

## Seed priming with ZNPs reduced expression of salinity tolerance genes in *Glycine max* L. and improved yield traits

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### Seed priming with ZNPs reduced expression of salinity tolerance genes in *Glycine max* L. and improved yield traits

**Abstract:** Little has been done to evaluate the molecular role of ZnO nanoparticles (ZNPs) in regulating biochemical processes and plant yield in response to salt-induced stress. In this study, the molecular response of salt-stressed soybean ('Giza111') was assessed under different concentrations of ZNPs (25, 50, 100, and 200 mg l<sup>-1</sup>) by measuring some osmolytes, yield parameters, and Na<sup>+</sup> and K<sup>+</sup> content. The impact of salinity on the mRNA expression levels of three key salt-tolerance related genes (*GmCHX1*, *GmPAP3*, and *GmSALT3*) using qRT-PCR was also determined. The high level of salinity (250 mM NaCl) led to a significant increase in Na<sup>+</sup> content, total soluble proteins, and total soluble carbohydrates and significantly upregulated gene expression of *GmCHX1*, *GmPAP3*, and *GmSALT3*, while reducing K<sup>+</sup> content, K<sup>+</sup>/Na<sup>+</sup> ratio and all yield parameters compared to control plants. Soaking soybean seeds in various ZNP concentrations, on the other hand, increased K<sup>+</sup> content and K<sup>+</sup>/Na<sup>+</sup> ratio while decreasing Na<sup>+</sup> content, total soluble proteins, and total soluble carbohydrates in stressed plants, particularly at 50 mg l<sup>-1</sup> ZNPs. Furthermore, *GmCHX1*, *GmPAP3*, and *GmSALT3* expressions were all downregulated at 50 mg l<sup>-1</sup> ZNPs, which ultimately improved soybean yield parameters. Accordingly, these results recommend the application of 50 mg l<sup>-1</sup> ZNPs for improving the productivity of soybean cultivated in saline soils.

**Key words:** ZnO; nanoparticles; salinity; soybean; gene expression; qRT-PCR; productivity

### Predtretiranje semen s cinkovimi nano delci je zmanjšalo izražanje genov tolerance na slanost pri soji (*Glycine max* L.) in izboljšalo lastnosti pridelka

**Izvleček:** Malo je bilo narejenega za ovrednotenje molekularne vloge nano delcev ZnO (ZNPs) pri uravnavanju biokemičnih procesov in pridelka rastlin kot odziva na slanostni stres. V tej raziskavi je bil ocenjen molekularni odziv na solni stres pri soji ('Giza111') pri uporabi različnih koncentracij ZNPs (25, 50, 100, in 200 mg l<sup>-1</sup>) z meritvami nekaterih osmotikov, parametrov pridelka in vsebnosti Na<sup>+</sup> in K<sup>+</sup>. Vpliv slanosti na količino mRNK treh ključnih s toleranco na slanost povezanih genov (*GmCHX1*, *GmPAP3*, in *GmSALT3*) je bil določen z uporabo qRT-PCR metode. Velika slanost (250 mM NaCl) je vodila k znatnemu povečanju vsebnosti Na<sup>+</sup>, celokupnih topnih beljakovin, celokupnih topnih ogljikovih hidratov in značilno povečala izražanje genov *GmCHX1*, *GmPAP3*, in *GmSALT3*, med tem ko, je zmanjšala vsebnost K<sup>+</sup>, razmerja K<sup>+</sup>/Na<sup>+</sup> in vse parameter pridelka v primerjavi s kontrolo. Namakanje semen soje v različnih koncentracijah ZNP je povečalo vsebnost K<sup>+</sup> in razmerje K<sup>+</sup>/Na<sup>+</sup> v rastlinah pod stresom in hkrati zmanjšalo vsebnost Na<sup>+</sup>, celokupnih topnih beljakovin in celokupnih topnih ogljikovih hidratov, še posebej pri uporabi 50 mg l<sup>-1</sup> ZNPs. Dodatno je bilo pri tem obravnavanju zmanjšano izražanje genov *GmCHX1*, *GmPAP3*, in *GmSALT3*, kar je na koncu izboljšalo parametre pridelka soje. Skladno s temi rezultati priporočamo uporabo 50 mg l<sup>-1</sup> ZNPs za izboljšanje pridelka soje, gojene na slanih tleh.

**Ključne besede:** ZnO; nano delci; slanost; soja; izražanje genov; qRT-PCR; produktivnost

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## 1 INTRODUCTION

Soybean (*Glycine max* L.) is one of the important food and industrial crops worldwide because of its content of cholesterol-free oil (30 %) and proteins (40 %), which are similar in their nourishing value to animal proteins (Van Zanten et al., 2016). The fractions and derivatives of soybean seeds have major economic importance in a wide range of industrial, food, pharmaceutical, and agricultural products (Chen et al., 2012).

Salinity of the soil is a serious problem all over the world. It has been estimated that around 954 million hectares are already salinized (Qadir et al., 2014). It usually causes a reduction of water potential, ion imbalances or disturbances in ion homeostasis, resulting in a reduction of plant growth and crop productivity (Han et al., 2019). Mittler (2002) observed that the oxidative demolition of the cell (oxidative stress) occurs by injuring membranes (lipid peroxidation), proteins, RNA, and DNA molecules as a result of elevated ROS levels in the cells. DNA damage is caused by OH<sup>•</sup> and O<sup>2-</sup> radicals, and this damage results in heritable changes (Fatima et al., 2017). Moreover, these signals play an important role in the adaptation process of plants to abiotic stress (Choudhury et al., 2017).

Plant tolerance to salinity stress includes physiological and molecular changes such as accumulation of organic solutes, antioxidant enzymes, and inorganic ions as well as gene expression responses (Ahanger et al., 2017). These alterations include either the induction of some polypeptides, the disappearance of others, or the overexpression of other sets of proteins (El-Mashad et al., 2012). Therefore, linking the expression of a gene to a higher degree of tolerance within a genotype offers an imperative argument for a role in plant adaptation (Abreu et al., 2013). Numerous reports suggest that the harmful effect of salinity stress was manifested by relatively higher expression of salt-related genes in soybean, such as *GmP5CS*, *GmDREB1a*, *GmGOLS*, *GmBADH* and *GmNCED1* (Liu et al., 2017), *GmERF3* (Zhang et al., 2009), *GmMYB* genes, *GmMYB76*, *GmMYB92* and *GmMYB177* (Liao et al., 2008), *GmPAP3* (Liao et al., 2003), *GmCHX1* (Patil et al., 2016) and *GmSALT 3* (Guan et al., 2014).

Previous studies in soybean determined that a QTL on chromosome 3 is the major genomic region that dictates salinity tolerance in soybean (Patil et al., 2016; Chen et al., 2018). This gene locus carries the dominant functional sodium/hydrogen exchanger family gene in wild (*GmCHX1*) and cultivated soybean (*GmNcl/GmSALT3*), which explains more than 64 % of the phenotypic variation (Qi et al., 2014). Normally, the *GmCHX1* gene is expressed under high salt conditions in root stellar cells and limits salt transport to shoot tissues (Guan et al., 2014). It

has been described that the full-length *GmSALT3* protein is closely correlated to the *Arabidopsis thaliana AtCHX20* (a Cation/Proton Exchanger), which is a functionally characterized member of the *CPA2* (Cation/Proton Antiporter2) family of transporters (Padmanaban et al., 2007; Qu et al., 2020). Functional studies of *AtCHXs* have shown that they might play a role in modulating cation and pH homeostasis within the endomembrane system (Chanroj et al., 2011). The ER-localized *AtCHX20* was suggested to be an endomembrane K<sup>+</sup> transporter involved in the osmoregulation of guard cells (Padmanaban et al., 2007). Purple acid phosphatases (PAPs) represent a diverse group of acid phosphatases in animals, microorganisms, and plants (Vogel et al., 2001; Olczak et al., 2003). The primary biochemical reaction of PAPs is to catalyze the hydrolysis of phosphate esters and anhydrides. The physiological role of *GmPAP3* might be related to the adaptation of soybean to NaCl stress, possibly through its involvement in reactive oxygen species (ROS) forming and/or scavenging or stress-responding signal transduction pathways (Liao et al., 2003; Soleimani et al., 2017).

Zinc (Zn) is a metallic cofactor for more than 300 enzymes. The Zn-finger proteins that attach to deoxyribonucleic acid (DNA) are clear evidence of the usefulness of Zn in biological systems (Hezaveh et al., 2019). Zinc is a structural component of ribosomes and is essential for their structural integrity. On the other hand, it has other indirect effects on the control of stomatal opening and closing and ROS detoxification (Haliloglu et al., 2020). Currently, nanotechnology has broad perspectives in all fields of science (Dewdar et al., 2018). The application of nanoparticles to plants can be beneficial for growth and development due to their greater absorbance and high reactivity (Fraceto et al., 2016). ZnO nanoparticles (ZNPs) are one of the most frequently used nanoproductions (Samei et al., 2019). Interestingly, priming of seeds with ZNPs positively affected the yield traits in salt-stressed plants, whereas ZNPs stimulated natural auxin (IAA), thus activating cell division and enlargement and also increasing K<sup>+</sup> ion content, which increases storage of food in seeds (Ali and Mahmoud, 2013), maintaining the structural integrity of biomembranes (He et al., 2015), improving protein synthesis and DNA replication (Landa et al., 2015), scavenging free oxygen radicals and decreasing the uptake of excess Na<sup>+</sup> and Cl<sup>-</sup> (Farhangi-Abriz and Torabian, 2018), as well as augmentation of photosynthesis, total soluble proteins, total soluble carbohydrates, and total phenols in stressed plants (Abdel Latef et al., 2017).

Transcription factors are the primary regulators of gene expression in a variety of genes that are involved in reducing and/or protecting against cellular stress damage

(Linh et al., 2020). The catalytic activity of RNA polymerases, which is essential for gene expression, is well known to require  $Zn^{2+}$  ions. Zn stabilizes several structural motifs in transcriptional regulatory proteins, such as Zn finger domains (Albert et al., 1998). Zn has been shown to upregulate gene expression, particularly in Zn-controlled genes, in numerous studies. Plants treated with ZnO, for example, had the highest *OsZIP1* expression in their roots after 7 days when compared to no-zinc controls (Selvaraj and Dananjeyan, 2016). Recently, ZNPs boosted the expression of the wheat drought-tolerance genes *DREB2* and *Wdhn13*, catalase activity (*CAT1*), proline biosynthesis (*P5CS*), and proline biosynthesis (*P5CS*) genes (Raeisi Sadati et al., 2022).

It was concluded that priming with ZNPs, particularly at  $60\text{ mg l}^{-1}$ , improved photosynthetic pigments, altered osmoregulation, and decreased MDA and Na concentrations in lupine plants (Abdel Latef et al., 2017). So, the current study was conducted to investigate the effect of seed-priming using different concentrations of ZNPs on the expression of three salinity-tolerance genes. In addition, their impacts on alleviating salinity stress and improving productivity in soybean plants were assessed.

## 2 MATERIALS AND METHODS

### 2.1 PLANT MATERIALS

Seeds of a soybean cultivar ('Giza 111') were provided by the Food and Legumes Research Department, Field Crops Research Institute, Agricultural Research Center, Giza, Egypt.

### 2.2 SYNTHESIS AND CHARACTERIZATION OF ZNO NANOPARTICLES

In this study, ZnO nanoparticles were synthesized using the chemical bath deposition (CBD) method as described by El-Shaer et al. (2018). The crystalline structure and optical properties of the prepared ZnO nanostructures were examined with X-ray Diffraction (XRD, Shimadzu 6000), while the samples' morphology was investigated using a scanning electron microscope (SEM, JSM-6510LV). As shown in Fig. 1A, ZnO nanostructures are formed as nano-rods with a hexagonal wurtzite crystal structure. These nano-rods accumulate to form the surface morphology of grains, similar to flowers. The XRD pattern of ZnO nano-rods is shown in Fig. 1B. The diffraction peaks at  $32^\circ$ ,  $34.5^\circ$ ,  $36.4^\circ$ ,  $47.5^\circ$ ,  $57^\circ$ ,  $62.7^\circ$ ,  $67.9^\circ$ , and  $69.3^\circ$  correspond to the (100), (002), (101), (102), (110), (103), (112), and (201) lattice planes, respectively (Fig. 1B).

### 2.3 PLANT GROWTH CONDITIONS

Priming and growing of the soybean seeds ('Giza 111') were performed as described by Gaafar et al. (2020). The seeds were sterilized with 70 % ethanol for 5 min and sodium hypochlorite (10 %) for 10 min, followed by washing several times with distilled water. Four concentrations of ZnO nanoparticles (ZNPs) of 25 (ZNPs25), 50 (ZNPs50), 100 (ZNPs100), and 200 (ZNPs200)  $\text{mg l}^{-1}$  were used to prime the seeds for two hours at room temperature, and distilled water was used as a control (0). Previous studies have found that low concentrations of

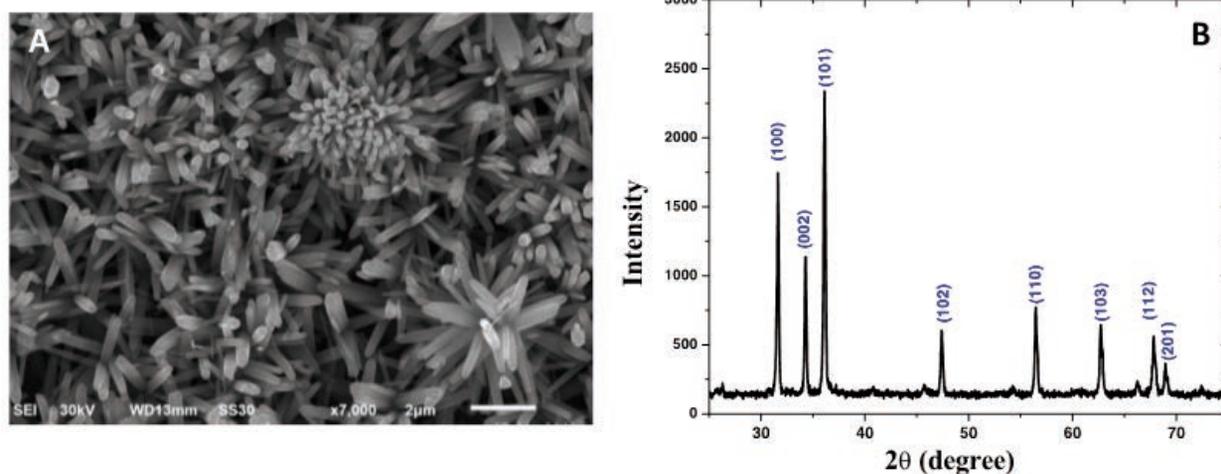


Fig. 1: (A) SEM image and (B) XRD chart of ZnO nanostructures prepared by CBD method

ZnO NPs are beneficial to plant growth, whereas concentrations equal to or greater than 200 mg l<sup>-1</sup> are detrimental. Therefore, the used ZNP concentrations were chosen (Liu et al., 2015; Abdel Latef et al., 2017). After priming, the seeds were sown (20 seeds/pot) in plastic pots (45 cm x 40 cm) filled with 24 kg of 2:1 (clay: sandy) soil. Based on the preliminary experiment results, 250 mM NaCl (S) was chosen as a sub-lethal salinity level and used in this study.

The pots were irrigated with tap water until seed germination (emergence), then with tap water and with 250 mM NaCl solution to 80 % field capacity for 21 days (seedling stage) and 90 days (yield stage). Three pots were used as replicates for each treatment. The germinated soybean seeds were let to grow in the green house under the following environmental conditions: 29 ± 2 °C/25 ± 2 °C day/night and 16h/8h light/dark regimes. The 21-day-old seedlings of all treatments were collected, washed, and used for further analyses, and the productivity of yielded seeds was determined on 90-day-old plants.

#### 2.4 DETERMINATION OF SODIUM AND POTASSIUM CONTENT

According to Allen et al. (1974), the mixed acid digestion method was used for element determination. The concentration of Na<sup>+</sup> and K<sup>+</sup> (mg g<sup>-1</sup> d.m.) was determined by using Inductively Coupled Plasma (ICP, STI) at the central laboratory of Tanta University.

#### 2.5 QUANTITATIVE ESTIMATION OF TOTAL SOLUBLE PROTEINS AND TOTAL SOLUBLE CARBOHYDRATES

The total soluble proteins were extracted according to the method described by Naguib et al. (1968). Then the protein content was determined as described by

Bradford (1976), and the phenol-sulfuric acid method has been used for estimation of total soluble carbohydrates according to Dubois *et al.* (1956).

#### 2.6 QUANTITATIVE REAL TIME PCR (QRT-PCR) RNA EXTRACTION AND PURIFICATION

For the extraction of total RNA, approximately 100 mg of ground plant fresh leaves were used, and RNA was extracted using the RNeasy Plant Mini Kit (Qiagen, Germany) according to the manufacturer's protocol. The total RNA was then quantified and assessed for quality using a Nanodrop (ScanDrop, Analytik, Jena, Germany). Total RNA samples were kept at -80 °C until further analysis.

#### 2.7 CDNA SYNTHESIS

The cDNA synthesis was performed using the SensiFAST cDNA synthesis kit (Ameridian Life Science, USA) using the protocol of the manufacturer. The cDNA synthesis reaction contained the following components: 1 µg total RNA, 1 µl reverse transcriptase enzyme, 4 µl 5 × Trans Amp buffer, which was completed to a total volume of 20 µl. The conditions for cDNA synthesis were as follows: primer annealing for 10 min at 25 °C, reverse transcription for 15 min at 42 °C and finally 5 min at 85 °C for enzyme inactivation. After being diluted in 10 mM Tris-HCl (pH = 8) and 0.1 mM EDTA, the cDNA reaction products were stored at -20 °C.

#### 2.8 GENE EXPRESSION ANALYSIS (QRT-PCR)

In order to measure the gene expression of the three targeted genes, the reaction mix was prepared by mixing 10 µl of TOP real qPCR2x premix (SYBR Green with low ROX), 1 µl of each of the cDNA template, forward and

**Table 1:** List of sequences of the primers used for gene expression study by qRT-PCR

Primer name	Sequence (5'→3')	Length (bp)	Annealing temp. (°C)	Reference
GmPAP3F	GTGGCCGGCAGTTGACATCC	20	55.5	Liao et al. (2003)
GmPAP3R	GCTGTGCCCTGGCTCTTCTGTG	22	55.5	
GmCHX1F	GATTTGTTTTCGGGCTAACG	20	49.5	Gutierrez Gonzalez et al. (2010)
GmCHX1R	ATCCACCACGCTTCGTAAC	20	49.5	
GmSALT3F	CGGTTGATGAAGGGAAAAC	19	48.5	Hu et al. (2009)
GmSALT3R	TCCTTGACGCTTGGAGTGTT	20	48.5	
GmTublinF	GAGAAGAGTATCCGGATAGG	20	50	Gutierrez Gonzalez et al. (2010)
GmTublinR	GTTTCCGAACACTCAAGCTC	20	50	

reverse primer (10 pmol  $\mu\text{l}^{-1}$ ) and was completed up to 20  $\mu\text{l}$ . The Rotor-Gene Q5 plex (Qiagen, Germany) was used, and the PCR conditions were as follows: an initial denaturation step at 95 °C for 10 min; a denaturation step at 95 °C for 10 s; an annealing step at 60 °C for 15 s; and an elongation step at 72 °C for 15 s. The thermal cycler steps were repeated 35 times. The sequences of the primers used for qRT-PCR analysis are shown in Table 1. The relative gene expression was calculated using the  $2^{-\Delta\Delta\text{CT}}$  method according to Livak and Schmittgen (2001).

## 2.9 YIELD TRAITS

The yield parameters, including length of pods/plant, mass of pods/plant, the mass of 1000 seeds, the number of pods/plant, the number of seeds/pod, the mass of seeds/pod, and the mass of seeds/plant, were determined at the end of the growing season. (approximately 3 months from cultivation). The maturity (number of viable - nonviable seeds in pods \* 100) and the productivity of soybean (weight of the yielded seeds/pot in grams) were also calculated.

## 2.10 STATISTICAL ANALYSIS

The statistical analyses were carried out according

to a completely randomized design (CRD) using analysis of variance. The significance was determined using LSD values at  $p = 0.05$  and  $0.01$  according to Bishop (1983). The results were analyzed using a one-way ANOVA test to determine the degree of significance. The statistical analyses were performed using CoStat Software version 6.311 (CoHort Software, CA, USA). The heatmap of the gene expression data and Pearson correlation were constructed using R software (ver. 4.1.1).

## 3 RESULTS

### 3.1 SODIUM AND POTASSIUM CONTENT

The results in Table 2 show the effect of salinity stress on mineral ion content ( $\text{Na}^+$ ,  $\text{K}^+$ , and  $\text{K}^+/\text{Na}^+$ ) in 21-day old soybean seedlings after soaking of soybean seeds in different concentrations of ZNPs (0, 25, 50, 100, and 200  $\text{mg l}^{-1}$ ). The salinity stress (250 mM NaCl) severely decreased the content of potassium by 68 % compared to control. Similarly, it reduced the  $\text{K}^+/\text{Na}^+$  ratio by 90 % compared to control. In contrast, the content of  $\text{Na}^+$  was highly increased by 2.16-fold compared to control. On the other hand, the combination of ZNPs50+S (50  $\text{mg l}^{-1}$  + 250 mM NaCl) significantly increased the content of potassium by 1.67-fold compared to salt-stressed seedlings and ameliorated the harmful effect of salinity

**Table 2:** Effect of salinity (S = 250 mM NaCl) on the content of  $\text{Na}^+$ ,  $\text{K}^+$  and  $\text{K}^+/\text{Na}^+$  ratio of 21-day old soybean ('Giza 111') seedlings grown in clay-sandy soil (2:1 w/w) after soaking of soybean seeds in four different concentrations of ZnO nanoparticles (ZNPs) (ZNPs25 = 25, ZNPs50 = 50, ZNPs100 = 100, and ZNPs200 = 200 mg/L)

Treatments		$\text{K}^+$ (mg g <sup>-1</sup> d. m.)	$\text{Na}^+$ (mg g <sup>-1</sup> d. m.)	$\text{K}^+/\text{Na}^+$ ratio
Salinity level (0 mM NaCl)	Control	4.287 ± 0.04 <sup>a</sup>	2.346 ± 0.0008 <sup>f</sup>	1.827
	ZNPs25	4.043 ± 0.04 <sup>b</sup>	2.160 ± 0.007 <sup>f</sup>	1.871
	ZNPs50	4.242 ± 0.01 <sup>a</sup>	1.761 ± 0.022 <sup>g</sup>	2.409
	ZNPs100	3.942 ± 0.04 <sup>b</sup>	2.247 ± 0.009 <sup>f</sup>	1.754
	ZNPs200	3.756 ± 0.04 <sup>c</sup>	2.286 ± 0.048 <sup>b</sup>	1.642
Salinity level (S = 250 mM NaCl)	Salinity (S)	1.336 ± 0.01 <sup>g</sup>	7.419 ± 0.08 <sup>a</sup>	0.180
	ZNPs25+S	2.539 ± 0.14 <sup>e</sup>	5.425 ± 0.13 <sup>d</sup>	0.468
	ZNPs50+S	3.569 ± 0.11 <sup>d</sup>	4.061 ± 0.03 <sup>e</sup>	0.878
	ZNPs100+S	2.011 ± 0.008 <sup>f</sup>	6.629 ± 0.28 <sup>c</sup>	0.303
	ZNPs200+S	1.118 ± 0.004 <sup>h</sup>	7.120 ± 0.06 <sup>b</sup>	0.157
	F-value	689.8	894.8	-
	LSD (0.05)	0.138	0.226	-
Significance	*	-	-	

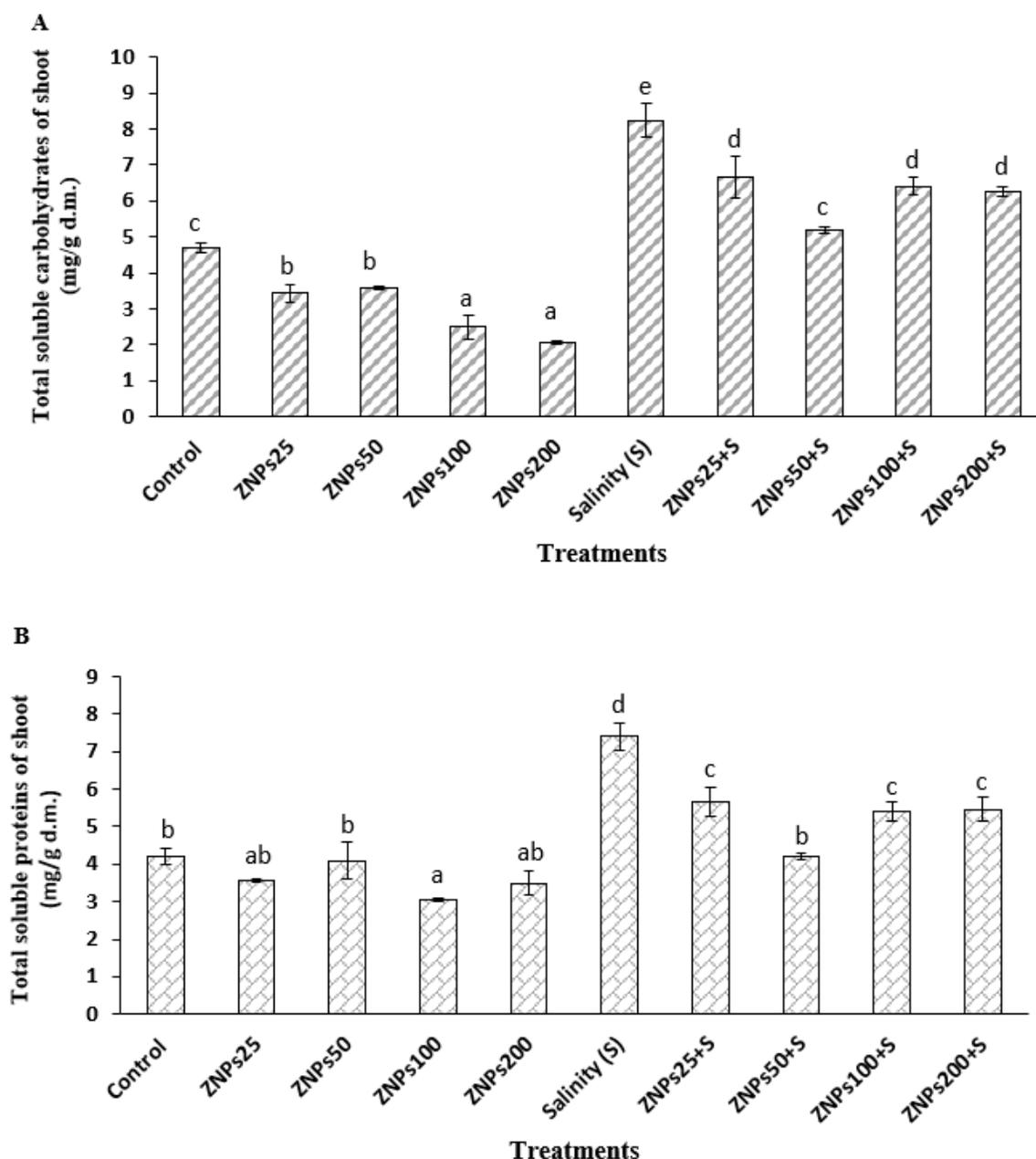
Values are the mean of three replicates ± SD. Values within the same column for each factor designated by different letters are significant at  $p \leq 0.05$ , while values with identical letters are non-significant. \*: significant at  $p \leq 0.05$

stress. Also, the combination of ZNPs25 + S (25 mg l<sup>-1</sup> + 250 mM NaCl) increased the content of potassium ions by only 1.07-fold.

Moreover, results indicated that the combination of ZNPs200 + S (200 mg l<sup>-1</sup> + 250 mM NaCl) exhibited a severe harmful effect compared to other treatments; thus, it reduced the potassium and K<sup>+</sup>/Na<sup>+</sup> ratio content by 16 % and 12 %, respectively, compared to salt stressed seedlings (Table 2).

### 3.2 TOTAL SOLUBLE PROTEINS AND TOTAL SOLUBLE CARBOHYDRATES

The results in Figure 2 (A and B) indicated that high salinity stress (S = 250 mM NaCl) caused a highly significant increase in total soluble carbohydrates and protein content by 75.1 % and 76.1 %, respectively, compared to control. However, the results showed a general decrease in total soluble carbohydrates and protein content for all



**Fig. 2:** Effect of NaCl (S = 250 mM) on the total soluble carbohydrates (A) and total soluble proteins (B) of 21-day old soybean ('Giza 111') seedlings grown in clay-sandy soil (2:1 w/w) after soaking of soybean seeds in four different concentrations of ZnO nanoparticles (ZNPs) (ZNPs25 = 25, ZNPs50 = 50, ZNPs100 = 100, and ZNPs200 = 200 mg l<sup>-1</sup>)

ZNPs (25, 50, 100, and 200 mg l<sup>-1</sup>) combined with salinity, except for the combination of ZNPs25+S (25 mg l<sup>-1</sup> + 250 mM NaCl), which exhibited the least reduction in total soluble carbohydrates and protein content with a percentage of 10 % and 23 %, respectively, compared to control plants, which were irrigated with water. The highest reduction in total soluble carbohydrates and protein content was recorded in the case of ZNPs50+S (50 mg l<sup>-1</sup> + 250 mM NaCl) with 29 % and 43 %, respectively, compared to control plants irrigated with salt only (Fig. 2A and B).

### 3.3 GENE EXPRESSION ANALYSIS (QRT-PCR)

#### 3.3.1 *GmCHX1*

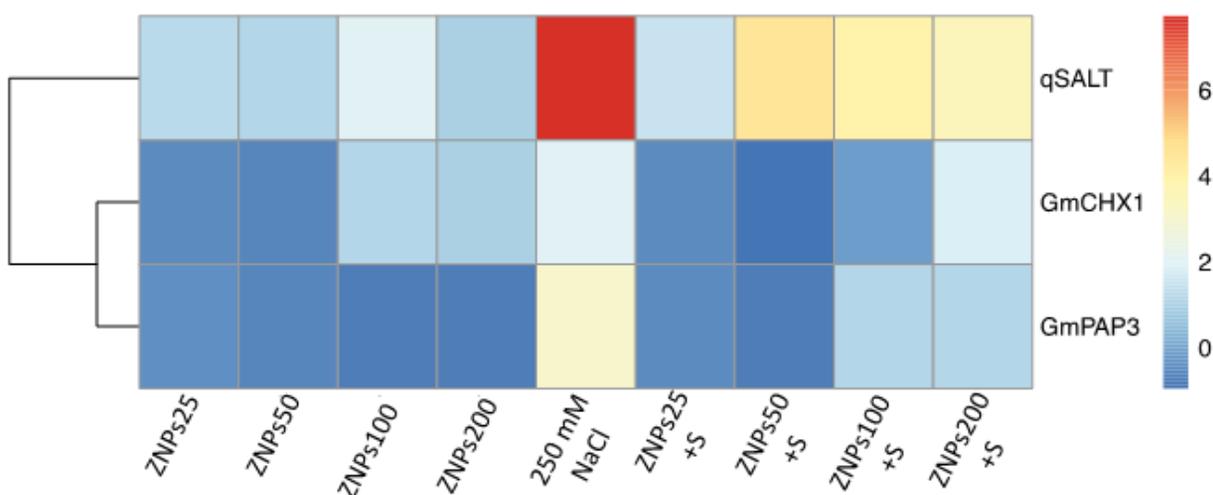
The results of qRT-PCR analysis showed that *GmCHX1* expression was increased by ZNPs alone and the highest increase was with ZNPs100+S (100 mg l<sup>-1</sup> + 250 mM NaCl) compared to control (no salinity) by about 1.2-fold (Fig. 3). Also, 250 mM NaCl (S) alone showed the highest increase in *GmCHX1* gene expression by 1.9-fold compared to control (no salinity and no ZNPs). However, ZNPs25+S (25 mg l<sup>-1</sup> + 250 mM NaCl) decreased gene expression by 0.4-fold, and then it was increased with ZNPs50+S (50 mg l<sup>-1</sup> + 250 mM NaCl). Also, *GmCHX1* gene expression was decreased by 0.08-fold with ZNPs100+S (100 mg l<sup>-1</sup> + 250 mM NaCl). In contrast, ZNPs200+S (200 mg l<sup>-1</sup> + 250 mM NaCl) showed an increase of 1.8-fold, which was similar to that of salinity (S = 250 mM NaCl) (Fig. 3).

#### 3.3.2 *GmPAP3*

*GmPAP3* expression was slightly increased by ZNPs treatments, and the two concentrations (ZNPs100 and ZNPs200 mg l<sup>-1</sup>) showed the highest increases of about 0.73 and 0.70-fold, respectively, compared to control (no salinity and no ZNPs) (Fig. 3). In the case of salt treatment (S = 250 mM NaCl), *GmPAP3* expression increased by 3-fold. ZNPs25+S (25 mg l<sup>-1</sup> + 250 mM NaCl), ZNPs50 + S (50 mg l<sup>-1</sup> + 250 mM NaCl), ZNPs100 + S (100 mg l<sup>-1</sup> + 250 mM NaCl), and ZNPs200 + S (200 mg l<sup>-1</sup> + 250 mM NaCl) all reduced *GmPAP3* expression. The highest decrease was by 0.46-fold and was recorded with ZNPs25+S (25 mg l<sup>-1</sup> + 250 mM NaCl) treatment (Fig. 3).

#### 3.3.3 *GmSALT3*

In the case of *GmSALT3*, gene expression was increased by ZNP treatment using a concentration of 100 mg l<sup>-1</sup> compared to control (no salinity and no ZNPs) by about 2-fold, as shown in Fig. 3. However, salinity stress (250 mM NaCl) showed the highest increase in *GmSALT3* gene expression by 7.7-fold. The concentration of 25 mg l<sup>-1</sup> of ZNPs with salinity decreased gene expression by 1.56-fold, and then it was increased by the concentration of 50 mg l<sup>-1</sup> of ZNPs with salinity by 4.5-fold. Then, in comparison to the salinity stress alone (250 mM NaCl), gene expression decreased by 3.9-fold with ZNPs100 + S (100 mg l<sup>-1</sup> + 250 mM NaCl) treatment and by 3.5-fold with ZNPs200 + S (200 mg l<sup>-1</sup> + 250 mM NaCl). In contrast to the salinity stress alone (250 mM NaCl), which increased gene expression by 8-fold (Fig. 3).



**Fig. 3:** Heatmap of relative expression of three soybean salinity-linked genes (*GmPAP3*, *GmCHX1*, and *GmSALT3*) in 21-day old soybean ('Giza 111') seedlings after soaking of soybean seeds in four different concentrations of ZnO nanoparticles (ZNPs) (ZNPs25 = 25, ZNPs50 = 50, ZNPs100 = 100, and ZNPs200 = 200 mg l<sup>-1</sup>)

### 3.4 YIELD PARAMETERS

The results given in Table 3 show the effect of 250 mM NaCl and ZNPs (25, 50, 100, and 200 mg l<sup>-1</sup>) treatments on yield parameters. These results revealed an observable increase in all measured yield parameters, specifically in the case of ZNPs25 (25 mg l<sup>-1</sup>) and ZNPs50 (50 mg l<sup>-1</sup>) treatments without salinity, where these treatments increased the pod length, pod mass, number of pods/plants, and mass of pods/plant, number of seeds/pods, mass of seeds/pods, and mass of seeds/plant. The most significant increase was with ZNPs50 (50 mg l<sup>-1</sup>) by 29 %, 27.8 %, 62.8 %, 39.9 %, 15.3 %, 47.8 %, and 78.8 %, respectively, compared to control. In contrast, results showed that the application of 200 mg l<sup>-1</sup> ZNPs (ZNPs200) caused a highly significant decrease in all measured yield parameters: pod length, pod mass, number of pods/plant, mass of pods/plant, number of seeds/pod, mass of seeds/pods, and mass of seeds/plant by 37.8 %, 16.1 %, 13.89 %, 20.9 %, 26.9 %, 11.5 %, and 18.1 %, respectively compared to control.

Similarly, a remarkable increase in all measured yield parameters in the case of treatments ZNPs25 + S, ZNPs50 + S, and ZNPs100 + S was observed (Table 3). The most significant increases in pod length, pod mass, number of pods/plants, and mass of pods/plant, number of seeds/pods, mass of seeds/pods, and mass of seeds/plant were with ZNPs50+S (50 mg l<sup>-1</sup> + 250 mM NaCl) by 50.11 %, 85.4 %, 42.6 %, 47.7 %, 341.6 %, 100 %, and 119 %, respectively, compared to control. Whereas, a decrease in these yield parameters was observed with ZNPs200+S (200 mg l<sup>-1</sup> + 250 mM NaCl) by 53.2 %, 15.2 %, 23.2 %, 14.5 %, 100 %, 15.7 %, and 4.7 %, respectively, compared to control.

Similarly, the most significant increases in mass of the seeds (g/plant), mass of 1000 seeds, maturity percentage, and productivity index (g/pot) were with treatment ZNPs50 + S (50 mg l<sup>-1</sup> + 250 mM NaCl) by 14.8 %, 31.5 %, 32.6 %, and 118.9 %, respectively, compared to control. In contrast, treatment with ZNPs200 + S (200 mg l<sup>-1</sup> + 250 mM NaCl) decreased these parameters by 3 %, 14.2 %, 30.3 %, and 17.8 %, respectively, compared to control. These results proved the efficiency of 50 mg l<sup>-1</sup> (ZNPs50) for increasing the productivity of the soybean plant under high salinity levels (S = 250 mM NaCl).

### 3.5 PEARSON CORRELATION ANALYSIS

As shown in Figure 4, the Na<sup>+</sup> was only positively corrected with Zn (r = 1.0<sup>\*</sup>), total proteins (r = 0.89<sup>\*</sup>), and total carbohydrates (r = 92<sup>\*</sup>), while it was negatively corrected with the rest of the studied characters, with dif-

ferent correlation coefficients. The pod weight was positively correlated with the weight of seeds/plant (r = 0.9<sup>\*</sup>), K<sup>+</sup> (r = 0.91<sup>\*</sup>), K<sup>+</sup>/Na<sup>+</sup> ratio (r = 0.88<sup>\*</sup>), and the number of seeds/pod (r = 0.79<sup>ns</sup>).

## 4 DISCUSSION

As sessile organisms, plants have adapted a variety of signal perception mechanisms as well as pathways to control molecular responses in order to respond effectively to abiotic stress situations (Dudziak et al. 2019). Exposure of soybean seedlings to high salinity stress (250 mM NaCl) imposed a significant depletion in K<sup>+</sup> content and in K<sup>+</sup>/Na<sup>+</sup> ratio by 68 % and 90 %, respectively, compared to control. Similar findings were also found by Tafouo et al. (2009) and Khan et al. (2017) in cowpea and soybean, respectively. Contrarily, the amount of Na<sup>+</sup> was 2.16-fold more than it was in the control plants. It is possible that high salinity promoted the uptake of Na<sup>+</sup> due to its adverse effects on membrane integrity. In this regard, a similar conclusion was also made by Abdel Latef et al. (2017) on lupine plants.

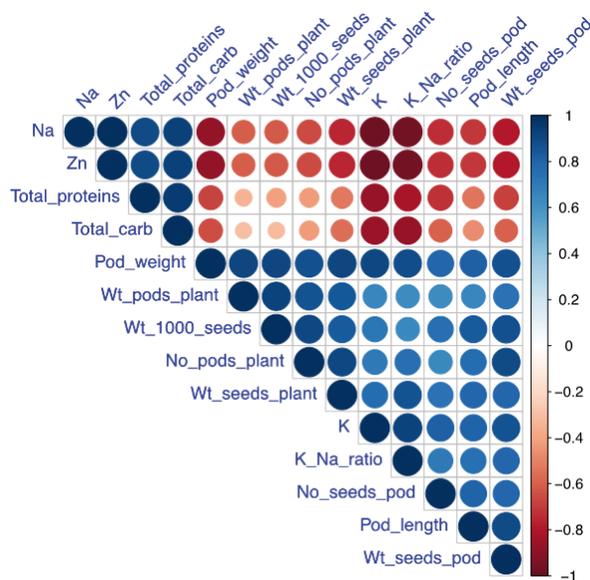
According to reports, K<sup>+</sup> is required for maintaining osmotic balance and is an essential co-factor for many enzymes. Therefore, K<sup>+</sup> reduction negatively affects the growth and productivity of plants (Hauser and Horie, 2010). The results of this study indicated that K<sup>+</sup> content in soybean seedlings showed a highly significant reduction under acute salinity stress. However, treatment with ZNPs increased K<sup>+</sup> by 1.67-fold and decreased Na<sup>+</sup> by 45 % compared to salt-stressed seedlings, which is comparable to the findings of Abdel Latef et al. (2017) on lupine plants (*Lupine termis* L.). This is due to the fact that Zn<sup>+2</sup> helps in maintaining the structural and functional integrity of root cell membranes and therefore controls the influx and efflux of Na<sup>+</sup> across the plasma membranes (Rezaie and Abbasi, 2014).

The application of ZnO is associated with a remarkable increase in K<sup>+</sup> uptake from soil to roots (Weisany et al., 2012; Soliman et al., 2015). As a consequence of enhanced K<sup>+</sup> uptake, plants treated with ZnO had greater K<sup>+</sup>/Na<sup>+</sup> ratios than those under salinity stress alone. A high K<sup>+</sup>/Na<sup>+</sup> ratio is often reported as a good indicator of a high tolerance to salt stress conditions (Khan et al., 2017). Thus, applications of ZNPs could be a useful strategy for achieving increased macronutrient uptake by plants (Dimkpa and Bindraban, 2016), which is similar to what was observed in this study, where ZNPs50+S (50 mg l<sup>-1</sup> + 250 mM NaCl) treatment significantly increased the content of potassium by 1.67-fold compared to salt-stressed seedlings and ameliorated the harmful effect of salinity stress.

**Table 3:** Effect of salinity (S = 250 mM NaCl) on the yield parameters of 90-day old soybean (cv. Giza 111) plants grown in clay-sandy soil (2:1 w/w) after soaking of soybean seeds in four different concentrations of ZnO nanoparticles (ZNPs) (ZNPs25 = 25, ZNPs50 = 50, ZNPs100 = 100, and ZNPs200 = 200 mg l<sup>-1</sup>)

Treatments	Pod length (cm)	Pod mass (g)	No. of pods/plant	M. of pods/plant (g)	No. of seeds/pod	M. of seeds/pod (g)	M. of seeds/plant (g)	M. of 1000 seed (g)	Percentage of maturity (%)	Percentage of Productivity index (g/pot)
Control	6.71 ± 0.12 <sup>e</sup>	2.41 ± 0.05 <sup>d</sup>	5.11 ± 0.44 <sup>d</sup>	8.42 ± 0.17 <sup>d</sup>	5.2 ± 0.18 <sup>c</sup>	1.38 ± 0.12 <sup>e</sup>	134.72 ± 0.22 <sup>c</sup>	61.12 ± 4.5 <sup>h</sup>	36 ± 4.1 <sup>f</sup>	5.42 ± 0.17 <sup>d</sup>
ZNPs25	7.34 ± 0.26 <sup>c</sup>	2.92 ± 0.05 <sup>b</sup>	6.16 ± 0.44 <sup>c</sup>	10.66 ± 0.25 <sup>b</sup>	5.8 ± 0.16 <sup>ab</sup>	1.58 ± 0.03 <sup>d</sup>	154.22 ± 0.13 <sup>b</sup>	92.33 ± 4.4 <sup>b</sup>	55.8 ± 1.6 <sup>b</sup>	8.37 ± 0.11 <sup>b</sup>
ZNPs50	8.68 ± 0.17 <sup>a</sup>	3.08 ± 0.13 <sup>a</sup>	8.32 ± 0.68 <sup>a</sup>	11.78 ± 0.16 <sup>a</sup>	6.0 ± 0.27 <sup>a</sup>	2.04 ± 0.12 <sup>a</sup>	175.8 ± 0.15 <sup>a</sup>	94.65 ± 4.8 <sup>a</sup>	69.9 ± 3.2 <sup>a</sup>	10.48 ± 0.14 <sup>a</sup>
ZNPs100	7.72 ± 0.20 <sup>b</sup>	2.12 ± 0.15 <sup>e</sup>	4.6 ± 0.93 <sup>e</sup>	6.30 ± 0.32 <sup>f</sup>	4.6 ± 0.43 <sup>d</sup>	1.68 ± 0.06 <sup>c</sup>	124.4 ± 0.20 <sup>d</sup>	74.24 ± 4.8 <sup>d</sup>	29.4 ± 3.0 <sup>g</sup>	4.35 ± 0.11 <sup>g</sup>
ZNPs200	4.17 ± 0.10 <sup>i</sup>	2.02 ± 0.06 <sup>f</sup>	4.4 ± 0.44 <sup>f</sup>	6.66 ± 0.41 <sup>e</sup>	3.8 ± 0.18 <sup>e</sup>	1.22 ± 0.06 <sup>f</sup>	117.0 ± 0.12 <sup>f</sup>	66.34 ± 4.4 <sup>f</sup>	27.1 ± 3.4 <sup>h</sup>	4.64 ± 0.17 <sup>f</sup>
Salinity (S)	4.51 ± 0.11 <sup>h</sup>	1.31 ± 0.1 <sup>h</sup>	4.43 ± 0.44 <sup>f</sup>	6.62 ± 0.11 <sup>e</sup>	1.2 ± 0.12 <sup>g</sup>	0.95 ± 0.11 <sup>h</sup>	106.2 ± 0.42 <sup>h</sup>	70.12 ± 0.63 <sup>e</sup>	41.9 ± 2.6 <sup>c</sup>	3.65 ± 0.36 <sup>h</sup>
ZNPs25+S	5.22 ± 0.12 <sup>g</sup>	1.87 ± 0.1 <sup>g</sup>	3.46 ± 0.44 <sup>h</sup>	8.46 ± 0.13 <sup>d</sup>	4.3 ± 0.15 <sup>de</sup>	1.01 ± 0.02 <sup>gh</sup>	113.4 ± 0.20 <sup>g</sup>	74.23 ± 0.48 <sup>d</sup>	39.3 ± 3.0 <sup>d</sup>	5.35 ± 0.41 <sup>e</sup>
ZNPs50+S	6.77 ± 0.15 <sup>d</sup>	2.43 ± 0.11 <sup>c</sup>	6.32 ± 0.68 <sup>b</sup>	9.78 ± 0.16 <sup>c</sup>	5.3 ± 0.11 <sup>bc</sup>	1.9 ± 0.15 <sup>b</sup>	122.0 ± 0.72 <sup>e</sup>	92.22 ± 0.40 <sup>c</sup>	55.6 ± 3.3 <sup>b</sup>	7.99 ± 0.46 <sup>c</sup>
ZNPs100+S	5.55 ± 0.11 <sup>f</sup>	1.15 ± 0.12 <sup>i</sup>	3.6 ± 0.93 <sup>g</sup>	4.30 ± 0.33 <sup>h</sup>	4.6 ± 0.13 <sup>d</sup>	1.08 ± 0.03 <sup>g</sup>	107.0 ± 0.30 <sup>h</sup>	66.1 ± 1.0 <sup>g</sup>	37.6 ± 3.4 <sup>c</sup>	4.64 ± 0.47 <sup>f</sup>
ZNPs200+S	2.11 ± 0.13 <sup>j</sup>	1.11 ± 0.12 <sup>j</sup>	3.4 ± 0.44 <sup>h</sup>	5.66 ± 0.23 <sup>g</sup>	2.4 ± 0.18 <sup>f</sup>	0.80 ± 0.04 <sup>i</sup>	103.0 ± 0.31 <sup>i</sup>	60.1 ± 0.31 <sup>i</sup>	29.2 ± 0.31 <sup>g</sup>	3.0 ± 0.31 <sup>i</sup>
F-value	114655.2	14568.5	1794.3	8091.5	62.9	257.5	4036.3	252664.1	5384.5	15567.3
LSD (0.05)	0.017	0.017	0.108	0.077	0.562	0.077	1.08	0.077	0.562	0.056
Significance						*				

Values are the mean of three replicates ± SD. Values within the same column for each factor designated by different letters are significant at  $p \leq 0.05$ , while values with identical letters are non-significant



**Fig. 4:** Correlogram based Pearson correlation analysis of  $\text{Na}^+$ ,  $\text{K}^+$ ,  $\text{K}^+/\text{Na}^+$ , Zn and yield parameters of 90-day old soybean ('Giza 111') plants grown in clay-sandy soil (2:1 w/w) after soaking of soybean seeds in different concentrations of ZNPs (25, 50, 100, and 200  $\text{mg l}^{-1}$ ). On the right hand side of the correlogram, the legend color shows the correlation coefficients and the corresponding colors. The positive correlations are displayed in blue, while the negative correlations are shown in red. The color intensity and the size of the circle are proportional to the correlation coefficients

In this study, high salinity stress (250 mM NaCl) caused a highly significant increase in total soluble proteins and carbohydrates content by 76 % and 75 %, respectively, in salt-stressed soybean seedlings. Similar results were reported by Sadeghipour (2017) in *Vigna unguiculata* (L.), Karimi et al. (2019) in *Vitis vinifera* (L.), and Cardoso et al. (2019) in two varieties of cowpea. It is well known that osmotic stress induced by salt stress leads to the synthesis of proteins, which play an important role in plant salt tolerance through cytosolic calcium signal. This signal activates the calcium sensor protein for activation of the protein kinase to regulate  $\text{Na}^+/\text{H}^+$  antiporter in plasma membranes and tonoplasts, thus the osmo-sensory histidine kinase regulates osmotic homeostasis and ROS scavenging (Chinnusamy et al., 2005; Abdel Latef et al., 2017). In addition, total soluble carbohydrates are key osmolytes in the osmotic adjustment of all plants, ROS scavenging, and maintaining ion homeostasis under salinity stress (Chen and Jiang, 2010) and have a direct relationship with physiological processes in plants (Tombesi et al., 2019).

In this study, treatment with ZNPs reduced total soluble proteins and total soluble carbohydrates content under salinity stress, and the highest reduction was re-

corded in the case of ZNPs50+S (50  $\text{mg l}^{-1}$  + 250 mM NaCl) with 43 % and 36 %, respectively. This result indicates that ZNPs alleviated the harmful impacts of salinity stress. The ZNPs treatment might cause an inhibition of oxidative stress, decreasing the content of  $\text{Na}^+$  in the shoot tissues (Haidera et al., 2019). Indeed, ZNPs have been shown to increase  $\text{CO}_2$  fixation, photosynthetic pigments, photosynthetic efficiency, and plant growth restoration in response to salt stress (Soliman et al., 2015; Kasim et al., 2017; Mathur et al., 2019).

In addition, in this study, the expression levels of three key salt-tolerance related genes (*GmCHX1*, *GmPAP3*, and *GmSALT3*) were determined under 250 mM of NaCl salt alone. The gene expression was increased for all three genes (*GmCHX1*, *GmPAP3*, and *GmSALT3*) by 1.9-, 3-, and 7.7-fold, respectively. Generally, stress results in changes in the cellular program that involve significant transcriptional alterations aimed at increasing the chances of survival (Diédhiou et al., 2008). A study by Dang et al. (2014) proved that overexpression of *GmPAP3* improved rice salt tolerance by increasing the ROS-scavenging ability and decreasing oxidative damage. Similarly, a possible tolerance role of *GmPAP3* under oxidative stress was demonstrated in soybean, indicating that the *GmPAP3* gene expression is regulated by salinity, osmotic, and oxidative stresses (Liao et al., 2003; Li et al., 2008b; Soleimani et al., 2017). It can be concluded that salinity induces the formation of ROS, which in turn activates *GmPAP3*, leading to an increase in ROS degradation till reaching the proper level in the mitochondria, at which point the activity of the *GmPAP3* gene is decreased (Francisca, 2005; Li et al., 2008a).

As mentioned above, the results of this study revealed that *GmCHX1* expression was increased under salinity stress by 1.9-fold, which is parallel to the results of Patil et al. (2016), who reported that salinity stress (200 mM NaCl) significantly induced the expression of the *GmCHX1* gene in soybean, maintaining ion homeostasis by lowering the  $\text{Na}^+/\text{K}^+$  ratio. This result is also consistent with data from this study, which showed a high reduction in  $\text{K}^+/\text{Na}^+$  ratio by 90 % compared to control. Furthermore, the *GmCHX1* gene was highly expressed in the leaves and roots of soybean seedlings in response to salinity stress (Do et al., 2016). It was reported that low  $\text{Na}^+$  accumulation in shoot tissues of soybean plants may be due to the powerful function of the *GmCHX1* gene, which was highly expressed in salt-stressed soybean roots, forming  $\text{Na}^+$  exclusion proteins in root tissues and preventing  $\text{Na}^+$  entrance from soil to roots (Guan et al., 2014; Qu et al., 2020). This function of the *GmCHX1* gene has been documented in other plant species such as cotton (Wu et al., 2004), rice (Ren et al., 2005), *Arabidopsis* (Møller et al., 2009) and wheat (Munns et al., 2012).

Moreover, the results of this study indicated that *GmSALT3* gene expression was significantly increased in response to salinity stress by 7.7-fold in salt-stressed soybean seedlings. It was reported that the *GmSALT3* gene is the major salt tolerance gene in soybean belonging to the cation/H<sup>+</sup> exchanger (CHX) family (Patil et al., 2016), which is mainly expressed in root cells associated with the phloem and xylem, leading to limiting the accumulation of sodium ions in leaves (Pardo et al., 2006), which improved the physiological and morphological parameters and ultimately increased soybean yield under saline conditions (Do et al., 2016). As *GmSALT3* is localized in the endoplasmic reticulum (ER), it plays a direct role in the retrieval of salt from the xylem (Padmanaban et al., 2007; Cao et al., 2019). It has been reported that *GmSALT3* exerts a positive effect on soybean salt tolerance by exclusion of Na<sup>+</sup> in plant shoots and therefore prevents the toxic accumulation of Na<sup>+</sup> in photosynthetic tissues (Maathuis et al., 2014). Furthermore, Do et al. (2016) suggest that *CHX1/GmSALT3* controls Na<sup>+</sup>, K<sup>+</sup>, and Cl<sup>-</sup> accumulation and may function as a cation-chloride co-transporter.

Application of nanoparticles alters the levels of expression of certain transcription factors, making it possible to modify plant tolerance to salinity stress (Yamaguchi et al., 2013). In particular, application of ZNPs could upregulate or downregulate the stress-tolerant genes depending on their function by cascade reactions, thereby enhancing salt tolerance (Jonak et al., 2002).

The results of this study showed that application of ZNPs in combination with salt-stress downregulated the expression of the three studied salinity-tolerant genes in soybean seedlings compared to salt-stressed ones. The expression of *GmCHX1*, *GmPAP3*, and *GmSALT3* was decreased by 0.4, 0.46, and 1.56-fold, respectively, particularly with 25 mg l<sup>-1</sup> ZNPs in combination with high salinity stress (250 mM NaCl). Interestingly, this finding confirms the ameliorative role of ZNPs in improving soybean plant tolerance in response to salinity, which was reflected in enhancement effects on mineral uptake, total soluble proteins, total soluble carbohydrates, and yield characteristics. This finding is in accordance with that of Almutairi (2019) and Alharby et al. (2016) in tomato plants, where ZNPs imposed a positive response on plant metabolism under salt stress. It was reported that the differential response of *GmPAP3* expression in soybean to different ZNPs treatments under salinity stress could be as a result of reverted effects caused by NPs (Zhang et al., 2020) by excluding sodium ions from the roots, thus preventing the accumulation of toxic concentrations in the stem and leaves (Munns and Tester, 2008; Zhang et al., 2020).

## 5 CONCLUSIONS

The results of the present study indicated the importance of Zn<sup>+2</sup> in increasing soybean tolerance to salt stress. Soaking seeds of soybean cultivar Giza 111 in ZNPs at 50 mg l<sup>-1</sup> reduced oxidative damage caused by salinity stress, downregulated salt-tolerant gene expression, and increased soybean plant yield under high salinity stress (250 mM NaCl). Additionally, gene expression analysis of *GmCHX1*, *GmPAP3*, and *GmSALT3* confirmed their roles in salt tolerance in the soybean cultivar Giza 111. Moreover, the significant downregulation of these genes under combined treatments (250 mM NaCl and ZNPs) suggests that soybean plants favor ZNPs as an antioxidant. Therefore, application of low concentrations of ZNPs, particularly 50 mg l<sup>-1</sup>, is recommended before planting as a nano-fertilizer stimulator and could be a strategy to energize the growth and economic yield in plants growing in salinized soils, where it increases the adaptation capability of soybeans under such conditions.

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