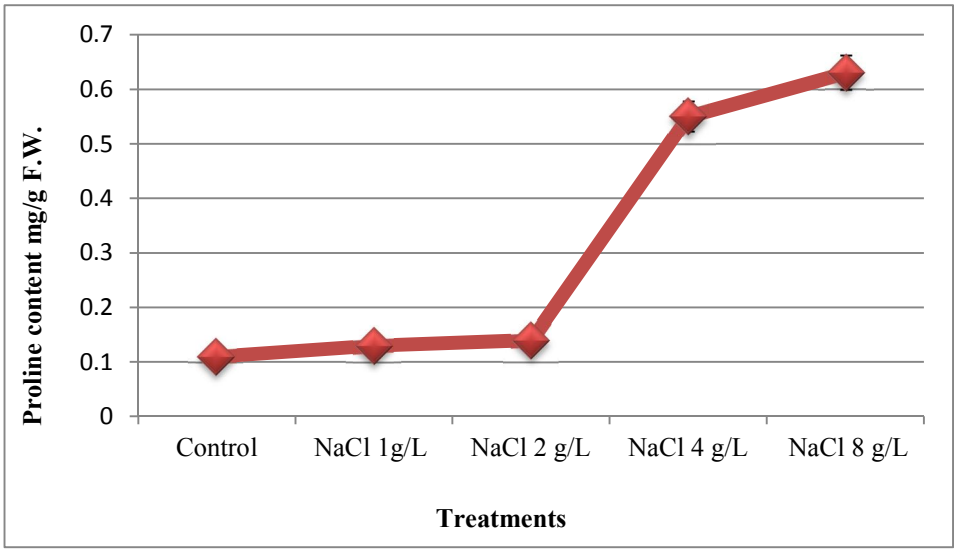

Fig. 4: Effect of various concentrations of NaCl levels on proline content of *Moringa olifera*

4-Effect of salt stress on Chlorophylls (a,b) and total carotenoids contents

Fig. (5) showed that adding NaCl at 1g/L to the culture medium significantly increased chlorophyll a content to the highest value (60.92 mg/100g.F.W.),while using of NaCl at 8g /L declined this value to the lowest one (13.16 mg/100g. F.W.) and significantly reduced Chl.a as compared to control. Decreasing the concentration of NaCl to 1 g/lin the culture medium significantly increased Chl.b as well as carotenoid contents giving the highest values with lowest concentration of NaCl, whereas using NaCl at 8g/L significantly decreased these values as compared to control. These results were confirmed by Sayed *et al*., (2014) on *Tagetes erecta* L. who mentioned that Chlorophylls a, b and carotenoids exhibited reduction under higher concentration of NaCl. Soliman *et al*., (2015) studied that total chlorophyll (a, b) and carotenoid contents were significantly low in plants grown under salt stress condition on *Morenga oleifera* plant. The accumulated amount of ions enter the plant through the transpiration stream, thereby causing cells injury in the transpiring leaves which may cause further reductions in photosynthesis processes thereby leading to growth reduction (El-Fouly *et al*., 2002; Munns *et al.,* 2006)

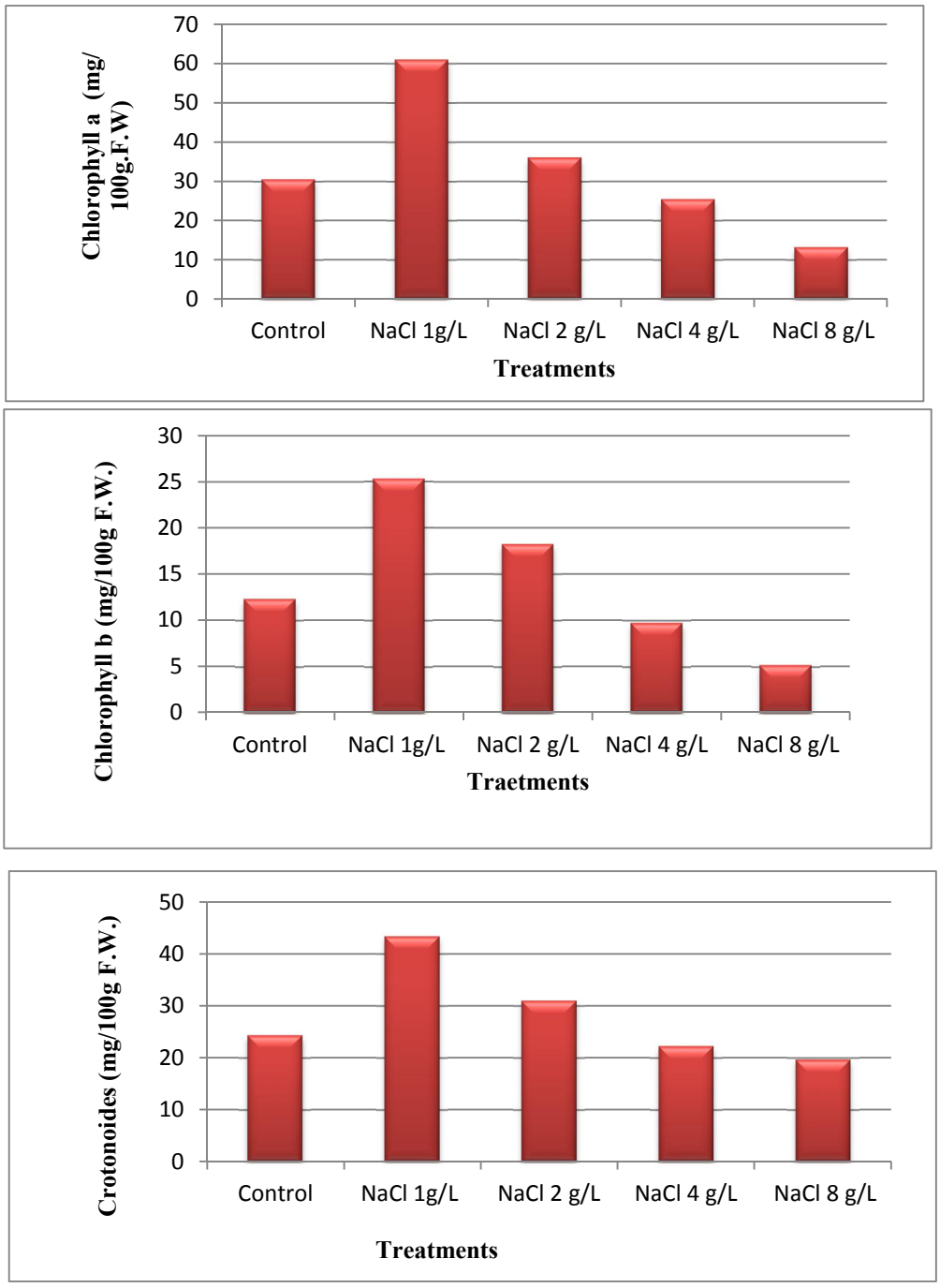

Fig. 5 (A, B and C): Effect of salt stress on Chlorophylls (a, b) and total carotenoids contents of *Moringa olifera*

5- Effect of salt stress and iron oxide NPs on micropropagation ability

In the present study, the effects of salinity and iron oxide NPs at various concentrations on *in vitro* growth ability were presented in Table (3) mentioned that, the iron oxide NPs decreased the effect of salt stress on *in vitro* growth of Moringa plants. The data showed that, using iron oxide NPs at 10mg/l with NaCl at 1 g/L gave the best results such as the highest survival percent (100%),number of shootlets /explant (10.62), shootlet length (46.07mm), and root percentage (66.66%),root number (4.89)and root length (21.62mm) as compared to other treatments and used NaCl only without iron oxide NPs. Moreover, using iron NPs at 5.0g/L or 10g/L gave positive effect on induction of roots with high concentration of NaCl (8g/l) comparing with using NaCl alone. In finely, it can notice that iron oxide NPs promote root initiation under salinity stress in the current study on *Moringa oliferera* L.

These results were in agreement with Ozturk *et al*. (2004) on lemon balm (*Melissa officialis* L.) who observed that iron NPs can decrease the effect of salinity in plants.

Soliman *et al*. (2015) on *Moringa peregrine* found that spraying plants with iron NPs increased growth parameters under salt stress.

Nano- technology can offer opportunities to enhance yield and counter environmental stress by using NPs (Anonymous, 2009).The application of NPs to plants can be beneficial (seedling growth and development) or non-beneficial (prevent root growth) (Zhu *et al*., 2008)

Table 3: *In vitro* shooting and rooting behaviors of *Moringa olifera* under effect of salt stress levels and iron oxide NPs.

Characters / Treatments	Survival (%)	No.of shoolet/ explant	Shoot Length (mm)	Rooting (%)	Number of roots/ shootlet	Root length (mm)
Control	100.0 a	14.6 a	41.8 bc	66.6 a	5.0 a	29.3 a
NaCl 1 g/L + 5.0 mg/L Fe NPs	100.0 a	10.0b	45.8 a	66.6 a	4.3 a	18.2 c
NaCl 1g/L + 10 mg/L Fe NPs	100.0 a	10.6 b	46.0 a	66.6 a	4.8 a	21.6 b
NaCl 2 g/L + 5.0 mg/L Fe NPs	100.0 a	8.0 bcd	44.1 ab	55.5 b	4.0 a	15.2d
NaCl 2g/L + 10 mg/L Fe NPs	100.0 a	9.0 bc	42.8 bc	55.5 b	3.8 ab	14.0 d
NaCl 4 g/L+ 5.0 mg/L Fe NPs	100.0a	6.0 de	41.3 c	44.4 c	2.6 bc	12.4 e
NaCl 4 g/L+ 10 mg/L Fe NPs	100.0 a	7.0 cd	38.8 d	44.4 c	2.3 cd	12.0 e
NaCl 8g/L + 5.0 mg/L Fe NPs	88.8 b	4.5 e	38.5 d	33.3 d	1.3 de	6.9 f
NaCl 8 g/L + 10 mg/L Fe NPs	77.7 c	5.0 e	36.0 e	33.3 d	1.0 e	5.3 g

6- Effect of salt stress and iron oxide NPs on proline content

In this present, the data in Fig.6 mentioned that, the proline content was increased gradually with increasing NaCl concentrations. The plants which were treated by 1 g/L in culture media gave the lowest content of proline (0.26 mg/g F.W.), but the plants that were treated by 8 g/L gave the highest value of proline (2.24 mg/g F.W.).

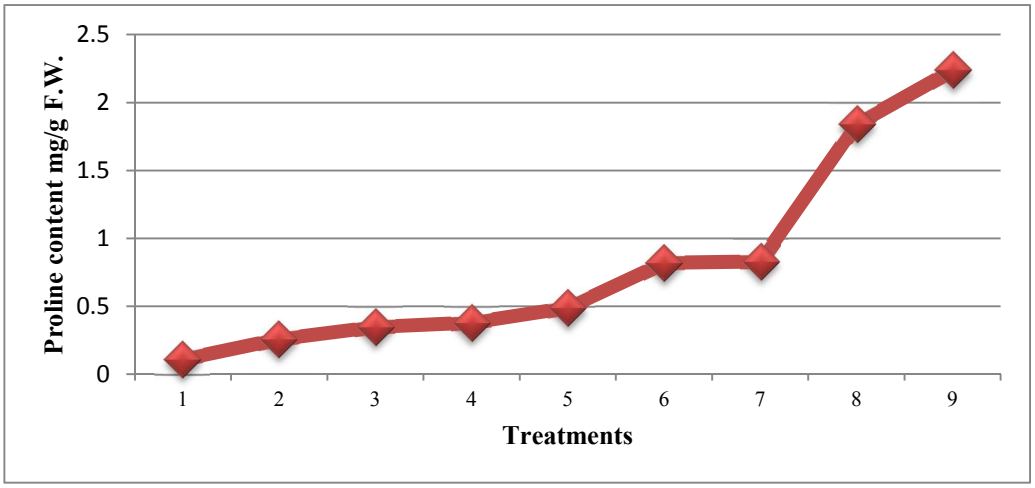

Fig. 6: *In vitro* proline content of *Moringa olifera* plant under effect of various concentrations of NaCl and iron oxide NPs.

1 (Control), 2 **(**Control+ NaCl (1 g/L) +5.0 mg/L Fe NPs), 3 (Control+ NaCl (1 g/L) +10 mg/L Fe NPs),4 (Control+ NaCl (2 g/L) +5.0 mg/L Fe NPs),5 (Control+ NaCl (2 g/L) +5.0 mg/L Fe NPs),6 (Control+ NaCl (4 g/L) +5.0 mg/L Fe NPs),7 (Control+ NaCl (4 g/L) +10 mg/L Fe NPs),8 (Control+ NaCl (4 g/L) +10 mg/L Fe NPs),9 (Control+ NaCl (4 g/L) +10 mg/L Fe NPs)

The application of iron oxide NPs (5.0 and 10 mg/L) gave a positive effect on salt stress with most concentrations of NaCl. Application 10 mg/L iron oxide NPs was more effective than 5 mg/L. plants treated by 8 g/L NaCl with 10 mg/L iron oxide NPs in culture media gave the highest proline content (2.24 mg/g F.W.). Meanwhile, plants treated with 8 g/L NaCl with 5.0 mg/L iron oxide NPs gave proline less than plants treated by 8 g/L NaCl with 10 mg/L iron oxide NPs. Using iron oxide NPs in culture medium increased proline more than using NaCl alone.

The positive effect of appropriate Fe NPs concentrations on plant under NaCl stress could be explained by the replacement of Fe with Nano forms (Wang *et al.,* 2011).

7- Effect of salt stress and iron oxide NPs on Chlorophylls (a, b) and total carotenoids content.

Indicated the influence of NaCl and iron oxide NPs at various concentrations was shown in Fig.(7) showed that treating *Moringa olifera* explant with NaCl1.0 g/ and 5.0 mg/L Fe NPs increased total chlorophyll (a and b) and carotenoids content to the highest values (91.37,38.45 and 66.15 mg/g.F.W) as compared to using NaCl only. While, using NaCl 8.0 g/L and 10 mg /Fe NPs declined this value and significantly reduced total chlorophyll (a and b) and carotenoids content as compared to control and other treatments. It can be noticed that using iron oxide NPs enhanced chlorophyll and carotenoids content under salinity stress as compared to use NaCl only.

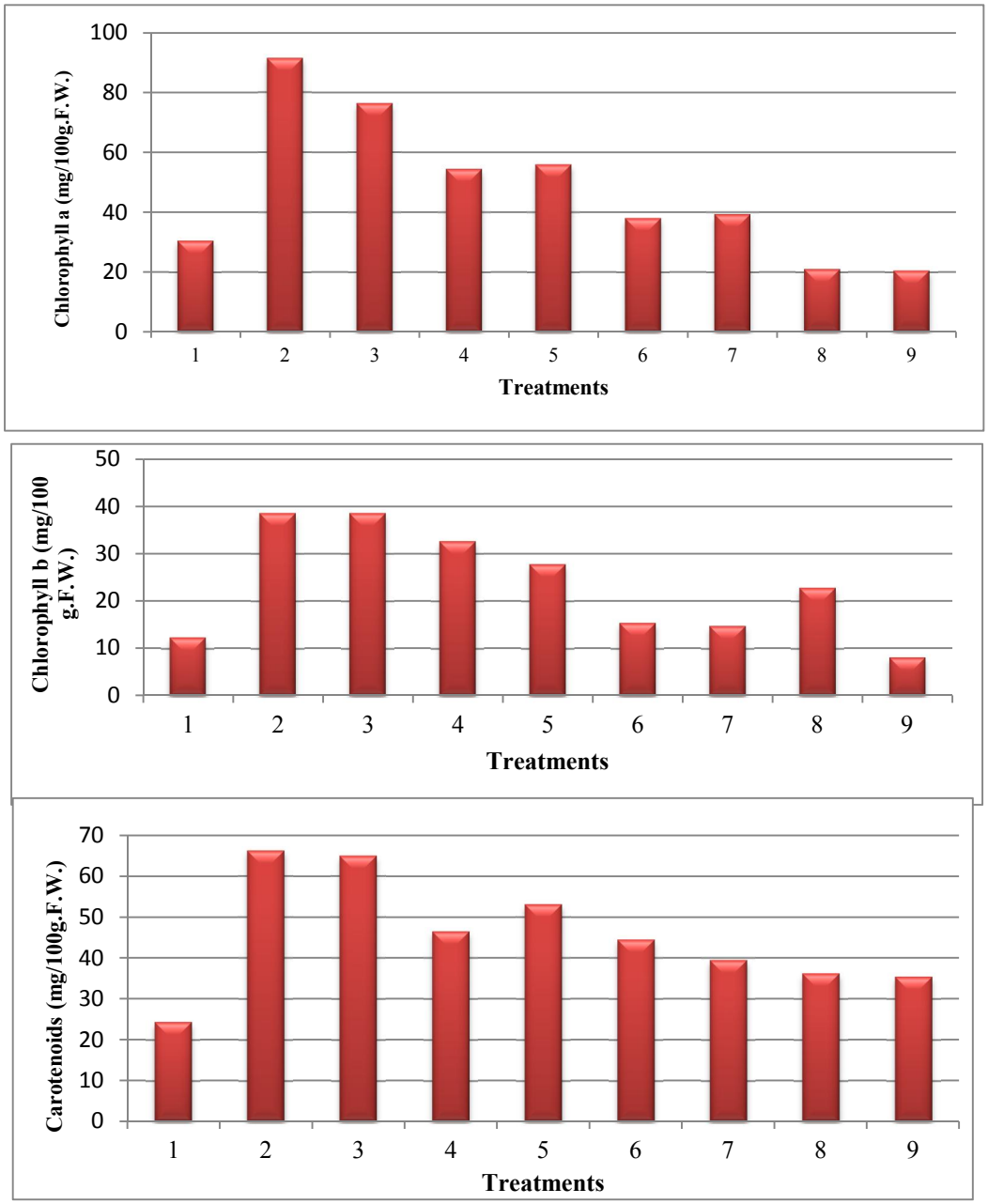

Fig. 7: Effect of salt stress and iron oxide Nano -practicalon Chlorophylls (a,b) and total carotenoids content.

1 (Control), **2 (**Control+ NaCl (1 g/L) +5.0 mg/L Fe NPs), **3** (Control+ NaCl (1 g/L) +10 mg/L Fe NPs), **4** (Control+ NaCl (2 g/L) +5.0 mg/L Fe NPs), **5** (Control+ NaCl (2 g/L) +5.0 mg/L Fe NPs), **6** (Control+ NaCl (4 g/L) +5.0 mg/L Fe NPs), **7** (Control+ NaCl (4 g/L) +10 mg/L Fe NPs), **8** (Control+ NaCl (4 g/L) +10 mg/L Fe NPs), **9** (Control+ NaCl (4 g/L) +10 mg/L Fe NPs)

Fig. 8: *In vitro* shooting and rooting ability of *Moringa olifera*: (A): cultures on 1 g/LNaCl+10 mg/L Fe NPs, (B): prepared rooted plantles for the acclumization stage and (C, D): Acclimatization to greenhouse.

These results are in agreement with the findings of Liu *et al*. (2005) who concluded that iron oxide NPs facilitated photosynthesis and iron transfer to leaves of peanut when compared to organic materials and iron citrate.

Iron also activates several enzymes and contributes in RNA synthesis and improves the performance of photosystems (Malakouti and Tehrani, 2005). In addition iron oxide NPs have been reported as facilitators for iron and photosynthetic transfer to the leaves of peanut (Liu *et al.*, 2005).

Conclusion

In this study, the results showed that Moringa plants could overcome the effect of salt stress by using iron oxide NPs. The ability of *in vitro* shooting and rooting and chemical composition was increased by using iron oxide NPs under salt stress.

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