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MITIGATION OF SALINITY STRESS ON *IN VITRO* GROWTH OF *EUSTOMA GRANDIFLORUM* USING ZINC NANOPARTICLES

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Abstract

In this work, an *in vitro* experimental system has been developed to study the harmful effect of NaCl on shooting and rooting abilities of *Eustoma grandiflorum* as well as the role of ZnO NPs in mitigating this bad effect of salt stress on the plant. Three salt concentrations (2.0, 4.0 and 6.0 g/L) comparing with control were applied. Zinc nanoparticles were used at 5.0, 10.0 and 20 mg/L and combined with each salinity level. Data showed that salt stress had a negative effect on shootlets number formed per explant during shoot multiplication stage whereas, shootlet length was significantly increased due to apply salt condition at levels of 2 and 4 g/L. The results of current study demonstrated that ZnO NPs have a significant role in alleviating salt stress especially when it used at low concentration. Photosynthetic pigments content such as chlorophyll (a) was significantly decreased as NaCl concentration was increased above 2 g/L while, chlorophyll (b) was reduced with increasing salinity stress. With evaluating enzymes activity, saline condition appeared to increase both POD and SOD as compared to control while, CAT activity was decreased. Addition of ZnO NPs to salt treatment decreased the POD activity but, it increased the activity of both SOD and CAT enzymes.

Key words : *Eustoma grandiflorum*, Tissue culture, Salt stress, zinc nanoparticles, antioxidant enzymes activity.

Introduction

Eustoma grandiflorum Gris is an ornamental cut flower within Gentianaceae family, native to southern parts of the United States (Popa *et al.,* 2004). The name of the plant is derived from two Greek words that is are Eu (beautiful) and stoma (mouth). It is an annual or biennial plant that tolerates mild cold (Roh and Lawson, 1988). Moreover, the flower of plant is characterized by postharvest longevity that reaches to 12-25 days (Uddin *et al.,* 2013). There is a huge international demand for this flower that has the potential to be a promising addition to the flower mark (Ahmed *et al.,* 2017).

Salinity is one of the major environmental stresses affecting the performance of many crop plants. Salinity has various effects on plant physiological processes such as increased respiration rate and ion toxicity, decreased leaf net CO_2 assimilation rate (Hajlaoui *et al.,* 2006). High salinity has been reported to induce ROS (reactive oxygen species) formation and accumulation in plant cells (Chawla *et al.,* 2013). Oxidative stress defenses occur through enzymatic by antioxidant mechanism including catalase (CAT), superoxide dismutase (SOD) and peroxidase (POX). One nanoparticle-signaling molecule, zinc oxide (ZnO), appears to play an active role in regulating various mechanisms involved in recognition and response to abiotic stresses in plants (Alharby *et al.,* 2016.). Tissue culture is a powerful tool for the screening of plant growth and development under well-defined and controlled environmental conditions (Bahmani *et al.,* 2012).

The objective of the present study was to examine the growth ability of *Eustoma grandiflorum* under various levels of saline condition and the effectiveness of ZnO NPs application to mitigate the harmful effect of salinity on the propagated plant via tissue culture technique.

Materials and Methods

The experimental work was conducted on *Eustoma grandiflorum* plant during years 2018-2019 at Tissue

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Plant materials and surface sterilization

Plants of Lisianthus (*Eustoma grandiflorum*) were subjected to initial sterilization of lateral buds as explants with few drops of hand washing liquid for 20 min then washed under running tap water for one hour. Explants were surface sterilized in 70% (v/v) ethanol for 1 min, then in 15% commercial sodium hypochlorite solution and one drop of tween 20 (polyoxy ethylene sorbitonmonolaurate) for 10 min followed with 7-min. in 0.1 g/L $HgCl_2$. After each disinfection treatment, the explants were rinsed three times with sterile distilled water.

Culture media and culture conditions

The explants were cultured on MS medium supplemented with 0.4 mg/l of BA (6-benzyl amino purine) as standard proliferation medium (Abou Dahab *et al.,* 2017) enriched with 25 g/L sucrose and 8 g/L agar then adjusted to pH 5.7 ±0.1, the medium was then autoclaved at 121°C under 100 kPa for 20 min. The cultures were incubated at 23 ±2°C, under 16/ 8 h photoperiod with light intensity of 30 umol $m^{-2}s^{-1}$. The obtained shootlet nodal explants were used for the experimental work after two months from *in vitro* culture.

Experiment procedure

For testing the *in vitro* shooting ability of explants under salinity stress, five concentrations of NaCl (0, 2, 4 and 6 g/l) were added to MS culture media. Each of these salt concentrations was combined with three concentrations of ZnO Nanoparticles (NPs) (5, 10 or 20 mg/l), each treatment combination was replicated 7 times (one jar/replicate) in a completely randomized design. The *in vitro* grown shootlets were re-cultured after two months with the same treatments supplemented to IBA (indole- 3- butyric acid) at 1 mg/l and activated charcoal (AC) at 1g/L to examine the *in vitro* rooting ability of the plant under saline condition for another two months. Data recorded were number of shootlets/explant, shootlet length (cm) and number of leaves/shootlet for shooting stage as well as, rooting %, number of roots/shootlet and length of roots (cm) for rooting stage.

Specification of used ZnO Nanoparticles in the experiment was indicated as following: (Table 1 and image 1&2)

Table 1: Specification of used Zinc Oxide Nanoparticles.

Specification	Test method	
Phase	ZnO	XRD
Particle size	<30 nm	TEM
Surface area	>20m^2/gm	BET (P/Po: up to 0.35)

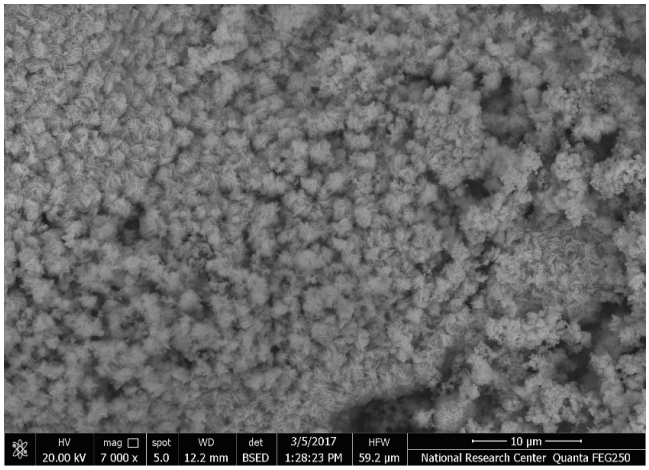

Image 1: Scanning electron microscopy image of Nano-ZincOxide.

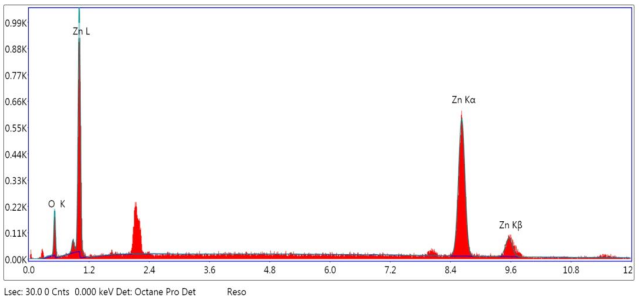

Image 2: XRD peak pattern for Nano- Zinc Oxide.

Chemical constituents

Photosynthetic pigments

Chlorophylls A and B and carotenoids were determined according to the method described by Smith and Benitez (1955)

Inorganic ion concentration

For the quantification of inorganic ions (N^-, P^+, K^+, Na^+, and Cl^-), microshoots were randomly harvested from jars for each treatment and dried in an oven at 60°C. Half a gram of the ground aerial parts was acid-digested with H_2SO_4 overnight with careful heating on a hot plate at 100°C. The beaker was cooled, then 3-5 drops of H_2O_2 were added, heated again, concentrated by evaporation and 50 mL of distilled water was added to dilute the samples. The concentration of Na^+ and K^+ were determined by flame emission spectrophotometry according to (Jackson, 1958), Cl^- was measured by silver

nitrate method according to (Richards, 1954), while P was determined calorimetrically using the ammonium phosphorus vanadomolybdate method **(**Jackson, 1958**).** Nitrogen was determined by micro-Kjeldahl apparatus.

Proline concentration

Proline content in aerial pares were measured via reaction with ninhydrin (Bates *et al.,* 1973). For colorimetric determinations, a solution of proline, ninhydrin acid and glacial acetic acid (1:1:1) was incubated at 90 ºC for 1 h. Then, the reaction was cooled in an iced bath. The chromophore was extracted using 2 ml of toluene and its absorbance at 520 nm was determined by a Bio Mate spectrophotometer (Thermo Spectronic, USA).

Determination of antioxidant enzymes

Preparation of plant extracts

Plant materials were homogenized at ratio of 1:2 (w/ v) with 0.1 M potassium phosphate buffer, pH 7.0 and then they were centrifuged for 30 min at 12000x under cooling (4^0C).

Enzyme activity assay

Superoxide dismutase (SOD, EC 1.15.1. 1) activity was evaluated by the kinetic colorimetric method of Marklund & Marklund, (1974). This determination based on the generation of superoxide anions by pyrogallol (1, 2, 3 benzenetriol) auto-oxidation in aqueous solution. Generation of superoxide anions decreases the overall rate of pyrogallol autooxidation. The SOD activity calculated by measuring the amount of generated superoxide anions radical (O_2^{-}) scavenged by SOD. Catalase activity (CAT, EC 1.11.1.6) was determined according to the method of Aebi, (1983) in which the disappearance of peroxide is followed spectrophotometrically at 240 nm. The method is based on the catalytic function of the enzyme where it catalyzes the decomposition of H_2O_2 into water and oxygen. The activity of POD (EC 1.11.1.7) determination was recorded by detecting the absorbance increasing at 470 nm. The coefficient of absorbance 26.6 mM^{-1} cm^{-1} was used for estimation of POD activity. One unit of POD activity was defined as the amount of enzyme required to form 1 uM of tetraguaiacol per minute at room temperature (Angelini *et al.,* 1990).

Statistical analysis

The data of the experiments were averaged and statistically analyzed using analysis of variance. The Dancan‘s multiple range test was done for comparison among means at 5% level according to Steel and Torrie (1980).

Results and Discussion

Shooting ability

As shown in table 2, data revealed that salt stress had a negative effect on *E. grandiflorum* shootlet number formed per explant during shoot multiplication stage. The shootlet number was decreased significantly when explants were exposed to 2 g/L salinity comparing with control. It could be noticed also that shootlet number/ explant was significantly decreased with increasing salt concentration from 2 to 4 g/L, and in significantly from 4 to 6 g/L. Adding ZnO NPs at 5.0 mg/L to MS culture medium alleviated the bad effect of all salinity levels (Image 3). Length of the formed shootlets was significantly increased when MS culture medium was supplemented with NaCl (2 or 4 g/L) comparing with control. Adding ZnO NPs at 5 mg/L to MS medium contained 4 g/L of NaCl had a positive effect on cell elongation which lead to the highest significant shootlet length (6.27 cm). Number of leaves formed per shootlet was significantly affected by salinity and ZnO NPs in which the highest significant number of leaves (52.67) was recorded for explants growing on medium enriched with 4 g/L salinity and 5.0 mg/L ZnO NPs. While, the minimum number (23.33) was counted for those grown on medium with the high levels of salinity (6 g/L) and ZnO NPs (20 mg/L). Number of leaves/ shootlets exposed to 2 g/L salinity is so closed to that of control. Increasing salinity from 2 to 4 g/L and from 4 to 6 lead to significant decrease of leaves number from 50 to 40.67 and to 26.33. Under salinity conditions, leaves number reversely related with ZnO NPs levels. Generally, salt stress had a negative effect on *in vitro* growth parameters of plants. In this concern, Bahmani *et al*. (2012) mentioned that explants of MM. 106 apple rootstock were seriously affected by salinity treatments that resulted in reduction in shoot growth (shoot number, length). Similar results have been reported that, at low concentrations of NaCl exerts a significant positive effect on shoot proliferation *in vitro* due to the increased osmolarity (Flowers and Lauchli, 1983). The results of current study demonstrated that ZnO NPs have a significant role in alleviating salt stress especially with low concentrations and this is due to that it is an essential micronutrient for all organisms and this is in agreement with that of Xu *et al*. (2014). Increasing concentrations of saline and Zn produced a decline in the ecophysiological of the plants, with observable synergistic effects on parameters like shootlet number/ explant and leaves number/ shootlet. This may be due to that Zn uptake is increased with salinity and may resulted in plant toxicity by zinc and this is in agreement with results that were mentioned by Hirpara *et al.,* (2005) who stated that there

Table 2: Effect of ZnO NPs concentration on *in vitro* shooting ability of *Eustoma grandiflorum* under different levels of salinity stress.

Treatments	Number of shootlets/ explant	Shootlet length(cm)	Number of leaves/shootlet
Control	10.67 a	2.67 fg	49.67 b
NaCl 2 g/L	6.67 bc	5.17 b	50.00 ab
NaCl 4 g/L	4.00 ef	4.00 cd	40.67 d
NaCl 6 g/L	3.67 e-g	2.17 g	26.33 gh
NaCl 2 g/L + 5 mg/L ZnO NPs	8.00 b	3.13 ef	49.33 b
NaCl 2 g/L + 10 mg/L ZnO NPs	4.00 ef	4.10 c	44.00 c
NaCl2 g/L + 20 mg/L ZnO NPs	3.00 fg	3.97 cd	28.33 fg
NaCl 4 g/L + 5 mg/L ZnO NPs	10.33 a	6.27 a	52.67 a
NaCl 4 g/L + 10 mg/L ZnO NPs	6.67 bc	3.37 e	34.67 e
NaCl 4 g/L + 20 mg/L ZnO NPs	5.67 cd	3.10 ef	29.67 f
NaCl 6 g/L + 5 mg/L ZnO NPs	4.67 de	3.50 de	36.67 e
NaCl 6 g/L + 10 mg/L ZnO NPs	2.33 e	3.00 cf	24.67 hi
NaCl 6 g/L + 20 mg/L ZnO NPs	2.67 fg	2.17 g	23.33 i

Means within a column having the same letters are not significantly different according to Duncan's Multiple Range Test (DMRT) at 5% level.

Table 3: Effect of ZnO NPs concentration on *in vitro* rooting ability of *Eustoma grandiflorum* under different levels of salinity stress.

Treatments	Rooting %	Number of roots/ shootlet	Length of roots (cm)
Control (IBA at 1 mg/L+ AC at 1 g/L)	57.33 e	1.33 gh	9.33 c
NaCl 2 g/L	83.67 b	3.67 b-d	14.50 b
NaCl 4 g/L	42.67 g	1.00 h	4.50 g
NaCl 6 g/L	36.00 h	2.00 f-h	9.17 c
NaCl 2 g/L + 5 mg/L ZnO NPs	71.33 c	4.00 bc	8.83 cd
NaCl 2 g/L+ 10 mg/L ZnO NPs	71.00 c	4.33 b	6.50 e
NaCl 2 g/L + 20 mg/L ZnO NPs	50.00 f	3.33 b-e	5.37 fg
NaCl 4 g/L + 5 mg/L ZnO NPs	83.33 b	2.33 e-g	17.00 a
NaCl 4 g/L + 10 mg/L ZnO NPs	66.00 d	2.67 d-f	6.33 ef
NaCl 4 g/L + 20 mg/L ZnO NPs	50.33 f	2.33 e-g	4.83 g
NaCl 6 g/L + 5 mg/L ZnO NPs	100.00 a	8.00 a	8.00 d
NaCl 6 g/L + 10 mg/L ZnO NPs	50.33 f	3.00 c-f	6.00 ef
NaCl 6 g/L + 20 mg/L ZnO NPs	49.67 f	2.33 e-g	6.50 e

Means within a column having the same letters are not significantly different according to Duncan's Multiple Range Test (DMRT) at 5% level.

was a significant increase in the concentration of Zn in leaves, stems, tap roots and lateral roots of *Butea monosperma* seedlings in response to increase in salt stress.

Rooting ability

Data in table 3 illustrated that salinity significantly affected the *in vitro* rooting parameters of *E. grandiflorum* shootlets that were transferred to MS medium contained IBA at 1.0 mg/L and activated charcoal (AC) at 1.0 g/L. Rooting percentage was decreased significantly with salinity level above 2.0 g/L comparing with control. Adding ZnO NPs had a positive effect on rooting percentage with increasing salinity. The highest significant rooting percentage (100%) was recorded for plants culturing on MS medium added with 6 g/L of salinity plus 5 mg/L ZnO NPs. It seemed that adding ZnO NPs at the low concentration (5 mg/L) caused high increment of rooting at both salinity levels 4 and 6 g/L comparing with these obtained on salinity levels individually. Number of roots at 2 g/L of salinity was significantly increased as compared with control. Adding 5 mg/L of ZnO NPs to 6 g/L salinity had a synergistic effect on rooting by which leads to 100% rooting and 8 roots per shootlet which were successfully survived when the rooted plantlets were acclimatized in peatmoss combined with perlite and sand (2:1:1 v/v) in the greenhouse (Image 3). Moreover, using this concentration (5 mg/L) resulted in longest roots length as compared to the same salinity level without ZnO NPs while, further increase in ZnO NPs concentration resulted insignificant reduction in root length.

In this respect, Abed Alrahman *et al.,* (2005) reported that increasing salinity significantly decreased root length of Cucumber microshoot. Similarly, a significant reduction in number of roots per shoot and root length were observed with the increase of NaCl concentration (Bahmani *et al.,* 2012).

Photosynthetic pigments content

Chlorophyll a content in leaves of shootlets was significantly decreased as NaCl concentration in the culture medium was increased above 2 g/L while, chlorophyll b was insignificantly decreased with increasing salinity stress. Carotenoid contents of leaves were significantly decreased as NaCl concentration was increased to 2 or 4 g/L and insignificantly when shootlets exposed to 6 g/L of NaCl (Table 4). Similar result was

Table 4: Effect of ZnO NPs concentration on photosynthetic pigments content (mg/ 100g F.W) of *Eustoma grandiflorum* shootlets under different levels of salinity.

Treatments	Chl. A	Chl. B	Carotenoid
Control	29.53 b	15.13 b	94.13 a
NaCl 2 g/L	19.53 b-d	14.07 b	66.50 c-e
NaCl 4 g/L	13.3 d	13.67 b	66.20 c-e
NaCl 6 g/L	13.73 d	11.6 b	82.10 ab
NaCl 2 g/L + 5 mg/L ZnO NPs	16.33 cd	18.07 b	51.17 f
NaCl 2 g/L + 10 mg/L ZnO NPs	13.5 d	6.17 b	52.73 ef
NaCl 2 g/L + 20 mg/L ZnO NPs	13.23 d	6.17 b	60.47 d-f
NaCl 4 g/l + 5 mg/L ZnO NPs	23.73 b-d	11.63 b	67.63 cd
NaCl 4 g/L + 10 mg/L ZnO NPs	16.23 cd	9.83 b	54.27 d-f
NaCl 4 g/L + 20 mg/L ZnO NPs	14.03 cd	7.13 b	47.93 f
NaCl 6 g/L + 5 mg/L ZnO NPs	50.87 a	35.13 a	89.57 ab
NaCl 6 g/L + 10 mg/L ZnO NPs	26.73 bc	16.5 b	78.27 bc
NaCl 6 g/L + 20 mg/L ZnO NPs	23.00 b-d	11.27 b	77.70 bc

Means within a column having the same letters are not significantly different according to Duncan's Multiple Range Test (DMRT) at 5% level.

Table 5: Effect of nano-zinc on elements content of *Eustoma* grown *invitro* under salt stress.

ElementTreatment	N (%)	P (%)	K (%)	Na (%)	Cl (mg/100g)
Control	3.26 a	0.54	11.92 a-c	0.72 de	0.87 d
NaCl 2g/L	2.63 bc	0.60	10.63 d	0.78 cd	1.17 c
NaCl 4g/L	2.43 c	0.59	10.60 d	0.92 bc	2.05 b
NaCl 6g/L	1.33 d	0.54	10.50 d	1.93 a	2.10 b
NaCl 2g/L + 5 mg/L ZnO NPs	3.10 a	0.49	12.21 a	0.58 e	1.17 c
NaCl 2g/L + 10 mg/L ZnO NPs	2.84 b	0.45	11.35 b-d	0.78 cd	2.90 a
NaCl 2g/L + 20 mg/L ZnO NPs	2.67 bc	0.45	11.06 cd	0.75 cd	1.25 c
NaCl 4g/l + 5 mg/L ZnO NPs	3.17 a	0.59	12.07 ab	0.78 cd	1.17 c
NaCl 4g/L + 10 mg/L ZnO NPs	2.87 b	0.83	12.07 ab	0.89 cd	0.88 d
NaCl 4g/L + 20 mg/LZnO NPs	2.87 b	0.83	11.92 ab	0.89 cd	0.88 d
NaCl 6g/L + 5 mg/L ZnO NPs	2.81 b	0.82	12.36 a	0.74 cd	0.63 e
NaCl 6g/L + 10 mg/L ZnO NPs	2.65 bc	0.74	12.01ab	0.89 cd	0.59 e
NaCl 6g/L + 20 mg/L ZnO NPs	2.68 bc	0.82	12.26 ab	0.90 cd	0.48 e

Means within a column having the same letters are not significantly different according to Duncan's Multiple Range Test (DMRT) at 5% level.

reported by Hasaneen *et al*. (2009) on *Lactuca sativa.* This might be due to decrease of nitrogen availability that could be one of the reasons for decreased chlorophyll content because of salinity stress (Parashar and Verma, 1993). Reddy and Vora (1986) and Singh and Dubey (1995) suggested another reason for the reduction of total chlorophyll content under salt stress that was probably related to the enhanced activity of the enzyme chlorophyllase and inducing the destruction of chloroplast structure and the instability of pigment protein complex. In this study, adding ZnO NPs had no significant effect on plant pigments content (chlorophylls a and b and carotenoid) except this of plants grown on medium with 6 g/L NaCl in the presence of ZnO NPs at 5.0 mg/L which recorded the highest significant value of chlorophyll contents a and b (50.8 and 35.13 mg/ 100 g fresh weight, respectively). There are many authors studied the effect of zinc on chlorophyll content and they suggested that Zn probably maintains chlorophyll synthesis through sulphydryl group protection, a function primarily associated with Zn (Cakmak, 2000). Moreover, it participates in the synthesis of chlorophyll (Li *et al.,* 2006).

Proline content

Proline content was insignificantly affected by NaCl and ZnO NPs application (Fig. 1). Under 2 g/L of NaCl plus 20 mg/L ZnO NPs recorded the highest accumulation of proline (0.337 m/100 g F.W.). In this respect, there are reports mentioned that increasing the accumulation of proline maintain the water potential of tissue under saline condition, thus availability an important endurance strategy (Ahmed *et al*. 2015). The accumulation of proline might be improved through regulation of solute potential and so the water uptake (Ahmad, 2017).

Elements content

Data tabulated in table 5 showed the ionic contents (Na^+, N^-, P^-, K^+ and Cl^-) of *E. grandiflorum* shootlets grown under various concentrations of NaCl and ZnO NPs. Nitrogen content was significantly affected by various salt treatments. The highest significant nitrogen contents (3.26, 3.17 and 3.10 %) were recorded for control, 5.0 mg/L of ZnO NPs with both 4 g/L salinity and 2g/L salinity, respectively. While, exposing shootlets to different levels of salinity alone significantly reduced N concentration as compared to control especially, the highest level (6 g/L) which gave 1.33% of N. However, there are no significant differences were found between the different treatments and control treatment for

Table 6: Effect of ZnO NPs on antioxidant enzyme activity of *Eustoma* grown *in vitro* under salt stress.

Enzyme / Treatments	POD µm/min/g	SOD U/g	CAT µm/min/g
Control	3.09 d	5.59g	1.36fg
NaCl 2g/L	3.69 c	5.89g	1.17g
NaCl 4g/L	4.15b	6.61f	1.17g
NaCl 6g/L	4.51a	7.52e	3.17a
NaCl 2g/L + 5 mg/L ZnO NPs	3.46cd	8.09 cd	1.44 f
NaCl 2g/L + 10 mg/L ZnO NPs	3.35d	8.38bc	1.27 fg
NaCl 2g/L + 20 mg/L ZnO NPs	3.22d	8.66ab	1.77e
NaCl 4g/l + 5 mg/L ZnO NPs	3.51cd	8.46b	2.11cd
NaCl 4g/L + 10 mg/L ZnO NPs	3.49cd	8.63ab	2.09 d
NaCl 4g/L + 20 mg/L ZnO NPs	3.44 cd	8.55 b	2.39 c
NaCl 6g/L + 5 mg/L ZnO NPs	4.52 a	8.93 a	2.95 ab
NaCl 6g/L + 10 mg/L ZnO NPs	4.33ab	7.79de	3.18a
NaCl 6g/L + 20 mg/L ZnO NPs	4.29 b	7.82 de	3.19 a

Means within a column having the same letters are not significantly different according to Duncan's Multiple Range Test (DMRT) at 5% level.

Image 3: Development of *in vitro* growth sequence of *Eustoma grandiflorum* from shooting, rooting to acclimatization stages; (A): Control (0.0 salinity), (B): The obtained shootlets from NaCl 4 g/L + 5 mg/L ZnO NPs treatment, (C, D, E and F): Rooting of shootlets and acclimatization of obtained plantlets form the same treatment above.

phosphorus. Considerable differences were observed in K and Cl accumulation in the treated and untreated shootlets. Adding ZnO NPs to growth medium had a positive effect on K percentage that were significantly higher comparing with shootlets grown on medium added with the same levels of NaCl that applied to MS culture medium as compared to control. The addition of ZnO NPs under salinity stress led to reductions in the Na content. These results are in agreement with those of Babu *et al.* (2012), who showed that NaCl salinity caused a remarked reduction of mineral elements such as N and K. These decreases in shootlets traits are due to hyperosmotic stress leading to a reduction in water availability. The results of the current study demonstrated that application of ZnO NPs could reduce the harmful effect of salt stress. Similarly, a sufficient Zn supply could reduce Na accumulation and contribute to salt tolerance in plants (Alpaslan *et al.,* 1999; Alharby *et al.,* 2016).

Data in Fig. (2) showed that Na/K ratio was insignificantly differed as a result of salinity treatment except that of 6 g/L NaCl which showed a high ratio. This might be as a result of uptake a high amount of Na. Adding ZnO NPs at different concentrations to the same treatment of salinity (6 g/L) significantly reduced Na/K ratio. Our results are in agreement with that reviewed by Hussein and Abou-Baker (2018) on cotton plants.

Antioxidant enzyme activities

The present study was conducted to study the activity of antioxidative enzymes (POD, SOD and CAT) to determine whether ZnO NPs affects their activities in the salt-treated *E. grandiflorum* grown *in vitro* (Table 6). Salt treatment significantly increased the POD activity compared with control. However, addition of ZnO NPs decreased the POD activity in the leaves of shootlets grown on both 2 and 4 g/L salt. When shootlets were exposed to 6 g/L of salt, adding ZnO NPs had no significant effect tell its concentration increased up to 20 mg/L. The SOD activity increased in the leaves when shootlets were cultured on medium supplemented with salts compared with control. Additions of ZnO NPs lead to an increment of the SOD activity. The CAT activity was decreased insignificantly in the NaCl treated shootlets compared with control, and addition of ZnO NPs

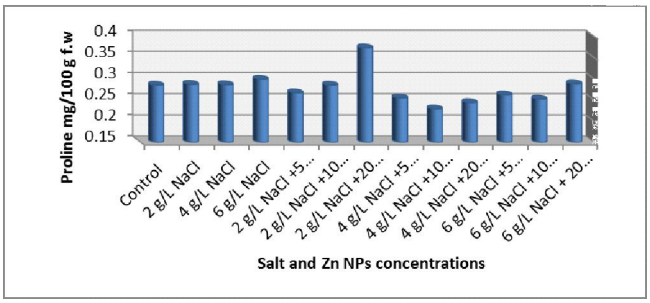

Fig. 1: Proline content of *E. grandiflorum* as affected by ZnO NPs under salt stress.

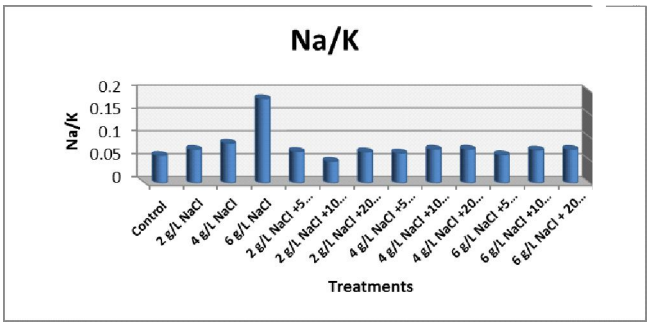

Fig. 2: Sodium/potassium ratio of *E. grandiflorum* as affected ZnO NPs under salt stress.

significantly increased its activity. The maximum CAT activity was measured in shootlets grown on medium supplemented with 6 g/L of salt plus ZnO NPs at different concentrations in this study. SOD is the primary scavenger involved in the detoxification of ROS in plants, and its function is to convert superoxide to H_2O_2 (Asada and Kiso 1973). CAT and POD play important roles in detoxifying H_2O_2 by catalyzing the reduction of H_2O_2 to H_2O (Foyer 1996). Several studies have demonstrated the effects of Zn on the activities of antioxidative enzymes (SOD, APX, and CAT) in plants (Wang *et al*. 2009). Daneshbakhsh *et al*. (2013) found that Zn application increases the root activities of CAT and SOD in wheat cvs. Rushan and Kavir. NaCl reduces the uptake of Zn and other minerals and causes nutritional disorders in plants. Further, Zn deficiency decreases antioxidative enzyme activities and so increases ROS accumulation and subsequent oxidative damage Xu *et al.,* 2014).

Conclusion

The *in vitro* growth ability of *Eustoma grandiflorum* was decreased when explants were exposed to salinity during shoot multiplication stage. Using ZnO NPs have a significant role in alleviating salt stress especially when it used at low concentration (5mg/l).

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