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IMPACT OF APPLICATION OF ZINC OXIDE NANOPARTICLES ON CALLUS INDUCTION, PLANT REGENERATION, ELEMENT CONTENT AND ANTIOXIDANT ENZYME ACTIVITY IN TOMATO (*SOLANUM LYCOPERSICUM* MILL.) UNDER SALT STRESS

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Abstract: The properties of nanomaterials and their potential applications have been given considerable attention by researchers in various fields, especially agricultural biotechnology. However, not much has been done to evaluate the role or effect of zinc oxide nanoparticles (ZnO-NPs) in regulating physiological and biochemical processes in response to salt-induced stress. For this purpose, some callus growth traits, plant regeneration rate, mineral element (sodium, potassium, phosphorous and nitrogen) contents and changes in the activity of superoxide dismutase (SOD) and glutathione peroxidase (GPX) in tissues of five tomato cultivars were investigated in a callus culture exposed to elevated concentrations of salt (3.0 and 6.0 g L^{-1}NaCl), and in the presence of zinc oxide nanoparticles (15 and 30 mg L^{-1}). The relative callus growth rate was inhibited by 3.0 g L^{-1} NaCl; this was increased dramatically at 6.0 g L^{-1}. Increasing exposure to NaCl was associated with a significantly higher sodium content and SOD and GPX activities. Zinc oxide nanoparticles mitigated the effects of NaCl, and in this application of lower concentrations (15 mg L^{-1}) was more effective than a higher concentration (30 mg L^{-1}). This finding indicates that zinc oxide nanoparticles should be investigated further as a potential anti-stress agent in crop production. Different tomato cultivars showed different degrees of tolerance to salinity in the presence of ZnO-NPs. The cultivars Edkawy, followed by Sandpoint, were less affected by salt stress than the cultivar Anna Aasa.

Key words: Nano biotechnology, *in vitro culture*, protein, salinity, superoxide dismutase, glutathione peroxidase

Abbreviations 2.4-D – 2,4-dichlorophenoxyacetic acid; dS – deci Siemens; BSA – bovine serum albumin; CFW – callus fresh weight; CSP – callus survival percentage; CRGR – callus relative growth rate; CWC – callus water content; CRD – completely randomized design; GPX – glutathione peroxidase; CV – coefficient of variation; m – million; MS – Murashige and Skoog; NPs – nanoparticles; NAA – naphthaleneacetic acid; SN – silver nitrate; SOD – superoxide dismutases; ZEA – zeatin; ZnO – zinc oxide

INTRODUCTION

Tomato (*Solanum lycopersicum* Mill. 2n = 12) is considered the second most important vegetable crop after potato, and in 2002 total its production was 116.5 million tons and increased to about 161.8 m tons in 2012. The largest producers are currently China (ca. 50.0 m tons), India (17.5 m tons), and the United States (13.2 m tons) [1]. Environmental stresses, including salt stress, generally adversely affect proteome and plant productivity of most tomato cultivars [2]. Compared to other vegetable crops, tomatoes are moderately sensitive to soil salinity with a maximum threshold of soil sodicity for yield loss of 2.5 dS m^{-1}. The availability of a large germplasm including numerous wild species is useful for introgressing resistance traits against many diseases as well as tolerance against soil salinity and drought [3].

Innovation in food production technology is one of the mainstays to overcome the lack of crop productivity. A selection process using modern agricultural

biotechnology, such as tissue culture, can play a key role in improving salt resistance [4] and the propagation of crops [5]. Cell and tissue culture systems have been considered for the selection of plant tolerance to salinity, drought and other stress factors [6] and an additional advantage of *in vitro* propagation of plants is the generation of high numbers of plants in a short time [7]. Tissue culture is a powerful tool for the screening of plantlets, and provides a unique opportunity for studying many aspects of plant growth and development under well-defined and controlled environmental conditions [8].

One of the new technologies to have recently emerged during is nanotechnology, and the development of nanodevices and nanomaterials is beginning to open up novel applications in plant biotechnology and agriculture [9]. The successful application of various nanoplatforms under *in vitro* conditions has generated interest in nanotechnology in agriculture. Applications of nanomaterials can help faster plant germination, production of improved plant resistance to abiotic and biotic stress, efficient nutrient utilization and enhanced plant growth, with reduced environmental impact compared to traditional approaches. Reynolds [10] demonstrated that micronutrients in the form of nanoparticles (NPs) can be used in crop production to increase yield. One nanoparticle-signaling molecule, zinc oxide (ZnO), appears to play an active role in regulating various mechanisms involved in recognition of and response to abiotic stresses in plants. It has been found that zinc has an important role in the management of reactive oxygen species (ROS) and protection of plant cells against oxidative stresses [11]. Prasad et al. [12] studied the effect of nanoscale ZnO on the germination, growth and yield of peanut, and observed significantly greater growth and yield. There are many references to the interaction between salinity and ZnO in higher plants, but there is currently no information available about the possible beneficial effects of ZnO-NP application to reduce salt stress damages. Thus, the aim of the present study was to examine the impact of different doses of ZnO-NPs on callus growth responses of tomato explants under two different levels of salinity.

Salinity can affect every aspect of plant growth, physiology and biochemistry because it causes both osmotic stress and ionic toxicity [13]. At the molecular level, one of the effects of salinity is impaired cellular function through the accelerated production of ROS [14]. To reduce the accumulation of ROS, plants produce antioxidant enzymes, such as superoxide dismutase (SOD) and glutathione peroxidase (GPX), to keep the ROS lower than the toxic limit [15]. There are a number of studies that have shown that antioxidant enzyme activities and antioxidant content increased in salt-tolerant plant species in response to salt more than in sensitive plants (e.g., wild tomato [16], *Plantago maritima* [17], radish [18], wheat [19])

The objective of the present study was to examine the effectiveness of ZnO-NP application as an advanced biotechnology method to evaluate available tomato germplasm under different treatments of salinity via tissue culture. The results are the first recorded on the effectiveness of ZnO-NP application to influence and improve salt stress in tomato.

MATERIALS AND METHODS

Germplasm

Experiments were conducted in the Biotechnology Laboratory, Faculty of Science, King Abdulaziz University, Jeddah, Saudi Arabia, in cooperation with the Tissue Culture Laboratory, Faculty of Science, Jeddah University, Jeddah, Saudi Arabia, during the period of February 2014 to May 2015. The seeds of four tomato (*Solanum lycopersicum* Mill) cultivars were kindly provided by the Leibniz Institute of Plant Genetics and Crop Plant Research (IPK), Gatersleben, Germany, while seeds of the Egyptian tomato cultivar Edkawy were obtained from the Agriculture Research Center (ARC), Giza, Egypt (Table 1).

Preparation of ZnO-NP suspension

Nanoparticles of ZnO with an average primary particle size of 30 nm were purchased from Sigma-Aldrich Company, California, USA. To prepare different concentrations of ZnO-NPs (15 and 30 mg L^{-1}), a bulk solution was prepared where 1.5 g of solid ZnO-NPs was dissolved in 1 L distilled water, and a sonicator was used to homogenize the solution at 100 W, 40 kHz for 30 min. Small magnetic stirrer bars were placed

Table 1. Accession code, commercial name, botanical name and origin of 5 tomato cultivars.

IPK Accession code*	Commercial name	Botanical name	Origin
LYC3028	Edkawy	*Solanum lycopersicum* Mill.	Egypt
LYC4112	Anna Aasa	*Solanum lycopersicum* Mill. convar. *infiniens* Lehm. var. *flammatum*	**Russia**
LYC3152	Australische Rosen	*Solanum lycopersicum* Mill	**Australia**
LYC4079	Sankt Ignatius	*Solanum lycopersicum* Mill. convar. *infiniens* Lehm. var. *commune*	**Italy**
LYC2493	Sandpoint	*Solanum lycopersicum* Mill. convar. *fruticosum* Lehm. var. *pygmaeum* Lehm.	**USA**

*: Accession code of the Leibniz Institute of Plant Genetics and Crop Plant Research (IPK)

in the suspensions which were automatically stirred thoroughly before use to avoid aggregation of the particles. The nanoparticle suspensions were then centrifuged (3000x*g* for 1 h) and filtered (0.7-µm glass filter) prior to being added to culture media [20].

Callus induction

Tomato seeds were washed three times in sterilized water and surface sterilized according to Metwali et al. [21]. The seeds were then cultured *in vitro* under aseptic conditions on a hormone-free germination medium (M1) composed of 4.4 g L^{-1} Murashige and Skoog (MS) salts [22], 3% sucrose, 100 mg L^{-1} myoinositol, 1.0 mg L^{-1} thiamin HCl and solidified with 0.25% (w/v) Phytagel. The media pH was adjusted to 5.7 by either 1 M NaOH or HCl, prior to autoclaving at 121°C for 20 min. Cultured seeds were kept in an incubator at 25±2°C in the dark for one week to induce seed germination. Subsequently, they were maintained in a 16-h photoperiod under 60 µmol m^{-2} s^{-1} illumination supplied by cool white fluorescent light for three weeks. Cotyledon explants were excised from 27-day-old seedlings cut into halves with a sterile scalpel blade and placed abaxial side facing down on callus induction medium as previously described [23]. Callus induction medium (M2) was prepared as previously described [24]. The cultures were then incubated under the controlled conditions described earlier.

In vitro salt and ZnO-NP treatments

Measurement of different callus traits

Nine treatments were prepared (T1=control; T2=3 g L^{-1} NaCl; T3=6 g L^{-1} NaCl; T4=15 mg L^{-1} ZnO-NPs; T5=30 mg L^{-1} ZnO-NPs; T6=3 g L^{-1} NaCl + 15 mg L^{-1} ZnO-NPs; T7=3 g L^{-1} NaCl + 30 mg L^{-1} ZnO-NPs; T8=6 g L^{-1} NaCl + 15 mg L^{-1} ZnO-NPs; T9=6 g L^{-1} NaCl + 30 mg L^{-1} ZnO-NPs) and added to callus proliferation medium (M2). The media pH was adjusted to 5.7 by either 1 M NaOH or HCl, prior to autoclaving at 121°C for 20 min. Healthy and actively growing calli pieces (4 per Petri dish) weighing 0.5 g from the callus induction cultures were selected and cultured on M2 medium; a total of 45 Petri dishes were used. The dishes were sealed with paraffin tape and subsequently arranged in a completely randomized design (CRD) with 5 replications; a total of 180 calli were tested per cultivar in an incubator for three weeks; the medium was refreshed every 7 days. At the end of three weeks the following traits were recorded: (i) CFW: Callus fresh weight; (ii) CSP: callus survival percentage = No. of callus survived/total no. of calli cultured x 100 according to [25]; (iii) CRGR: Callus relative growth rate = callus final weight – callus initial weight/callus initial weight according to [26]; (iv) CWC: Callus water content (%) = Callus fresh weight – Callus dry weight/Callus fresh weight x 100 according to the equation described previously [27]. Callus dry weight was gravimetrically determined after drying at 80°C for 48 h.

Plant regeneration

Friable and healthy calli were transferred to jars containing 25 mL of optimized regeneration medium (M3), comprising of the M1 medium supplemented with zeatin (ZEA, 1.0 mg L^{-1}), NAA (0.1 mg L^{-1}) and silver nitrate (SN, 5.0 mg L^{-1}). In this medium, different concentrations of ZnO-NPs and NaCl were added as described above for callus induction. The cultures were maintained in the growth chamber as above. After 4 weeks, the plant regeneration percentage (PRP) was obtained according to [25].

Measurement of inorganic ion concentration

For the quantification of major inorganic ions (Na^+, K^+, Cl^-, P^+ and Zn^+), calli were randomly harvested from Petri dishes for each treatment and dried in an oven at 60°C. Subsequent determination of the mineral composition was measured according to [28]. Half a gram of the ground calli was acid-digested with H_2SO_4 overnight with careful heating on a hot plate at 100°C. The beaker was cooled and 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 [29], Cl was measured by silver nitrate method according to [30], while P was determined calorimetrically using the ammonium phosphorus vanadomolybdate method [29] and Zn was determined by atomic absorption spectrophotometer (Thermo-Electron, S Series GE711838).

Measurement of antioxidant enzyme activities

Extraction

Selected plant organs or callus material (0.5 g fresh weight) was frozen in liquid nitrogen and then ground into a fine powder with a mortar and pestle and kept in a microfuge tube at -80°C until analysis. Crude protein extracts were prepared by incubating in frozen powder with 50 mM sodium phosphate buffer (pH 7.0) containing 0.1 mM EDTA, 0.1% (v/v) Triton X-100, 1 mM 2-mercaptoethanol and 1% (w/v) polyvinylpolypyrrolidone (PVPP) and homogenized with a chilled mortar and pestle. The homogenized samples were centrifuged (15 000x*g*) for 20 min at 4°C. After centrifugation, aliquots of the supernatant were removed and used as crude extract to determine enzyme activity. Protein was quantified as described by [31] using bovine serum albumin (BSA) as a standard.

Enzyme assay to measure the specific activities of SOD and GPX

Protein concentration in crude extracts was adjusted to 0.5 mg mL^{-1}. Total superoxide dismutase (EC 1.15.1.1; SOD) activity was assayed by its ability to inhibit photochemical reduction of nitroblue tetrazolium (NBT) according to a modified method [32]. Crude enzymatic extract (100 µL) was added to 3 mL of a reaction mixture, consisting of 50 mM potassium phosphate buffer (pH 7.8), 13 mM methionine, 0.1 mM EDTA, 75 µM NBT and 2.0 µM riboflavin as described [33]. The reaction was carried out in a chamber with a 15-W fluorescent lamp at 25°C. In this assay, 1 unit of SOD is defined as the amount required to inhibit the photoreduction of NBT by 50%. The specific activity of SOD was expressed as µmol mg^{-1} protein and SOD activity was measured spectrophotometrically at 560 nm. Glutathione peroxidase (EC 1.11.1.9; GPX) activity was also measured spectrophotometrically following the decrease in absorbance at 340 nm of NADPH. The assay mixture consisted of 0.1 M Tris-HCl buffer (pH 7.5), 1 mM EDTA and 10 mM $MgCl_2$, 0.15 mM NADPH and 50 µg of total protein (100 µL of 0.5 mg mL^{-1} crude extract) in 1-mL reaction tubes according to [34]. The mixture was pre-incubated at 37°C for 10 min. One unit of activity was defined as the consumption of 1.0 µmol NADPH per minute and the specific activity of GPX was expressed as µmol NADPH mg^{-1} protein.

Statistical analysis

The effect of different concentrations of NaCl and ZnO-NPs used was evaluated by analysis of variance (ANOVA) and the treatment means were compared using Duncan's multiple range test (DMRT) and each average was presented with the standard error. Significant differences between treatment means were denoted with letters and different letters denote statistical significance at $p \leq 0.05$ within the measurement.

RESULTS

Analysis of variance (ANOVA) indicated that the influence of the cultivars and treatments were significant ($p \leq 0.001$). The cultivar - treatment interaction was highly significant for all the traits ($p \leq 0.001$), except callus fresh weight, callus relative growth and sodium content, which recorded no significant differences (Table 2). Considering the significant cultivar - treatment interaction for most of the measured traits, comparison of means was performed only on combinations of two factors. This indicated that genotypic differences in response to NaCl and the ZnO-NP treatment in tomato do not produce similar trends to

Table 2. Analysis of variance for different traits of callus, plant regeneration, elements and antioxidant enzymes of 5 cultivars of tomato (*Solanum lycopersicum* Mill) under different treatments of salinity and zinc oxide nanoparticles.

SV	df	MS											
		CFW	CRGR	CWC	CSP	PRP	Na	K	N	P	Zn	SOD	GPX
Cultivars (C.)	4	0.304***	1.240***	1001.14***	455.96***	1952.98***	165.99***	28.51***	129.70***	7.697***	0.03149***	63.52***	64.80***
Treatments (T.)	8	1.275***	5.074***	2730.84***	2504.8***	3989.46***	49.13***	87.42***	98.35***	0.5001***	0.3083***	44.96***	47.93***
C. x T.	32	0.0375ns	0.1494ns	98.68***	39.58***	124.84***	6.67***	5.27***	12.81ns	0.224***	0.0022***	2.143***	4.448***
Error	180	0.0268	0.1075	12.22	0.3011	0.5914	1.539	0.0406	11.35	0.0187	6.5126	0.008	0.086
Total	224												
CV%		18.295	41.46	4.82	0.6609	1.011	3.491	0.3751	7.759	2.679	5.819	0.780	3.490

*Each column shows significant differences at p≤0.05 (*), p≤0.01(**), and p≤0.001 (***), by Duncan's multiple range test (DMRT); ns – non-significant difference. Also refer to abbreviations at the beginning of the article.

the genotypic differences under control conditions for most of traits measured in this investigation. Most of the traits exhibited a wide range of variability as indicated by the magnitude of the coefficient of variation (CV), which ranged from 0.3751% for potassium content to 41.46% for callus relative growth rate.

Impact of NaCl and ZnO-NPs on different callus traits and plant regeneration

In general, the lowest values of all callus traits, i.e. CFW, CSP, CRGR and CWC, and plant regeneration percentage (PRR) were recorded in the treatments with salinity stress compared with control or other treatments without salinity stress. Increasing salt in the tissue culture medium significantly decreased the CSP and lowered the CFW and therefore the CWC and CRGR (Table 3). The plant cultivars Anna Aasa and Sankt Ignatius were the most affected by the two salinity levels. The decreased rate in CFW ranged between 0.546 g for T2 (3 g L^{-1} NaCl), 0.545 g for T3 (6 g L^{-1} NaCl) and 0.66 g for T2 and 0.62 g for T3 in both cultivars, respectively. Under salt stress treatments, minimum values for CFW, CPS and CRGR were recorded in Anna Aasa and for CWC in Sankt Ignatius, while maximum values were observed in cv. Edkawy under T2, T3, T4, T6 and T7, Sandpoint under T8 and T9, Sankt Ignatius under T5 (Table 3). These results indicated that Edkawy followed by Australische Rosen and/or Sandpoint were more tolerant of salinity for most of the traits. Similar results were recorded in PRP in the presence of salinity, and the highest value of PRR was recorded in Sandpoint and Edkawy under T2 and T3, respectively. In contrast, all callus traits and PRP were increased when treated with the ZnO-NPs, especially under the higher concentration of ZnO-NPs (T5=30 mg L^{-1}), in all cultivars except Anna Aasa. The

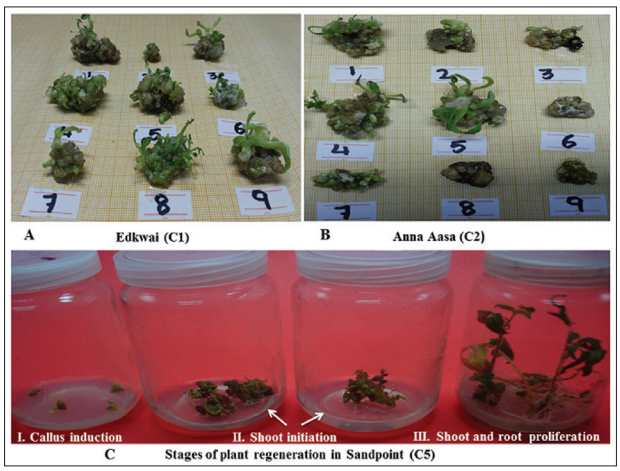

Fig 1. Effect of different levels of NaCl salt (3.0 and 6.0 g L^{-1}) and ZnO-NPs (15 and 30 mg L^{-1}) addition to control on callus growth in cultivar a) Edkawy and b) Anna Aasa. c) Plant regeneration stages (I. Callus induction, II. Shoot initiation, III. Shoot and root proliferation) of Sandpoint cultivar. (1) control; (2) 3 g L^{-1} NaCl; (3) 6 g L^{-1} NaCl; (4) 15 mg L^{-1} ZnO-NPs; (5) 15 mg L^{-1} ZnO-NPs; (6) 3 g L^{-1} NaCl + 15 mg L^{-1} ZnO-NPs; (7) 3 g L^{-1} NaCl + 30 mg L^{-1} ZnO-NPs; (8) 6 g L^{-1} NaCl + 15 mg L^{-1} ZnO-NPs; (9) 6 g L^{-1} NaCl + 30 mg L^{-1} ZnO-NPs.

ZnO-NPs at different concentrations T4 (15 mg L^{-1}) and T5 (30 mg L^{-1}) and in the both salt treatments (T2 and T3) caused a reduction in the effect of the salt stress in all measured traits (Table 3). Treatment T6 (15 mg L^{-1} ZnO-NPs + 3.0 g L^{-1} NaCl) recorded the maximum callus growth parameters and plant regeneration in most of the tomato cultivars, while for the higher level of salinity (T3) higher values were found in the callus treated with ZnO-NPs (15 mg L^{-1}) for both CRGR and PRP traits. For the rest of traits the effect was variable between the treatments. The best result in callus growth traits and plant regeneration in the presence of ZnO-NPs and salinity stress was observed in cultivars Edkawy, Australische Rosen and Sandpoint (Fig. 1).

Table 3. Callus growth and plant regeneration traits of 5 tomato cultivars grown *in vitro* under control, different levels of salt (3 and 6 g L^{-1}) and ZnO-NPs (15 and 30 mg L^{-1}) individual or in different combinations.

Parameters	Treatments	Tomato germplasm				
		Edkawy	**Anna Aasa**	**Australische Rosen**	**Sankt Ignatius**	**Sandpoint**
CFW	T1	1.21±0.117^{ab}	1.097±0.041^{ab}	1.04±0.273^{bc}	1.14±0.05^{a}	1.10±0.207^{a}
(g)	T2	0.85±0.037^{bc}	0.546±0.016^{d}	0.83±0.124^{d}	0.66±0.164^{b}	0.68±0.085^{c}
	T3	0.73±0.456^{C}	0.545±0.022^{d}	0.66±0.037^{d}	0.62±0.06^{b}	0.70±0.125^{c}
	T4	1.24±0.290^{ab}	1.16±0.090^{a}	1.19±0.098^{ab}	1.22±0.076^{a}	1.18±0.331^{a}
	T5	1.31±0.190^{a}	1.0±0.047^{b}	1.23±0.074^{a}	1.36±0.055^{a}	1.21±0.189^{a}
	T6	1.05±0.380^{abc}	0.70±0.094^{c}	0.890±0.138^{cd}	0.68±0.029^{b}	0.76±0.177^{bc}
	T7	0.82±0.267^{bc}	0.614±0.082^{cd}	0.70±0.096^{d}	0.63±0.031^{b}	0.73±0.093^{bc}
	T8	0.79±0.146^{bc}	0.619±0.024^{cd}	0.898±0.023^{cd}	0.69±0.064^{b}	0.95±0.021^{abc}
	T9	0.98±0.312^{abc}	0.71±0.034^{c}	0.71±0.008^{d}	0.66±0.008^{d}	1.01±0.0155^{ab}
	LSD 0.05	**0.294**	**0.0746**	**0.157**	**0.234**	**0.217**
CSP	T1	94.46±0.114^{c}	95.1±0.187^{b}	94.22±0.370^{b}	93.38±0.363^{b}	94.36±0.427^{b}
(%)	T2	84.36±0.240^{e}	74.3±0.223^{d}	85.22±0.668^{c}	76.46±0.966^{e}	77.9±0.655^{f}
	T3	70.44±0.456^{i}	61.44±0.336^{g}	73.28±0.944^{g}	67.6±0.245^{g}	70.9±0.685^{h}
	T4	96.58±0.192^{b}	95.52±0.376^{ab}	95.06±0.512^{ab}	93.12±0.481^{b}	94.32±0.722^{b}
	T5	97.48±0.349^{a}	96.14±0.391^{a}	95.54±0.702^{a}	96.12±0.349^{a}	96.95±0.785^{a}
	T6	85.28±0.389^{d}	74.34±0.230^{d}	83.08±0.589^{d}	80.24±0.397^{d}	81.62±0.576^{d}
	T7	80.12±0.178^{h}	64.46±0.304^{f}	77.26±0.622^{f}	70.32±0.342^{f}	74.74±0.472^{g}
	T8	83.5±0.244^{f}	76.48±0.178^{c}	84.64±1.165^{c}	80.94±0.626^{c}	84.5±0.122^{c}
	T9	80.96±0.192^{g}	66.02±1.33^{e}	80.78±0.909^{e}	75.9±0.418^{e}	80.42±0.34^{e}
	LSD 0.05	**0.363**	**0.669**	**0.971**	**0.652**	**0.729**
CRGR	T1	1.42±0.234^{abc}	1.19±0.083^{ab}	1.93±0.546^{bc}	1.29±0.077^{a}	1.21±0.414^{a}
	T2	0.71±0.074^{bc}	0.094±0.032^{d}	0.67±0.248^{d}	0.33±0.329^{b}	0.36±0.170^{b}
	T3	0.51±0.138^{c}	0.091±0.045^{d}	0.34±0.075^{d}	0.25±0.120^{b}	0.41±0.251^{b}
	T4	1.48±0.581^{ab}	1.33±0.181^{a}	1.39±0.196^{ab}	1.44±0.154^{a}	1.36±0.663^{a}
	T5	1.63±0.380^{a}	1.05±0.094^{b}	1.48±0.148^{a}	1.74±0.110^{a}	1.43±0.378^{a}
	T6	1.11±0.760^{abc}	0.42±0.194^{c}	0.78±0.277^{cd}	0.37±0.058^{b}	0.54±0.354^{b}
	T7	0.64±0.535^{bc}	0.23±0.164^{cd}	0.40±0.193^{d}	0.27±0.062^{b}	0.46±0.187^{b}
	T8	0.59±0.293^{bc}	0.24±0.047^{cd}	0.79±0.047^{cd}	0.38±0.129^{b}	0.90±0.043^{ab}
	T9	0.97±0.624^{abc}	0.43±0.068^{c}	0.42±0.017^{d}	0.33±0.074^{b}	1.01±0.229^{ab}
	LSD 0.05	**0.589**	**0.150**	**0.315**	**0.468**	**0.441**
CWC	T1	83.22±7.54^{ab}	81.04±0.687^{a}	83.88±0.954^{b}	85.89±2.44^{b}	83.59±4.14^{a}
(%)	T2	67.70±2.81^{d}	59.01±2.54^{c}	64.17±1.54^{g}	55.61±1.023^{d}	64.91±4.18^{b}
	T3	65.38±4.08^{d}	57.49±1.14^{c}	60.24±0.485^{h}	51.15±0.116^{e}	61.52±9.48^{b}
	T4	88.27±1.01^{a}	81.24±1.54^{a}	86.2±0.595^{a}	87.82±0.431^{ab}	84.24±6.02^{a}
	T5	89.80±1.93^{a}	81.1±0.744^{a}	86.57±2.55^{a}	89.85±0.393^{a}	88.58±2.01^{a}
	T6	77.55±6.22^{bc}	63.14±3.009^{b}	74.08±1.020^{d}	60.41±1.523^{c}	71.03±1.41^{b}
	T7	72.74±3.56^{cd}	58.25±1.30^{c}	66.14±1.21^{f}	59.88±1.092^{c}	66.11±4.04^{b}
	T8	75.74±3.93^{c}	57.16±2.28^{c}	76.35±0.925^{c}	60.86±3.38^{c}	84.03±2.19^{a}
	T9	73.14±6.47^{cd}	62.52±2.19^{b}	70.46±0.607^{e}	61.56±2.40^{c}	81.70±9.62^{a}
	LSD 0.05	**5.974**	**2.418**	**1.607**	**2.267**	**7.164**
PRP	T1	93.16±0.466^{c}	91.14±0.349^{a}	88.4±0.223^{c}	90.14±0.461^{a}	94.3±0.234^{a}
(%)	T2	80.66±0.207^{f}	56.32±0.294^{d}	69.9±0.547^{g}	65.8±0.534^{cd}	81.16±0.606^{f}
	T3	67.6±0.316^{i}	45.6±0.141^{f}	65.42±0.455^{h}	55.52±0.535^{f}	60.52±0.216^{i}
	T4	95.26±0.250^{a}	92.48±0.402^{a}	90.34±0.658^{b}	80.9±0.394^{b}	92.78±0.526^{b}
	T5	94.34±0.343^{b}	93.02±0.303^{a}	92.92±0.531^{a}	90.88±0.389^{a}	90.92±0.377^{c}
	T6	83.34±0.230^{a}	61.3±0.212b^{c}	77.46±0.270^{e}	64.56±0.404^{de}	83.12±0.912^{e}
	T7	71.58±1.60^{h}	50.32±0.205^{e}	70.14±0.477^{g}	56.62±0.370^{f}	67.48±0.148^{h}
	T8	82.32±0.216^{e}	64.58±0.148^{b}	78.16±0.230^{d}	67.7±0.474^{c}	87.64±0.581^{d}
	T9	72.48±0.230^{g}	58.26±0.130^{cd}	74.1±0.258^{f}	62.62±0.604^{e}	73.18±0.277^{g}
	LSD 0.05	**0.764**	**3.768**	**0.636**	**2.401**	**0.629**

Values are the means of five replications. Columns for each cultivar with a different lower-case letter were significantly different at $p<0.05$ compared to control as determined by Duncan's multiple range test.

T1= control; T2= 3 g L^{-1} NaCl; T3= 6 g L^{-1} NaCl; T4= 15 mg L^{-1} ZnO-NPs; T5= 30 mg L^{-1} ZnO-NPs; T6= 3 g L^{-1} NaCl + 15 mg L^{-1} ZnO-NPs; T7= 3 g L^{-1} NaCl + 30 mg L^{-1} ZnO-NPs; T8= 6 g L^{-1} NaCl + 15 mg L^{-1} ZnO-NPs; T9= 6 g L^{-1} NaCl + 30 mg L^{-1} ZnO-NPs.

Table 4. Ion concentrations in 5 tomato cultivars grown *in vitro* under control, different concentrations of NaCl (3 and 6 g L^{-1}) and ZnO-NPs (15 and 30 mg L^{-1}) and in different combinations.

Parameters	Treatments	Tomato germplasm				
		Edkawy	Anna Aasa	Australische Rosen	Sankt Ignatius	Sandpoint
Na content (g kg^{-1} dw)	T1	30.13±0.055^{h}	33.46±0.040^{a}	31.83±0.060^{b}	38.87±0.87^{e}	33.52±0.245^{e}
	T2	36.35±0.05^{c}	35.07±0.060^{a}	36.26±0.057^{ab}	40.13±0.152^{e}	34.08±0.107^{d}
	T3	39.63±0.305^{a}	32.77±0.095^{a}	40.20±0.100^{a}	42.16±0.152^{a}	39.65±0.080^{a}
	T4	30.33±0.351^{h}	34.41±0.080^{a}	35.20±0.095^{ab}	39.75±0.129^{d}	32.59±0.055^{f}
	T5	33.10±0.005^{f}	34.52±0.030^{a}	35.38±0.034^{ab}	39.05±0.05^{e}	32.47±0.076^{f}
	T6	32.31±0.017^{g}	32.17±0.155a	33.48±0.102^{b}	37.10±0.1006^{f}	32.58±0.023^{f}
	T7	33.53±0.075^{e}	32.60±0.155^{a}	34.02±0.155^{b}	39.71±0.160^{d}	33.46±0.235^{e}
	T8	35.68±0.023^{d}	33.20±0.136^{a}	36.1±0.1^{ab}	40.23±0.155^{c}	34.55±0.092^{c}
	T9	37.54±0.050^{b}	34.34±0.101^{a}	37.26±0.378^{ab}	41.18±0.023^{b}	37.21±0.182^{b}
	LSD 0.05	**0.275**	**3.529**	**3.346**	**0.201**	**0.253**
K content (g kg^{-1} dw)	T1	56.55±0.05^{a}	57.47±0.276^{a}	56.61±0.080^{a}	59.54±0.294^{b}	57.74±0.150^{a}
	T2	53.11±0.085^{c}	51.2±0.20^{e}	53.43±0.115^{b}	55.27±0.251^{e}	52.02±0.064^{g}
	T3	52.13±0.152^{d}	48.13±0.251^{g}	51.00±0.1^{f}	52.37±0.107^{h}	53.5±0.2^{e}
	T4	51.27±0.132^{e}	52.07±0.124^{d}	51.34±0.211^{e}	54.24±0.200^{f}	54.29±0.171^{d}
	T5	56.54±0.224^{a}	57.52±0.101^{a}	56.70±0.200^{a}	60.03±0.228^{a}	55.66±0.408^{c}
	T6	50.26±-0.251^{f}	52.5±0.1^{c}	52.23±0.152^{d}	56.36±0.208^{c}	52.65±0.080^{f}
	T7	55.4±0.30^{b}	54.34±0.144^{b}	53.23±0.251^{d}	55.86±0.152^{d}	56.09±0.215^{b}
	T8	48.73±0.251^{g}	51.46±0.163^{e}	51.4±0.1^{e}	50.06±0.158^{i}	52.06±0.417^{g}
	T9	53.43±0.23^{d}	50.31±0.225^{f}	52.83±0.208^{c}	53.44±0.191^{g}	54.36±0.115^{d}
	LSD 0.05	**0.348**	**0.3204**	**0.289**	**0.354**	**0.406**
N content (g kg^{-1} dw)	T1	44.11±0.095^{a}	45.18±0.170^{b}	47.51±0.250^{a}	48.46±0.351^{a}	46.2±0.264^{a}
	T2	30.31±0.128^{a}	40.30±0.260^{d}	43.46±0.135^{c}	46.6±0.458^{b}	42.37±0.402^{e}
	T3	41.18±0.131^{a}	37.06±0.208^{f}	39.99±0.115^{f}	44.73±0.378^{d}	41.96±0.208^{e}
	T4	44.18±0.051^{a}	45.99±0.110^{a}	47.5±0.346^{a}	48.63±0.378^{a}	46.6±0.3^{a}
	T5	44.6±0.264^{a}	46.3±0.173^{a}	47.30±0.128^{a}	48.56±0.251^{a}	46.1±0.360^{a}
	T6	41.26±0.251^{a}	40.41±0.162^{d}	40.46±0.256^{e}	45.7±0.2^{c}	43.66±0.230^{d}
	T7	43.16±0.416^{a}	40.85±0.259^{c}	44.42±0.150^{b}	47.13±0.416^{b}	45.26±0.305^{b}
	T8	41.03±0.450^{a}	37.30±0.260^{f}	39.31±0.3005^{g}	44.3±0.360^{d}	40.76±0.115^{f}
	T9	42.4±0.173^{a}	39.24±0.215^{e}	42.58±0.3008^{d}	45.03±0.305^{c}	44.26±0.550^{c}
	LSD 0.05	**12.882**	**0.357**	**0.404**	**0.605**	**0.559**
P content (g kg^{-1} dw)	T1	4.09±0.036^{ab}	5.11±0.01^{b}	5.41±0.189^{a}	5.5±0.05^{a}	5.93±0.148^{a}
	T2	3.91±0.017b	4.3±0.1^{c}	5.53±0.208^{a}	4.54±0.045^{c}	5.43±0.201^{bc}
	T3	4.4±0.3^{a}	4.04±0.051^{d}	5.18±0.170^{a}	4.86±0.057^{b}	5.83±0.051^{ab}
	T4	4.27±0.072^{a}	5.34±0.036^{a}	5.38±0.371^{a}	5.48±0.047^{a}	5.34±0.257^{c}
	T5	4.3±0.03^{a}	5.25±0.113^{ab}	5.18±0.170^{a}	5.57±0.102^{a}	5.53±0.108^{abc}
	T6	4.29±0.04^{a}	5.36±0.125^{a}	5.38±0.156^{a}	5.45±0.098^{a}	5.52±0.017^{abc}
	T7	4.276±0.011^{a}	5.24±0.1^{ab}	5.35±0.127^{a}	5.5±0.16^{a}	5.66±0.146^{abc}
	T8	4.273±0.148^{a}	5.37±0.025^{a}	5.25±0.055^{a}	5.47±0.088^{a}	5.63±0.221^{abc}
	T9	4.3±0.02^{a}	5.36±0.034^{a}	5.37±0.011^{a}	5.68±0.153^{a}	5.70±0.190^{abc}
	LSD 0.05	**0.199**	**0.133**	**0.329**	**0.169**	**0.286**
Zn content (g kg^{-1} dw)	T1	0.343±0.017^{f}	0.374±0.004^{e}	0.413±0.002^{e}	0.383±0.003^{d}	0.435±0.005^{e}
	T2	0.275±0.005^{g}	0.288±0.001^{g}	0.353±0.003^{f}	0.318±0.001^{f}	0.403±0.006^{g}
	T3	0.226±0.003^{h}	0.204±0.006^{i}	0.252±0.005^{g}	0.253±0.003^{g}	0.310±0.002^{i}
	T4	0.536±0.003^{c}	0.489±0.001^{c}	0.568±0.008^{c}	0.504±0.007^{c}	0.545±0.006^{c}
	T5	0.714±0.004^{a}	0.658±0.002^{a}	0.735±0.031^{g}	0.681±0.007^{a}	0.693±0.006^{a}
	T6	0.376±0.002^{e}	0.321±0.001^{f}	0.406±0.002^{e}	0.329±0.001^{f}	0.416±0.005^{f}
	T7	0.575±0.004^{b}	0.53±0.003^{b}	0.640±0.001^{b}	0.548±0.011^{b}	0.585±0.005^{b}
	T8	0.273±0.038^{g}	0.254±0.004^{h}	0.360±0.004^{f}	0.369±0.005^{e}	0.355±0.005^{h}
	T9	0.411±0.001^{d}	0.472±0.003^{d}	0.544±0.004^{d}	0.493±0.001^{c}	0.509±0.003^{d}
	LSD 0.05	**0.0233**	**0.00612**	**0.0202**	**0.0116**	**0.0095**

Values are the means of five replications. Columns for each cultivar with a different lower-case letter were significantly different at $p<0.05$ compared to control as determined by Duncan's multiple range test.

T1=control; T2=3 g L^{-1} NaCl; T3=6 g L^{-1} NaCl; T4=15 mg L^{-1} ZnO-NPs; T5=30 mg L^{-1} ZnO-NPs; T6=3 g L^{-1} NaCl + 15 mg L^{-1} ZnO-NPs; T7=3 g L^{-1} NaCl + 30 mg L^{-1} ZnO-NPs; T8=6 g L^{-1} NaCl + 15 mg L^{-1} ZnO-NPs; T9=6 g L^{-1} NaCl + 30 mg L^{-1} ZnO-NPs.

Impact of NaCl and ZnO-NPs on elemental content of callus

The Na, N, P, K and Zn ionic contents in calli grown under different treatments of salt and ZnO-NPs was estimated and expressed as g kg^{-1} dry weight (Table 4). Considerable differences were observed in ion accumulation in the treated and untreated calli. Sodium significantly increased with increases in the levels of NaCl applied compared to control calli, while ion concentration reduced appreciably in the salt-stressed calli in comparison to control with a strong reduction observed at the higher level of NaCl (T2). The addition of ZnO-NPs under salinity stress led to significant reductions in the Na content, with the average Na content in calli tissue across all tomato cultivars 35.39 g kg^{-1} dw, as compared to 37.63 g kg^{-1} dw under the salt-stress treatment only. On the other hand, no significant differences were found between the ZnO-NPs and control treatment for K, N and P content. The highest K content was observed for the combination treatment, salinity stress and ZnO-NPs, in cultivars Sandpoint (56.09; 54.36 g kg^{-1} dw) and Edkawy (55.4; 53.43 g kg^{-1} dw) under T7 and T9 treatment, respectively in both cases. The results show that nitrogen accumulation increased in callus tissue under treatment with 30 mg L^{-1} ZnO-NPs compared to 15 mg L^{-1} ZnO-NPs in the presence of salt (3.0 g L^{-1} and 6.0 g L^{-1}). The lowest values of nitrogen accumulation (40.41; 40.85 g kg^{-1} dw) and (37.30; 39.24 g kg^{-1} dw) were recorded for cultivar Anna Aasa at both levels of ZnO-NPs and salt. Data on phosphorous accumulation and its relation to salt treatment and ZnO-NPs are presented in Table 4. There were non-significant differences between the treatments in most of the cultivars. The results revealed that the highest mean value of P content was recorded for cultivar Sandpoint (5.83 g kg^{-1}) at T3 (6 g L^{-1}), Australische Rosen (5.53 g kg^{-1}) at T2 (3 g L^{-1} NaCl), Anna Aasa and (5.37 g kg^{-1}) at T8 (15mg L^{-1} ZnO-NPs + 6 g L^{-1} NaCl) and Sankt Ignatius (5.68 g kg^{-1}) at T9 (30 mg L^{-1} ZnO-NPs + 6 g L^{-1} NaCl). Zinc concentration in callus tissue was lowest in the absence of ZnO-NP treatment (Table 4). Increasing the NaCl concentration in the callus induction media from 0 to 3 to 6 g L^{-1} led to a significant decrease in Zn concentration in callus tissue. The addition of ZnO-NPs increased Zn accumulation in callus tissue even in the presence of added NaCl, and was greatest in T7 (30 mg L^{-1} ZnO-NPs + 3 g L^{-1} NaCl) followed by T9 (30 mg L^{-1} ZnO-NPs + 6 g L^{-1} NaCl). In general, maximum values for Na and N accumulation were observed in cv. Sankt Ignatius, for P in cv. Sandpoint, for K in both of cv. Sankt Ignatius and cv. Sandpoint, and for Zn in cv. Australische Rosen (Table 4).

Impact of NaCl salt and ZnO-NPs on protein content and antioxidant enzyme activation in callus tissue

The present study showed that salt stress decreased the total protein content of tomato calli in comparison with the untreated control. NaCl stress at 3 g L^{-1} caused a more pronounced effect than NaCl at 6 g L^{-1} in terms of protein content (Table 5). The callus grown in the presence of ZnO-NPs at 15 and 30 mg L^{-1} showed a statistically significant increase in protein content compared to calli grown in the presence of NaCl. There was also an interaction between NaCl and ZnO-NP application resulting in a significant increase in protein content in comparison with non-ZnO-NP treatments. The addition of NaCl induced a significant increase in SOD and GPX accumulation in comparison to control and ZnO-NP treatments (Fig. 2). The results with calli showed that even the low concentration of NaCl (3.0 g L^{-1}) upregulated SOD and GPX accumulation, however, the increase was genotype-dependent (Fig. 2). Accumulation of SOD (15.92, 14.306, 12.93 μmol mg^{-1} protein) and GPX (12.65, 9.90, 7.779 μmol mg^{-1} protein) was found to be highest in salt-tolerant cv. Sandpoint, Edkawy and Sankt Ignatius compared to the salt-sensitive cv. Anna Aasa (9.536 and 7.44 μmol mg^{-1} protein). For any given cultivar, the addition of ZnO-NPs at 15 mg L^{-1} triggered a similar impact to that of the control treatment on the SOD and GPX accumulation measured. Nonetheless, increasing ZnO-NP concentration to 30 mg L^{-1} was more effective on SOD and GPX accumulations, which were significantly increased compared to control treatments. In response to NaCl in combination with ZnO-NPs, data showed a small but significant increase in SOD and GPX accumulation in most of the combination treatments when cross-comparing with individual treatments (control, NaCl and ZnO-NPs). The highest SOD activity in this study was 16.69 μmol mg^{-1} protein in the salt-tolerant cv. Sandpoint, followed by Australische Rosen, while low SOD activity was observed in cv. Anna Aasa for all

Table 5. Protein content of 5 tomato cultivars grown *in vitro* under control, different levels of salt (3 and 6 g L^{-1}) and ZnO-NPs (15 and 30 mg L^{-1}) individual or in different combinations.

Parameters	Treatments	Tomato germplasm				
		Edkawy	Anna Aasa	Australische Rosen	Sankt Ignatius	Sandpoint
Protein content (mg g^{-1}fw)	T1	40.03±0.067^{b}	38.83±0.112^{a}	39.99±0.110^{a}	41.31±0.017^{a}	42.34±0.051^{a}
	T2	23.24±0.053^{h}	19.62±0.043^{h}	20.10±0.140^{g}	21.30±0.025^{h}	22.42±0.043^{i}
	T3	23.30±0.030^{h}	18.51±0.041^{i}	17.62±0.130^{h}	22.60±0.003^{g}	23.60±0.050^{h}
	T4	36.05±0.050^{d}	35.08±0.037^{c}	39.20±0.100^{b}	37.99±0.100^{c}	38.90±0.020^{d}
	T5	41.03±0.055^{a}	37.35±0.050^{b}	40.02±0.064^{a}	38.51±0.070^{b}	39.84±0.040^{c}
	T6	26.75±0.050^{g}	23.58±0.080^{g}	25.48±0.028^{f}	24.43±0.076^{f}	27.70±0.020^{g}
	T7	31.27±0.037^{f}	25.53±0.080^{f}	31.03±0.080^{e}	28.96±0.055^{e}	33.49±0.040^{f}
	T8	35.49±0.017^{e}	30.32±0.065^{d}	32.50±0.060^{d}	33.45±0.050^{d}	40.78±0.026^{b}
	T9	37.45±0.050^{c}	29.99±0.095^{e}	33.81±0.070^{c}	33.50±0.055^{d}	36.72±0.030^{e}
	LSD 0.05	**0.0817**	**0.1237**	**0.1470**	**0.1011**	**0.0646**

Values are the means of five replications. Columns for each cultivar with a different lower-case letter were significantly different at $p < 0.05$ compared to control as determined by Duncan's multiple range test.
T1=control; T2=3 g L^{-1} NaCl; T3=6 g L^{-1} NaCl; T4=15 mg L^{-1} ZnO-NPs; T5=30 mg L^{-1} ZnO-NPs; T6=3 g L^{-1} NaCl + 15 mg L^{-1} ZnO-NPs; T7=3 g L^{-1} NaCl + 30 mg L^{-1} ZnO-NPs; T8=6 g L^{-1} NaCl + 15 mg L^{-1} ZnO-NPs; T9=6 g L^{-1} NaCl + 30 mg L^{-1} ZnO-NPs.

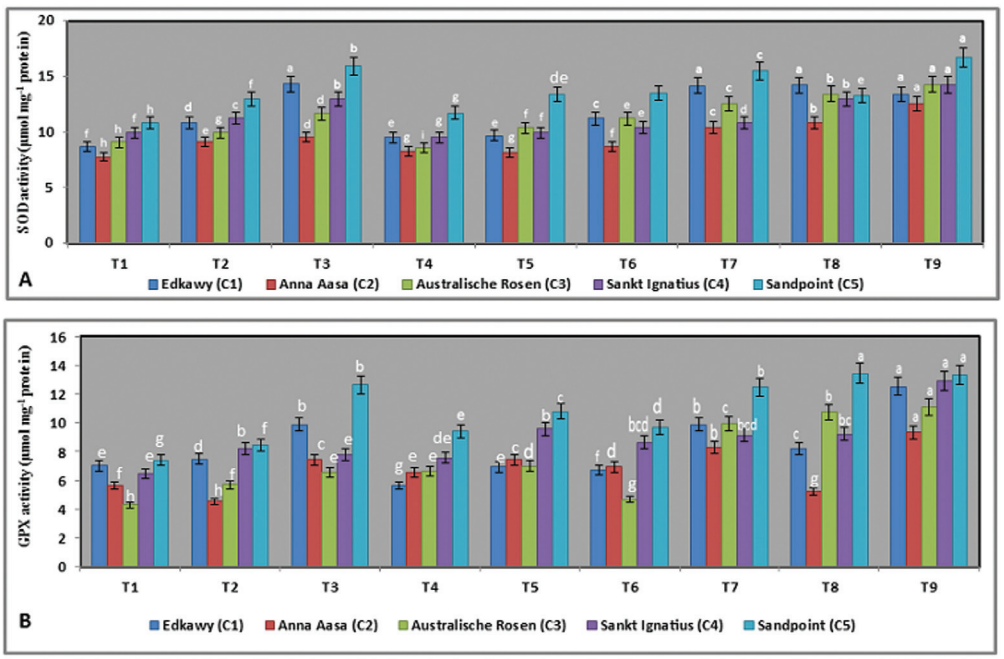

Fig 2. a) Superoxide dismutase and b) Glutathione peroxidase activity in calli of 5 tomato cultivars grown *in vitro* under control conditions, different levels of salt and ZnO-NPs. T1= control; T2=3 g L^{-1} NaCl; T3=6 g L^{-1} NaCl; T4=15 mg L^{-1} ZnO-NPs; T5=30 mg L^{-1} ZnO-NPs; T6=3 g L^{-1} NaCl + 15 mg L^{-1} ZnO-NPs; T7=3 g L^{-1} NaCl + 30 mg L^{-1} ZnO-NPs; T8=6 g L^{-1} NaCl + 15 mg L^{-1} ZnO-NPs; T9=6 g L^{-1} NaCl + 30 mg L^{-1} ZnO-NPs.

the treatments (Fig. 2). With respect to GPX activity, the results showed that cv. Sandpoint possessed the highest GPX activity (13.453 and 13.343 µmol mg^{-1} protein) under T8 (6.0 g L^{-1} NaCl + 15 mg L^{-1} ZnO-NPs) and T9 (6.0 g L^{-1} NaCl + 30 mg L^{-1} ZnO-NPs), respectively, followed by cv. Edkawy (Fig. 2). Low activity was recorded for Australische Rosen under all treatments except T4 and T9 (Fig. 2).

DISCUSSION

Most crop plants encounter insect infections, drought, salt, changes soil and solution pH, disease, exposure to harsh weather conditions and late harvesting. Biotechnological advancements in protection and crop nutrition strategies have attempted to provide some solutions to these challenges [35]. Of the newest techno-

logical innovations, nanotechnology offers an important opportunity and promises a prominent position in transforming agriculture by improving existing crop management techniques and food production practices [36]. In recent years, a considerable improvement in salinity tolerance has been achieved in some vegetable crop species by nanotechnology [37]. *In vitro* culture provides a controlled and uniform environment for studying the physiological and biological processes in plants, particularly at the cellular level, under different treatments of chemical compounds [38].

In the present study, the impact of the application of ZnO-NPs at two concentrations (15 and 30 mg L^{-1}) on different growth and biochemical parameters in tomato plants under salt stress (3 and 6 g L^{-1} NaCl) via tissue culture was investigated. In general, the results indicated that, as expected, salt stress adversely influenced most of the growth parameters, leading to Na accumulation, and plant tissues attempted to counteract this effect by antioxidant (SOD and GPX) accumulation (Table 3, 4 and Fig. 2). These results are in agreement with those of [39] and [40], who showed that NaCl salinity caused a marked reduction in callus growth, such as CWC, CSP and mineral elements such as K, in tomato. These decreases in callus traits are due to hyperosmotic stress leading to a reduction in water availability. Consequently, this reduced cell growth and impeded cell division as a result of which the CFW, CRGR, CWC and CSP of salt-stressed calli decreased in comparison to control calli. Such reduction in growth helps the plant to save energy for defense purposes but also limits the risk of heritable damage [41]. The results of the current study demonstrated that the concentration of zinc decreased with elevated soil salinity, but application of ZnO-NPs could reduce the harmful effect of salt stress (Table 4). Similarly, a sufficient Zn supply could reduce Na accumulation and contribute to salt tolerance in tomato plants [42]. Zinc plays a key role in controlling the generation and detoxification of free oxygen radicals, which can damage membrane lipids and sulfhydryl groups [43] and may help to limit lipid peroxidation rate since it is a protective and stabilizing component of biomembranes against activated oxygen species [44]. Adequate zinc also prevents the uptake and accumulation of Na in shoots by increasing membrane integrity of root cells.

The highest Zn average value recorded in the calli for all cultivars in this study was under treatment T4 (0.6962 g kg^{-1} dw) followed by T7 (0.5756 g kg^{-1} dw), T5 (0.5284 g kg^{-1} dw) and T9 (0.4858 g kg^{-1} dw) (Table 4). These levels are greater than the critical toxic level of 220 mg Kg^{-1} dw reported by [45], but did not adversely influence callus growth in the current study. Moreover, our results are in agreement with those of [46], who showed that to reach a 50% probability of Zn toxicity, leaf Zn concentrations must increase to >700 mg kg^{-1} in 6-week-old plants and >300 mg kg^{-1} in 8- to 10-week-old plants. The severity of Zn toxicity appeared to increase as plants matured and the proportion of plants exhibiting toxicity symptoms increased with plant age, indicating a cumulative metabolic toxicity. In another study [47], the availability of Zn declined as the pH rose, with the critical Zn toxicity point occurring between pH 5.5 to 6.5, and toxicity was reduced or eliminated when the pH rose above 6.0. Gall and Barnette [48] demonstrated that soil texture can also affect critical Zn levels and more soil Zn was required for Zn toxicity in clay than in sandy soils. Sokolov et al. [49] found that the sodium chloride matrix plays a key role in stabilizing the ZnO-NPs that could inhibit Zn toxicity. This relationship between plant age, medium pH, composition of the soil/culture medium and sodium chloride demonstrates the complexity in predicting Zn toxicity. An explanation of the non-toxic effect of high levels of Zn in our results is that the NPs modify or influence the toxic nature of the Zn ions such that the calli do not suffer metabolic inhibition. This hypothesis requires future exploration that needs to focus on the action of ZnO-NPs and the interaction between the previous factors inside the calli cells and plant cells levels, in either *in vitro* or *in vivo* culture conditions.

A similar increases in Na concentration and SOD and GPX enzyme contents in tomato plants in response to salt stress were reported [50]. Accumulation of sodium ions, SOD and GPX in stressed calli appears to be associated with osmotic adjustment under salt stress at the cost of reduced growth rate and water content [51]. There was strong evidence of cultivar differences, with strong accumulation of SOD and GPX in the salt-tolerant cultivars (Edkawy and Sandpoint) compared to the salt-sensitive cultivar (Anna Aasa), suggesting that the induction of antioxidant defenses is one component of the toler-

ance mechanisms to salt treatment. This increase in SOD activity can increase the ability of plant tissues to scavenge O_2 radicals, which might lessen membrane damage in the hypohydrated state [52]. Also, the expression of enhanced GPX levels in response to salinity was reported by [53] and the major function of GPX in plants appears to be the scavenging of phospholipid hydroperoxides and thereby the protection of cell membranes from peroxidative damage in tomato [54]. In the current study, we also observed a decrease in the protein content in calli grown under salt stress, probably as a result of salt stress-induced of the integrity of the cellular membrane, as well as cellular protein-containing components [55] and excessive ROS generation [56]. However, the increasing in protein content under ZnO-NPs in various combinations with or without salt stress suggests the synergistic effects of ZnO-NPs in the amelioration of salinity stress. The present observation is supported by the previous work [57] in which the authors found that zinc application could enhance protein content in pistachio plants under salinity stress. The promoting effects of zinc on protein content could be attributed to reducing ion leakage, thereby alleviating the damage normally caused by salt stress [58].

The inclusion of ZnO-NPs in culture media somewhat mitigated the deleterious effect of salinity and had a significant effect on most of the traits when compared to salt stress alone (Tables 3, 4 and Fig. 2). In the presence of ZnO-NPs, there was an improvement in all characters observed, except for K ,which continued to be adversely affected by Na. There were also no significant changes in N and P in the presence of ZnO-NPs. Under different combinations of NaCl and ZnO-NPs, the treatment with ZnO-NPs at 15 mg L^{-1}, in the presence of either 3 or 6 g L^{-1} NaCl resulted in the highest increase in CSP, CWC, CRGR, PRR and Na, SOD and GPX, as compared to ZnO-NPs at 30 mg L^{-1} + 3 g L^{-1} or 6 g L^{-1} NaCl in most cultivars. The improvements in callus growth traits and regeneration rate with ZnO-NPs in this study could be due to the role of Zn has in improving the plant water status, which is in agreement with [20], where the authors indicated that treatment with Zn and ZnO nanoparticles caused the successful establishment of tissue culture and increased the number of plantlets accompanied by suppuration of microbial contaminants in banana tissue cultures. Study of the effect of ZnO-NPs on seed germination and seedling growth in onion [59] revealed that seed germination increased at low concentrations of ZnO-NPs but decreased at higher concentrations. The positive effects of NPs on higher plants were explained [60]. Thus, when materials are transformed to a nanoscale, they change their physical, chemical and biological characteristics as well as their catalytic properties. There is an increase in chemical and biological activities such as the increase in nitrate reductase in plants, thereby enhancing the plant's abilities to absorb and utilize water and fertilizer, coupled with stimulation of antioxidant systems. The increase in SOD and GPX activity in salt-stressed tomato calli and sensitive cultivars was consistent with previously reported results [61]. In the present study, it was surprising that the higher concentration ZnO-NPs was inferior to the lower concentration, as it was found that the low dose was better at both salinity levels. This observation requires further work that needs to focus on the toxicity factors of ZnO-NPs, and the determination of the extent of effects on the intra- and extracellular milieu.

CONCLUSION

We conclude that the availability of high levels of sodium ions in growth media leads to salt stress through accumulation of sodium and an increase in osmotic stress. In response, tomato plant tissues upregulate antioxidant enzymes SOD and GPX in an attempt to offset the metabolic effects of salt stress. ZnO-NPs help to mitigate the effects of salt stress partly through further upregulation of SOD and GPX. Zinc oxide nanoparticles can help in alleviating the adverse effects of salt stress on tomato plants. However, further intensive studies are needed to define the role of ZnO-NPs in mediating stress response in plants in order to improve the ability of tomato plants to withstand stresses in a range of environments.

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measured the protein content; Ehab M extracted SOD and GPX antioxidant enzymes; Hesham F analyzed the element content; Ehab M, Hesham F and Amal A analyzed the data statistically; Ehab M, Hesham F and Mick F wrote the literature review; Mick F revised the English language; all authors read and approved the final MS.

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