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Ameliorating effect of nanoparticles and seeds' heat pre-treatment on soybean plants exposed to sea water salinity

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ABSTRACT

Impairing plant growth and reducing crop production, salinity is considered as major problem in modern agriculture. The current study aimed to investigate the role of seeds' heat pretreatment at 45 °C as well as application of two different nanoparticles nanosilica (N1) and nanoselenium (N2) in reducing salinity stress in three genotypes of Egyptian commercial soybeans (*Glycine* max L.). Two levels of salt stress using diluted sea water (1/12 and 1/6) were tested either alone or in combination with protective treatments. Obtained results revealed that salinity caused a significant reduction in all tested physiological parameters such as germination rate and membrane stability in soybean plants. A significant reduction in mitotic index and arrest in metaphase were recorded under both tested levels of salinity. It was also revealed that chromosomal abnormalities in soybean plants were positively correlated with the applied salinity concentrations. The fragmentation effect of salinity on the nuclear DNA was investigated and confirmed using Comet assay analysis. Seeds heat pre-treatment (45 °C) and both types of nanoparticles' treatments yielded positive effects on both the salt-stressed and unstressed plants. Quantitative real-time reverse transcription PCR (qRT-PCR) analysis for salt stress responsive marker genes revealed that most studied genes (*CAT*, *APX*, *DHN2*, *CAB3*, *GMPIPL6* and *GMSALT3*) responded favorably to protective treatments. The modulation in gene expression pattern was associated with improving growth vigor and salinity tolerance in soybean plants. Our results suggest that seeds' heat pretreatment and nanoparticle applications support the recovery against oxidative stresses and represent a promising strategy for alleviating salt stress in soybean genotypes.

1. Introduction

Water and soil salinization has a magnitude negative consequences including serious plant impairment, yield losses, deterioration in the potable water quality, negative impact on farmer and local communities' expenditure, employment opportunities, and supply

Abbreviations: chromosomal abnormalities (CA), mitotic index (MI).

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chains [1,2].

Therefore, new strategies should be developed to counteract the negative impact of salinity on the agriculture. Soybean (*Glycine* max L.) is an important crop for human nutrition and animal feeds because of its seeds contain about 40 % protein and 20 % edible oil as well as 30 % carbohydrates including 10 % of total sugar [3]. The global demand for soybean is ever increasing as it is used as input for numerous industries [4].

Salinity stress (the salinity of the soil and irrigated water) is one of the main abiotic stresses that exert negative effects on the agricultural productivity and quality [5–7]. More than 20 % of irrigated lands in the world are subject to salinization [8,9]. Plant growth and development are adversely affected by salinity through ion toxicity, reduced water uptake, hormonal disturbance and excessive formation of reactive oxygen species [6,7,10]. The increasing pace of arable land salinization shall exert disastrous impact on the worldwide agriculture [11]. Moreover, in recent years due to climate change and geopolitical conflicts, limitation of freshwater resources has been a major problem for national security. Thus, it has become a necessity to search for non-traditional sources of water in irrigation [12]. The concept of exploiting relatively saline or seawater in agriculture has been discussed since 1966 [13]. Furthermore, several strategies were suggested and evaluated, and some were applied to improve the crops' growth and tolerance under salinity conditions. Nanotechnology applications in Agriculture have been introduced as eco-friendly strategy that aims to reducing hazardous chemical applications, nutrient losses, pest control and improvement of crops' yield [5,14,15]. Moreover, the potential benefits of nanoparticle applications have been suggested as a new strategy to improve plant growth and performance under salinity stress [16,17]. In addition, modulation in the expression patterns of some defense involved genes was caused by some nanoparticle treatments [18–20].

The aims of this study were: i) evaluation of the salt stress negative effects on three local Egyptian soybean genotypes through assessment of changes in physiochemical and cytological parameters, and detection of the nuclear DNA damage using Comet assay analysis; ii) evaluation of the protective effects of seeds' heat pretreatment and two different nanoparticle applications, and their combined applications against salt stress in different soybean genotypes; iii) investigation of the underlying mechanism of salt stress alleviation via expression profiling of known salt stress responsive marker genes.

2. Materials and methods

2.1. Plant material

Three Egyptian commercial soybean genotypes (Giza 35, Carford, and Giza 111) were used in this study. The seeds of selected genotypes were provided by Giza agricultural experiment and research station, Agricultural Research Center (ARC), Giza, Egypt. The experiments were carried out during the summer season of 2018/2019 at the experimental farm of Faculty of Agriculture, Tanta University, Egypt.

2.2. Nanomaterials

Two nanomaterials used in this study were as follows.

i. Silica nanoparticles (N1) as silicon dioxide nanoparticles (SiO_2) with average size 50 ± 10 nm were used in concentration of 1 mM [21].
ii. Selenium nanoparticles (N2) as Se zero-valent-state [6,22] with average size 40 ± 5 nm in concentration of 0.075 mg/l.

These two nanomaterials were provided by NanoTech® Egypt for Photo-Electronics Communication Center. Characteristics and Transmission electron microscopy analysis (TEM) of used nanoparticles are shown in Table S1 and Fig. S1, respectively. The treatment of irrigation water with nanomaterials began with the sowing of seeds.

2.3. Salinity stress

The salinity stress was induced in soybean plants by irrigation with diluted sea water obtained from Al-Ajmi, Alexandria, Egypt with salinity concentration ranged from 37.51 to 39.71 ppt [23]. The experiments were performed using two dilutions of sea water; 1/12 sea water (S1) and 1/6 sea water (S2), in addition to the control group (C) where non-saline tap water was used according to Qados [24]. Treatment with a stressor began with sowing seeds. Plants were irrigated regularly three times a week, and the experiment was carried out for 35 days.

2.4. Experimental conditions

This study was designed as two main experiments. The non-pretreated seeds: the seeds were sown directly in the plastic pots (20 cm radius × 30 cm height). The pots were filled with 2 kg soil and six seeds were sown in each pot.

The 45 °C pretreated seeds: the seeds were placed on water-moistened paper tissues for 12 h. Subsequently, they were incubated at 45 °C for 30 min before being sown in the plastic pots.

All pots were kept under field conditions. The conditions were similar for both experiments and all the agriculture processes were followed as recommended for soybean cultivation and the two nanomaterials were added in the irrigation water. Each experiment was

conducted using the three studied genotypes, two salinity concentrations and two nanomaterials applications. All experimental variants were carried out in triplicate. The descriptions of all variants are shown in Fig. S2.

2.5. Rate of seed germination

The seed germination rate of three studied genotypes was calculated on the 7th day after sowing using the following equation:

Rate of seed germination (%) = [Number of germinated seeds / Total number of seeds sown] × 100

2.6. Electrolyte leakage (EL)

Electrolyte leakage (EL) was measured using the conductivity meter (Adwa-AD32, Romania). Five leaf discs (10 mm diameter) of 35-day-old plants from each variant were taken and weighted. The discs were placed in a vial containing 20 ml of distilled water, shaken, and the electrolyte conductivity of the solution was measured immediately (EL_0) and after 1 h (EL_1). Finally, each vial was placed in a boiling water for 1 h and left to cool down to room temperature and was measured again (EL_2) [20]. The electrolyte leakage rate was calculated as the ratio of the net electrical conductivity of the solution with leaf discs immersed for 1 h to the total electrical conductivity after boiling, calculated on a fresh weight basis and expressed as $\mu S\ cm^{-1}\ mg^{-1}\ FW\ h^{-1}$. The electrolyte leakage rate was calculated using the following equation:

Electrolyte leakage rate (EL) = $[(EL_1) - (EL_0) / (EL_2) - (EL_0)]/ FW$

2.7. Evaluation of lipid peroxidation

The concentration of thiobarbituric acid reactive substances (TBARS), equated with malondialdehyde (MDA) was used to evaluate the lipid peroxidation as previously described in Anjum et al. [25] with slight modifications. Half a gram of 35-day-old plants' leaf tissue was homogenized in 5 ml 5 % (w/v) trichloroacetic acid (TCA) and centrifuged at 4000 rpm and 5 °C for 10 min. The chromogen was formed by mixing 2 ml of supernatant with 3 ml of reaction mixture containing 20 % (w/v) TCA and 0.5 % (w/v) 2-thiobarbituric acid (TBA). The mixture was heated in a boiling water bath for 15 min and the reaction was stopped by rapid cooling in ice-water bath, followed by centrifugation at 4000 rpm and 5 °C for 10 min.

The amount of MDA was measured by the spectrophotometer (UV1901PC, Shanghai, China). A sample without plant extract was used as blank. The concentration of MDA was calculated using an extinction coefficient, 155 $mmol^{-1}\ cm^{-1}$ and expressed as nmol mg^{-1} FW using the following equation:

$[MDA] = [(Abs_{532} - Abs_{600}) - 0.0571 \times (Abs_{450} - Abs_{600})] /0.155$

2.8. Cytological analysis

Root tips of the Egyptian soybean genotype "Carford" were used to examine the toxicity effects of applied nanoparticles on soybean plants under control and salt-stress conditions. Root tips (1.5–2 cm) after 48 h of soaking in different treatment were cut off and fixed in Carnoy's solution (absolute ethanol and glacial acetic acid in the ratio of 3:1) for 24 h. Fixed root tips were kept in 70 % ethanol at 4 °C until analysis. Acetocarmine stain (2 %) was used for cytological preparations as previously described by Smith [26]. Mitotic index (MI), mitotic phases' indices (MP), and the numbers and types of chromosomal abnormalities were scored in at least 3000 examined cells per treatment (~1000 cells/replicate) using the light microscope (PT/Slope, Pearl, England). MI and the percentage of cells with chromosomal abnormalities (abnormal cells, AC) were calculated using the following formulas [19]:

MI = (Total dividing cells/Total dividing and non-dividing cells) × 100

AC = (Total abnormal cells/Total dividing cells) × 100

2.9. Comet assay analysis

Comet assay was performed to assess the extent of nuclear DNA damage in the cells caused by nanoparticles application with or without salt stress, both qualitatively and quantitatively. Frozen leaves of 35-day-old Carford genotype plants were used in this assay according to previously described protocol [27,28]. The migration patterns of DNA fragment of 100 cells for each sample were evaluated with the fluorescence microscope (Confocal microscope C2, Nikon, Japan) at magnification of $40^{\times}$ with excitation filter 420–490 nm. The length of DNA migration and the percentage of migrated DNA were analyzed using Komet 5 image analysis software

developed by Kinetic Imaging, Ltd. (Liverpoo1, UK) linked to a CCD camera. The length of comet tails was measured from the middle of the nucleus to the end of the tail.

2.10. Gene expression analysis

Plant material (leaves of 35-day-old plants) was collected and immediately flash frozen using liquid nitrogen and kept at −80 °C until processing for molecular analysis. Total RNA was extracted from soybean Egyptian genotypes Giza 35 and Giza 111 of the treatments: 0 sea water (Control), 0 sea water + 1 mM nanosilica (N1), 0 sea water + 0.075 mM nanoselenium (N2), 1/12 sea water (S1), 1/12 sea water + 0.075 mg/l nanoselenium (S1N2), 1/6 sea water (S2), 1/6 sea water + 1 mM nanosilica (S2N1), control + 45 °C seed pretreatment (CP), 1/12 sea water + 1 mM nanosilica with 45 °C seed pretreatment (S1N1P), and 1/6 sea water + 0.075 mg/l nanoselenium with 45C° seed pretreatment (S2N2P) using I Qeasy™ plus plant RNA extraction Mini Kit. For quality control the RNA was analyzed in 1 % agarose gel with RNase-free devices. The gel was stained with ethidium bromide 1 μg ml^{-1} and visualized by UV-trans-illuminator and photographed using digital camera with UV filter adaptor. RNA concentration and purity were measured using Nano-Drop (BioDrop μLITE, UK). Half μg of total extracted RNA of each sample were used for first-strand cDNA synthesis according to the protocol supported by HiSenScript™ RH cDNA Synthesis Kit (iNTRON Biotechnology). PCR was used to approve the primer specificity and annealing temperature for all studied genes. Studied genes, primer sequence, and annealing temperature (Ta °**C)** are shown in Table 1.

Quantitative real-time polymerase chain reaction (qRT-PCR) analyses were used to measure the changes in gene expression in response to the salinity stress in selected genotypes. qRT-PCR reactions were conducted using SYBR Green stain. $5^{\times}$ HOT FIREPoL® EvaGreen® qPCR Supermix was used. The reactions were carried out in 20 μl reaction volume containing 1 μl of cDNA, 0.5 μl of both forward and reverse primers, 4 μl of SYBR Green buffer and 14 μl of water. The reactions were carried out using Step One Plus™ Real Time PCR system (Applied Biosystem™). The actin gene from soybean (accession no. BW652479) was used as an internal control. All genes' specific primers were designed according to available data of soybean on National center on biological information (NCBI) using primer 3 software and blast online algorithm (Table 1). The reactions were performed under standard conditions using variflex option to adjust different annealing temperature for different genes on the same plate. Data are given as means ± SE of relative expression (RQ) for three biological replicates for each cDNA sample. RQs were calculated as $2^{-\Delta\Delta ct}$ calibrated with the internal control gene and control treatment [29].

2.11. Statistical analysis

All values shown in the figures and tables were expressed as the mean ± SD (mean ± SE for gene expression analysis) obtained from three independent experiments. Statistical package for social sciences (SPSS) software for Windows version 20 were applied on obtained data for one-way analysis of variance (ANOVA) followed by Duncan test and the results were considered significant at $p \leq 0.05$.

3. Results and discussion

3.1. Seed germination rate

The obtained results showed significant differences in the germination rates among the three soybean genotypes under salinity compared to non-salt stressed plants (Fig. 1). In the set test conditions, the higher salt concentration (S2) resulted in a significant reduction in the germination rate in the Giza 111 genotype compared to the control group. On the other hand, both types of nanoparticles tested in this study, as well as heat pretreatment, have significantly recovered the germination rate under the S2 level of salt stress (Fig. 1).

Table 1
List of primers used in qRT-PCR analysis.

Gene	Function	Accession. no	Sequence (5′ to 3′)	Ta °C
ACT	Actin, housekeeping gene	BW652479	ATCTTGACTGAGCGTGGTTATTC GCTGGTCCTGGTGTCTCC	59
CAB3	Chlorophyll *a*/b-binding protein	NM_001248354	GGTCCCAGATCTTCAGCGAG TAGGCCCAGGCATTGTTGTT	58
DHN2	Dehydrin	AM421515	TCGCAAGGTCGACGAGTATG TTCTTCTCGTGCTGACCACC	58
CAT	Catalase	AF035252	AGCATCTCACCTGAACTTGAA AGGTGAGAGGTTTGTGGCC	61
APX	Ascorbate peroxidase	L10292	CGTGACGATGATTGGGAAGT TGATAGTGATCTTTCGGACCT	61
GmPIPL6	Plasma membrane intrinsic protein	NM_001353638	AGGGTGTGAGAAGAGAATGGAG GGTGACCTGTTGACACCCAT	58
GmSALT3	Glycine max salt tolerance-associated gene	NM_001353420	GCTCGACTTTCGCACCATTC CACGTGTGGTCAGCAGTTTG	58

Furthermore, obtained results showed that the heat pretreatment improved germination rate in both nanoparticles treated or nontreated plants under both normal and salt stress conditions in Carford genotype (Fig. 1). It is known that seed germination is negatively correlated with salinity intensity [12,30–32]. Our results are in agreement with numerous studies reporting the positive effects of nanoparticles as well as heat pretreatments as promising strategies for improving crop productivity under saline stress conditions [16,17].

On other hand, in all three tested genotypes, the 45 °C heat pretreatment caused significant improvement in seeds' germination rate under the higher salt concentration (S2) compared to the non-heat-pretreated plants. However, the same outcome was not observed at the lower tested salt concentration (S1). The 45 °C heat pretreatment induced significant increase in germination percentage; it raised up to 100 % in most experimental variants off Giza111 genotype (Fig. 1). After treatment with nanoparticles, the protective status of plants improves and the oxidative load of ROS in stressed plants decreases [15,19].

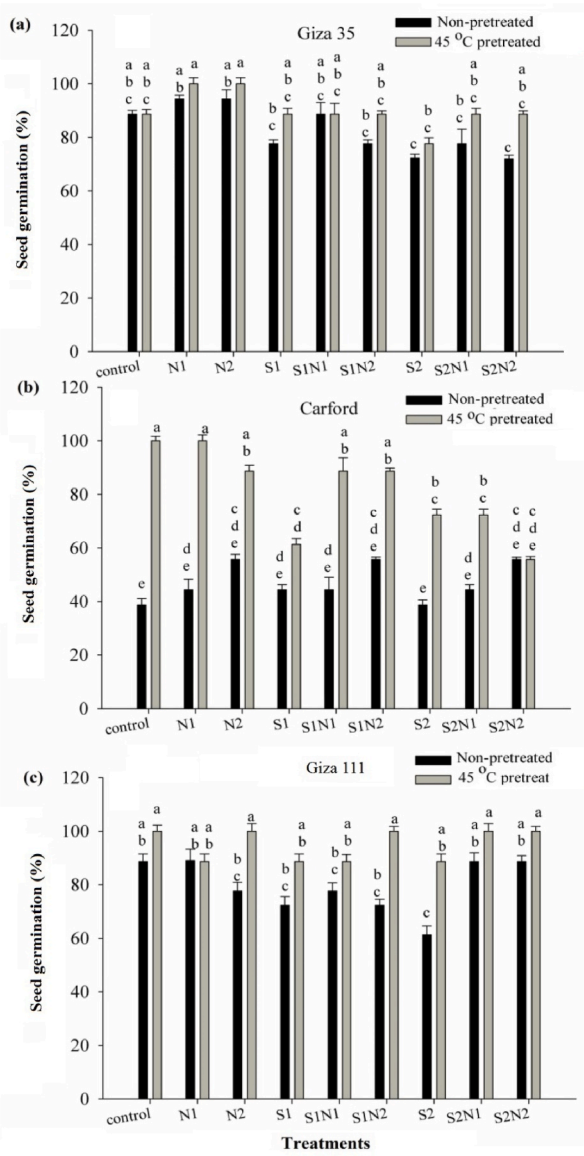

Fig. 1. Changes in seed germination rate of all studied genotypes under all experimental conditions. Control: 0 sea water (tap water), N1: 0 sea water + 1 mM nanosilica, N2: 0 sea water + 0.075 mg/l nanoselenium, S1: 1/12 sea water, S1N1: 1/12 sea water + 1 mM nanosilica, S1N2: 1/12 sea water + 0.075 mg/l nanoselenium, S2: 1/6 sea water, S2N1: 1/6 sea water + 1 mM nanosilica and S2N2: 1/6 sea water + 0.075 mg/l nanoselenium. Different letters indicate significant differences at $p \leq 0.05$ according to one-way ANOVA followed by Duncan test.

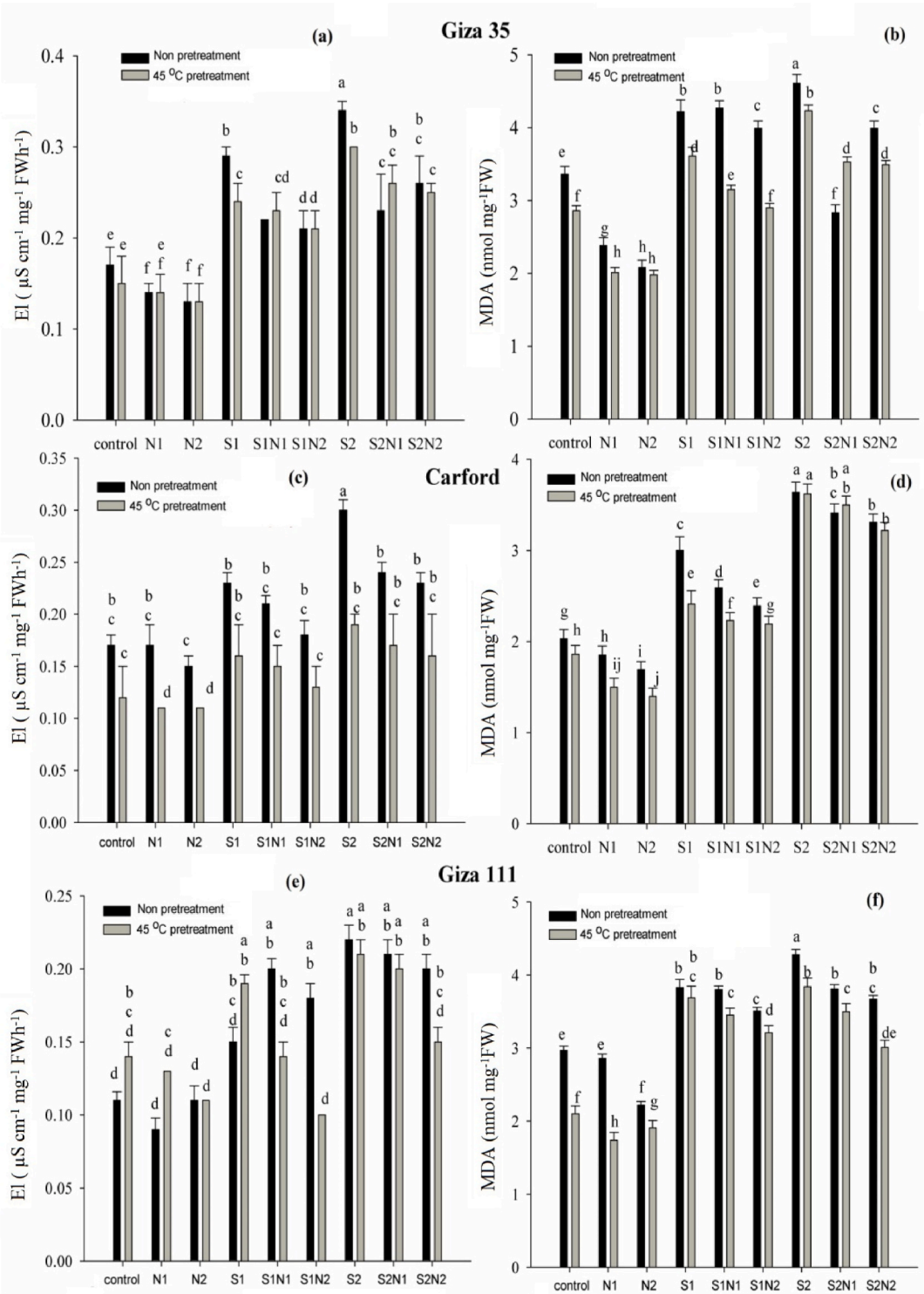

Fig. 2. Changes in electrolyte leakage rate (EL) and malondialdehyde (MDA) content for all genotypes under all experimental conditions. Control: 0 sea water (Tap water), N1: 0 sea water + 1 mM nanosilica, N2: 0 sea water + 0.075 mg/l nanoselenium, S1: 1/12 sea water, S1N1: 1/12 sea water + 1 mM nanosilica, S1N2: 1/12 sea water + 0.075 mg/l nanoselenium, S2: 1/6 sea water, S2N1: 1/6 sea water + 1 mM nanosilica, and S2N2: 1/6 sea water + 0.075 mg/l nanoselenium. Different letters indicate significant differences at $p \leq 0.05$ according to one-way ANOVA followed by Duncan test.

3.2. Rate of electrolyte leakage and MDA content

Changes in electrolyte leakage rate (EL) and MDA content are shown in (Fig. 2) for the three studied genotypes in control and salt-stress conditions for both 45 °C pretreated and non-pretreated seeds with and without nanoparticles application. In the set test conditions, the 45 °C pretreatment had a positive effect by reducing the EL and MDA values for Giza 35 and Carford genotypes compared to non-pretreated seeds. Also, the MDA values in the 45 °C pretreated plants of Giza-111genotype were lower compared to the non-pretreated plants.

Obtained results showed that both levels of salt stress (S1 and S2) increased EL and MDA values. These increases were more significant in Giza 35 Genotype, while it was less or non-significant in other two genotypes, Carford and Giza 111. The data also showed obvious positive correlation between EL values and MDA content with salt concentration for all studied genotypes (Fig. 2). It is known that oxidative stress provoked by excess salinity results in peroxidation of membranes' lipids and disturbs membrane barrier function, thus leads to increasing the rate of electrolytes leakage [21,23]. Similarly, it has been reported that exposing soybean plants for soil salinity for prolonged durations increased the EL values and MDA content by about two and three-fold, respectively, compared to their level in the control group [33].

The presented data are in agreement with numerous studies reporting that the EL and MDA values are heavily reliant on the genotypes in soybean [34–36]. For example, among the 11 soybean genotypes studied, BINA-03, followed by SBM-22 and BINA-01, was reported to have the highest MDA content under saline conditions [37]. Another study indicated that the influence of genotype was more pronounced with more severe salinity injury occurring, for example in Giza 82 and Giza 35, but not in Giza 111, where the effects of stress were less manifested [38].

In the set test conditions, the 45 °C pretreatment caused significant improvement in EL and MDA values of plants under both salt concentrations (S1 and S2) compared to the non-heat-pretreated plants except for EL value of Giza 111(Fig. 2E).

Treatments with both types of nanoparticles (N1 and N2) reduced the MDA value in most studied variants for heat pretreated and non-pretreated seeds under both level of salinity. In agreement with our findings, Abdel Latef et al. [39], reported that the lupine (*Lupinus termis*) plants exposure to 150 mM NaCl accumulated MDA by ~ 84 % more than the control plants, while priming with nanoparticles reduced MDA content. In addition, induced antioxidant enzymes' activity has been recorded in various crops treated with different types of nanoparticles [15,21,32].

3.3. Comet assay analysis

Comet assay analysis (also called, single cell' gel electrophoresis) was used to detect any DNA damage of Carford genotype plants under control and salt-stress conditions with and without nanoparticles treatments (Fig. 3 and Table 2).

Obtained results showed that both levels of salinity (S1 and S2) induced a significant increase in DNA damage comparing with one

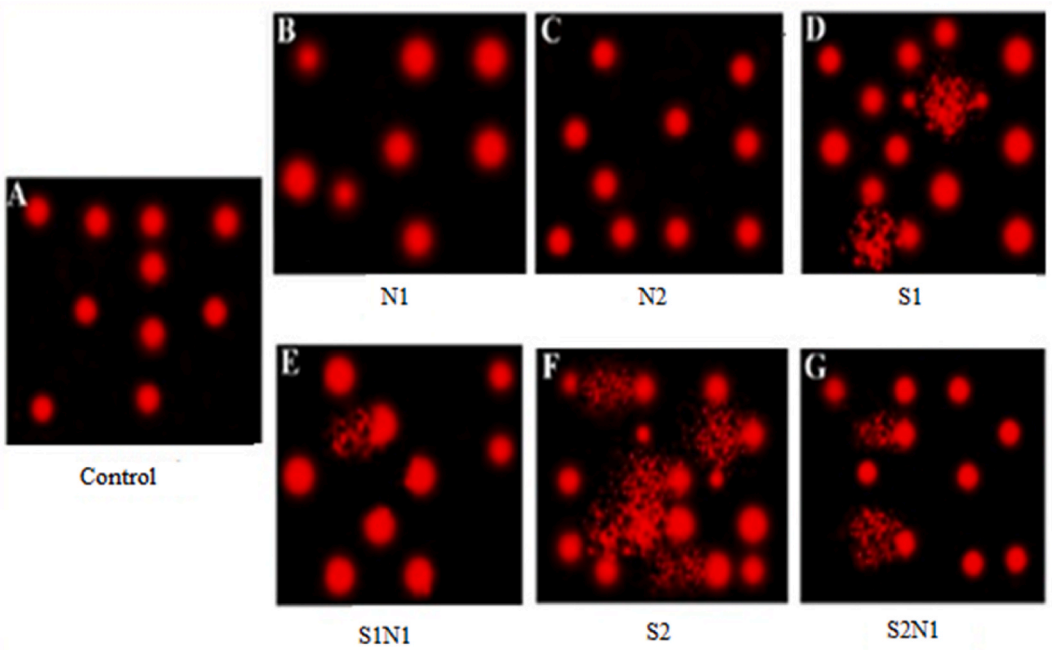

Fig. 3. Representative pictures of comet assay on DNA damage induced in root cells' of soybean plants (Carford genotype) under control and stress condition with and without nanoparticles treatment. Control: (A), N1: 1 mM nanosilica (B), N2: 0.075 mg/l nanoselenium (C), S1: 1/12 sea water (D), S1N1: 1/12 sea water + 1 mM nanosilica (E), S2: 1/6 sea water (F), S2N1: 1/6 sea water + 1 mM nanosilica (G).

Table 2
Comet assay analysis of soybean plants (Carford genotype) under control and different experimental conditions.

Group	Tailed nuclei (%)	Tails length (μm)	Tail DNA (%)	Tail moment
Control	2^{g}	1.31 ± 0.10^{d}	1.44^{d}	$\mathbf{1.89^{f}}$
N1	3^{e}	1.42 ± 0.11^{d}	1.52^{d}	$\mathbf{2.16^{e}}$
N2	2.75^{f}	1.37 ± 0.09^{d}	1.49^{d}	$\mathbf{2.04^{ef}}$
S1	10^{b}	3.15 ± 0.13^{b}	3.73^{b}	$\mathbf{11.75^{b}}$
S1N1	7^{d}	2.03 ± 0.10^{c}	2.49^{c}	$\mathbf{5.05^{d}}$
S2	18^{a}	5.27 ± 0.17^{a}	5.41^{a}	$\mathbf{28.51^{a}}$
S2N1	$\mathbf{9^{c}}$	$\mathbf{3.04 \pm 0.09^{b}}$	$\mathbf{3.55^{b}}$	$\mathbf{10.79^{c}}$

Means carrying different letters are significantly different at $p \leq 0.05$ according to one-way ANOVA followed by Duncan test. Control: 0 sea water (tap water), N1: 0 sea water + 1 mM nanosilica, N2: 0 sea water + 0.075 ml/l nanoselenium, S1: 1/12 sea water, S1N1: 1/12 sea water + 1 mM nanosilica, S2: 1/6 sea water, S2N1: 1/6 sea water + 1 mM nanosilica.

in the plants under control conditions (Fig. 3D and F). This damage was scored as an increase in tailed nuclei' percentage, DNA tail length, and tail moment (Table 2). The tailed nuclei' was increased from 2 % in control group, to 10 % and 18 % under both levels of salinity S1 and S2, respectively (Table 2). Similarly, both levels of salinity (S1 and S2) induced significant increase in tail length (μm) and tail moment of tested nuclei. Releasing free radicals such as ROS under oxidative stress induced by salinity was specified as a reason for increasing DNA breaks in salinity stressed plants [39]. Enhanced DNA damage under salinity was reduced after treatment with silica nanoparticles (N1) (Fig. 3E and G) as shown via percentage of tailed nuclei, tail length, and tail moment. Finally, the effect of both types of nanoparticles (N1 and N2) was meager on unstressed plants as shown in tail length and tail DNA percentage (Fig. 3B and C and Table 2). Recovery effects of nanoparticle treatments could be due to their role in inducing antioxidant enzymes subsequently reducing the oxidative stress in plants under salinity [32].

Our results revealed that the applied concentrations of both studied nanoparticles (N1 and N2) showed no signs of cytotoxicity or DNA damage on the soybean plant cells. Presented data are in agreement with previous studies when using comet assay was demonstrated low cytotoxicity of nano titanium dioxide (TiO_2) at different concentrations [40]. On the other hand, Abdelmigid et al. [41], reported that comet assay in pomegranate leaf samples displayed that the frequency of DNA damage increased steadily with increasing concentrations of nano phosphorus used as nano fertilizer, regardless of whether DNA damage was expressed as tail

Table 3
Mitotic index and mitotic phases' index in root meristems of Carford soybean genotype under control and different experimental conditions.

Treatment	Sum of analyzed cells in all replicates	Mitotic phase index (MP), % *{mitotic phases' proportion, %}*				Total no. of normal dividing cells	Mitotic index (MI), %
		Prophase	Metaphase	Anaphase	Telophase		
Control	3185	2.35 ± 0.113^{c} *{29.75}*	2.64 ± 0.081^{g} *{33.33}*	1.98 ± 0.05^{b} *{25}*	0.94 ± 0.04^{c} *{11.89}*	252	7.91 ± 1.32^{b}
N1	3094	3.10 ± 0.087^{b} *{28.81}*	4.27 ± 0.152^{a} *{39.64}*	2.23 ± 0.106^{a} *{20.72}*	1.16 ± 0.06^{a} *{10.81}*	333	10.76 ± 1.075^{a}
N2	3095	3.2 ± 0.089^{a} *{29.99}*	4.17 ± 0.214^{b} *{39.09}*	2.23 ± 0.09^{a} *{20.91}*	1.07 ± 0.032^{b} [46]	330	10.66 ± 1.03^{a}
S1	3139	0.095 ± 0.004^{h} *{3.53}*	1.91 ± 0.05^{h} *{71.09}*	0.67 ± 0.008^{e} *{24.9}*	0.01 ± 0.003^{h} *{0.372}*	84	2.69 ± 0.122^{h}
S1N1	3124	0.96 ± 0.035^{d} *{17.85}*	2.98 ± 0.072^{e} *{55.35}*	1.25 ± 0.05^{c} *{23.21}*	0.19 ± 0.002^{f} *{3.57}*	168	5.38 ± 0.79^{d}
S1N2	3097	0.48 ± 0.006^{g} *{6.94}*	3.58 ± 0.092^{c} *{51.39}*	2.23 ± 0.085^{a} *{31.93}*	0.68 ± 0.06^{d} *{9.72}*	216	6.97 ± 0.98^{c}
S2	3070	0.097 ± 0.007^{h} *{4.14}*	1.47 ± 0.081^{i} *{62.47}*	0.68 ± 0.008^{e} *{29.17}*	0.097 ± 0.003^{g} *{4.14}*	72	2.35 ± 0.13^{f}
S2N1	3094	0.78 ± 0.072^{e} *{15.99}*	2.81 ± 0.103^{f} *{57.98}*	1.07 ± 0.078^{d} *{21.99}*	0.19 ± 0.007^{f} *{3.98}*	150	4.85 ± 0.38^{e}
S2N2	3082	0.58 ± 0.009^{f} *{8.69}*	3.41 ± 0.127^{d} *{50.72}*	2.24 ± 0.182^{a} *{33.32}*	0.49 ± 0.009^{e} *{7.24}*	207	6.72 ± 0.71^{c}

Means carrying different letters are significantly different at $p \leq 0.05$ according to one-way ANOVA followed by Duncan test. Control: 0 sea water (tap water), N1: 0 sea water + 1 mM nanosilica, N2: 0 sea water + 0.075 mg/l nanoselenium, S1: 1/12 sea water, S1N1: 1/12 sea water + 1 mM nanosilica, S1N2: 1/12 sea water + 0.075 mg/l nanoselenium, S2: 1/6 sea water, S2N1: 1/6 sea water + 1 mM nanosilica, and S2N2: 1/6 sea water + 0.075 mg/l nanoselenium.

Table 4
Analysis of the mitotic division's chromosomal abnormalities (%) in plant roots of soybean (genotype Carford) under different conditions.

Treatments	No. of screened cells	Total no. of normal dividing cells	No. of abnormal cells	Abnormalities (%)	Mitotic abnormalities (%)										
					C-metaphase	Vagrant chromosome in Anaphase with chromosome bridge	Sticky prophase	Irregular Metaphase	Sticky Anaphase	Irregular telophase with unequal division	chromosome breakage	Chromosomal laggard in metaphase	Anaphase with laggard chromosome	Clumped metaphase	Anaphase with a single bridge and sticky chromosome
Control	1061.667	84	2	0.188^{e}	1.190^{a}	0.000^{e}	0.000^{e}	0.000^{c}	0.000^{e}	0.000^{e}	0.000^{b}	1.190^{d}	0.000^{c}	0.000^{e}	0.000^{d}
N1	1031.333	111	1	0.096^{g}	0.000^{c}	0.000^{e}	0.000^{e}	0.000^{c}	0.000^{e}	0.000^{e}	0.000^{b}	0.901^{d}	0.000^{c}	0.000^{e}	0.000^{d}
N2	1031.667	110	1	0.097^{f}	0.909^{b}	0.000^{e}	0.000^{e}	0.000^{c}	0.000^{e}	0.000^{e}	0.000^{b}	0.000^{e}	0.000^{c}	0.000^{e}	0.000^{d}
S1	1046.333	28	8	0.764^{b}	0.000^{c}	3.571^{b}	0.000^{e}	3.571^{b}	3.571^{a}	3.571^{a}	0.000^{b}	3.571^{b}	7.143^{a}	3.571^{b}	0.000^{d}
S1 N1	1041.333	56	5	0.480^{c}	0.000^{c}	1.786^{c}	0.000^{e}	0.000^{c}	1.786^{c}	0.000^{e}	0.000^{b}	1.786^{c}	0.000^{c}	1.786^{d}	1.786^{b}
S1 N2	1032.333	72	4	0.387^{d}	0.000^{c}	0.000^{e}	1.389^{d}	0.000^{c}	0.000^{e}	1.389^{c}	0.000^{b}	1.389^{d}	0.000^{c}	0.000^{e}	1.389^{c}
S2	1023.333	24	10	0.977^{a}	0.000^{c}	4.167^{a}	4.167^{a}	4.167^{a}	0.000^{e}	0.000^{e}	4.167^{a}	4.167^{a}	4.167^{b}	4.167^{a}	4.167^{a}
S2 N1	1031.333	50	5	0.484^{c}	0.000^{c}	0.000^{e}	2.000^{b}	0.000^{c}	2.000^{b}	2.000^{b}	0.000^{b}	2.000^{c}	0.000^{c}	2.000^{c}	0.000^{d}
S2 N2	1027.333	69	5	0.486^{c}	0.000^{c}	1.449^{d}	1.449^{c}	0.000^{c}	1.449^{d}	0.014^{d}	0.000^{b}	1.449^{d}	0.000^{c}	0.000^{e}	0.000^{d}

Means carrying different letters are significantly different at $p \leq 0.05$ according to one-way ANOVA followed by Duncan test. Control: 0 sea water (tap water), N1: 0 sea water + 1 mM nanosilica, N2: 0 sea water + 0.075 mg/l nanoselenium, S1: 1/12 sea water, S1N1: 1/12 sea water + 1 mM nanosilica, S1N2: 1/12 sea water + 0.075 mg/l nanoselenium, S2: 1/6 sea water, S2N1: 1/6 sea water + 1 mM nanosilica, and S2N2: 1/6 sea water +0.075 mg/l nanoselenium.

moment, percentage of tail DNA, or tail length. Taken together, these results suggest that the potential cytotoxicity of nanoparticles depends on the plant species and variety, type and concentration of nanoparticles, which, in turn, prompts further research to confirm safe compounds/dosages before large field applications.

3.4. Cytological analysis

Cytological analysis aimed to examine the possible cytotoxic effects of selected nanoparticles' type and concentrations on soybean plants (Carford genotype) under control and salt-stress conditions. In the root apical meristem were determined: the total number of mitotically active cells (mitotic index, MI), the number of cells in different phases of mitosis (mitotic phase index, MP), and the relative proportion of a certain mitotic phase from the sum of all mitotic phases (Table 3). It should be recalled here that the frequency of detection of one or another phase of mitosis in a population of dividing cells is proportional to the duration of this phase [42].

The results obtained showed (Table 3) that treatment with nanoparticles of both types (N1 and N2) increases mitotic indices (MI), while, based on the mitotic phases' proportion, it does not affect the entry of cells into mitosis, somewhat slowing down metaphase and speeding up anaphase by compared with the control group. This is consistent with the comet assay data, which showed no DNA damage from N1 and N2 nanoparticles (Fig. 3 and Table 2); because it is known that DNA damage, in particular during salt stress, initiates mechanisms leading to G2-M arrest of the mitotic cycle [43]. Both levels of salinity (S1 and S2) led to a significant decrease in MI, to a pronounced arrest of cells in metaphase, and S2 salinity also to a slight delay in anaphase (Table 3). Metaphase and metaphase-anaphase arrest of cells is usually associated with a violation of the mitotic spindle; inhibition of microtubule organization is the key actions of anti-mitotic drugs, which act at the metaphase/anaphase transition [44]. Although it is well known that salinity leads to disruption of the tubulin cytoskeleton and can lead to the death of some cells or to a significant change in their morphology and physiology [45,46], we did not find in the available literature information about a pronounced metaphase arrest of root apical meristem' cells under salt stress. Nevertheless, similar to our data, also reported that exposing *Hordeum vulgare* to high salt concentrations led to a significant decrease in the MI [40]; mitotic divisions were completely suppressed at high salt concentrations in root meristem cells of in vitro cultured optionally salt-tolerant plant, Centaurea ragusina [41]; in onion root meristem cells, the reduction in MI was positively correlated with tested salts concentrations and duration of treatment [42]. It should be noted that at both salinity levels (S1 and S2), the prophase proportion decreased more than seven times relative to the control (Table 3), which indicates a violation of the transition from the G2 phase of the mitotic cycle to mitosis. This fact is fully explained by the significant level of DNA damage under these salt stresses (Fig. 3 and Table 2), which, as already mentioned, should lead to G2-M arrest of the mitotic cycle [43].

In the set test conditions, the numbers of chromosomal abnormalities (CA) were examined in the root apical meristem (Table 4). The most frequent CAs were C-metaphase, vagrant chromosome in anaphase with chromosome bridge, sticky prophase, irregular metaphase, sticky anaphase, irregular telophase with unequal division, chromosome breakage, laggard chromosome in metaphase,

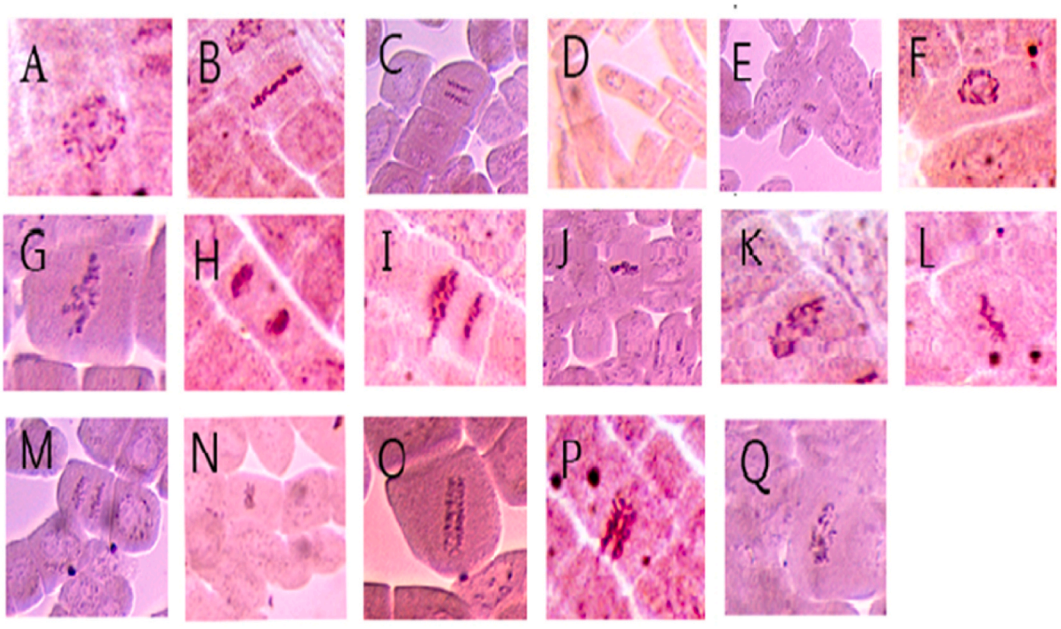

Fig. 4. Representative pictures for chromosomal abnormalities obtained during cytological examination of soybean plants (genotype Carford) under both control and experimental variants. (A) C-metaphase; (B) normal metaphase; (C) normal anaphase; (D) normal telophase; (E) vagrant chromosome in anaphase with chromosome bridge; (F) sticky prophase; (G) irregular metaphase; (H) sticky anaphase; (I) irregular telophase with unequal division; (J) chromosome breakage; (K) C-metaphase; (L) laggard chromosome in metaphase; (M) anaphase with laggard chromosome; (N) clumped metaphase; (O) normal anaphase; (P) anaphase with a single bridge and sticky chromosome; (Q) C-metaphase.

anaphase with laggard chromosome, clumped metaphase, anaphase with a single bridge and sticky chromosome (Fig. 4). Moreover, most of the detected CAs appeared at both tested salinity levels. The lowest value of CA was observed in the variant with silica nanoparticles (N1) followed by the selenium nanoparticles (N2) in the unstressed plants, and CA level was significantly higher in salt-stressed plants and positively correlated with salt concentration (Table 4). It was reported previously that increasing salt

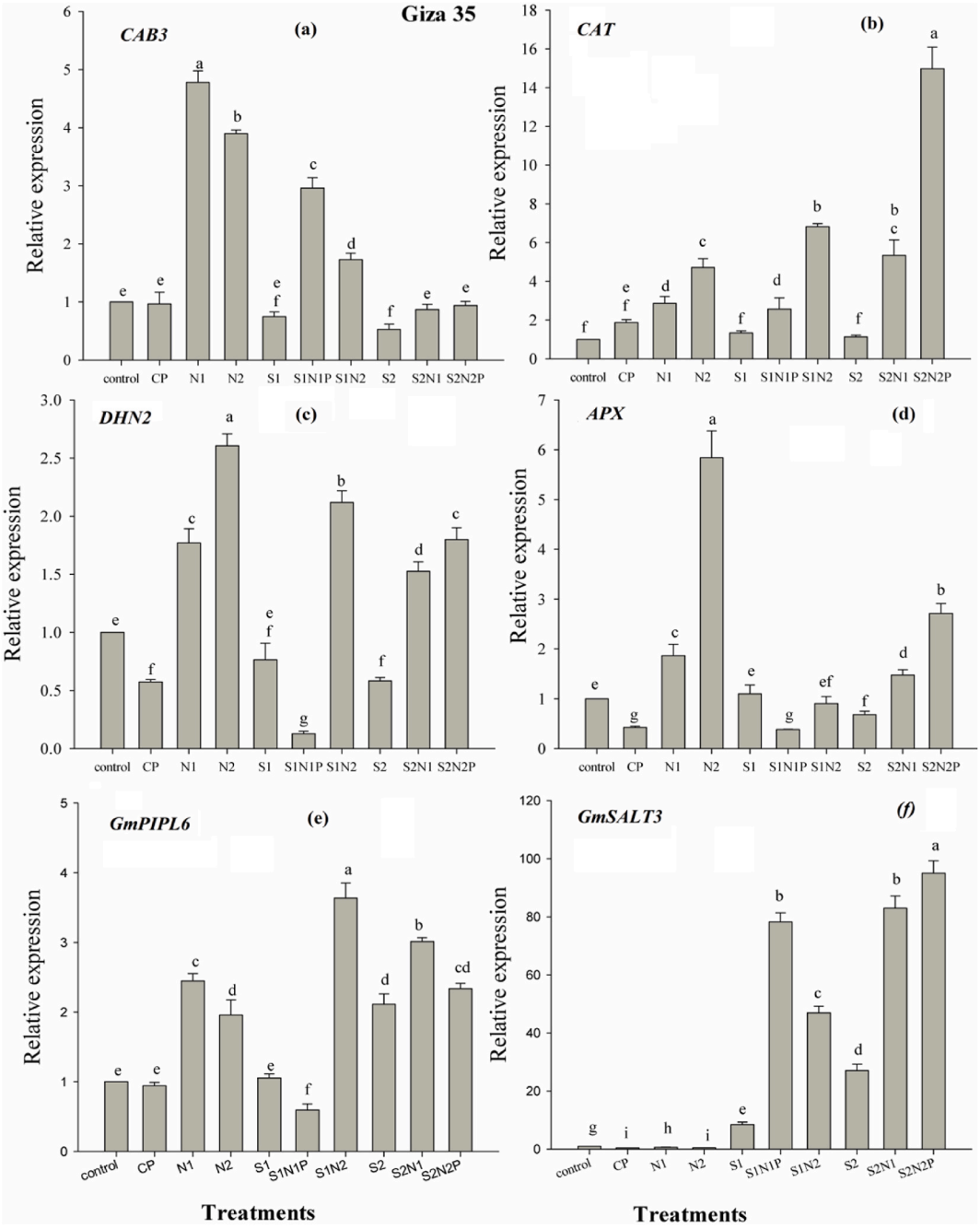

Fig. 5. Gene expression profile changes in selected salt-stress responsive and growth-related genes in seedlings of Giza 35 genotype. Control: 0 sea water, CP: control + heat pre-treatment, N1: 0 sea water + 1 mM nanosilica, N2: 0 sea water +0.075 mg/l nanoselenium, S1: 1/12 sea water, S1N1P: 1/12 sea water + 1 mM nanosilica with 45 °C pre-treatment, S1N2: 1/12 sea water +0.075 mg/l nanoselenium, S2: 1/6 sea water, S2N1: 1/6 sea water +1 mM nanosilica, and S2N2P: 1/6 sea water +0.075 mg/l nanoselenium with 45 °C pre-treatment. The expression was determined as changes in the transcript amount using qRT-PCR. Relative expression (RQ) was calculated as $2^{-\Delta\Delta ct}$ calibrated with actin gene (accession no. BW652479) as reference gene and control treatment as endogenous control. Different letters indicate significant differences at $p \leq 0.05$ according to one-way ANOVA followed by Duncan test. Data are given as means ± SE of relative expression for three biological replicates for each sample.

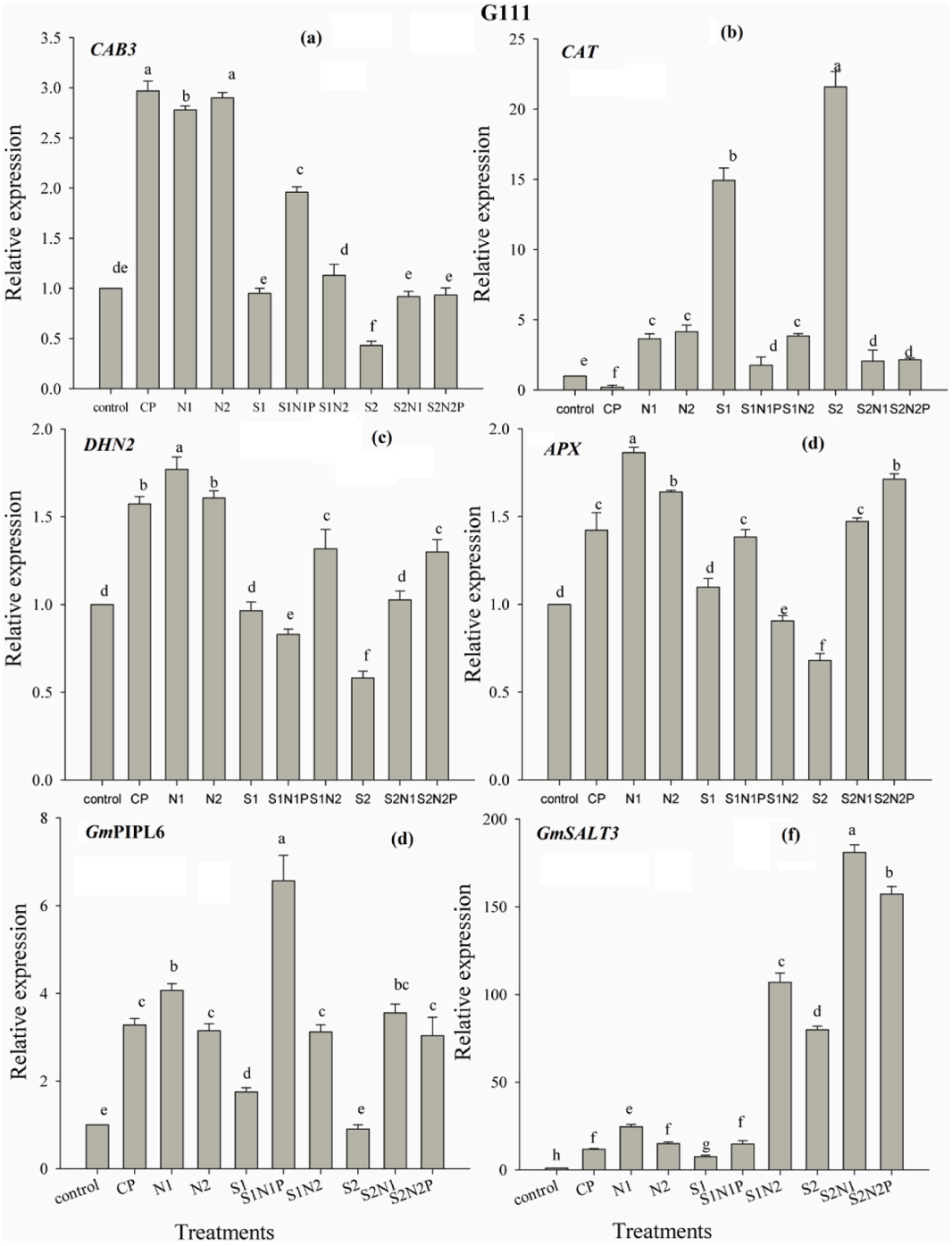

Fig. 6. Gene expression changes in some stress related genes in seedlings of Giza 111 genotype in the given set of experimental conditions. Control: 0 sea water, CP: control + heat pre-treatment N1: 0 sea water + 1 mM nanosilica, N2: 0 sea water +0.075 mg/l nanoselenium, S1: 1/12 sea water, S1N1P: 1/12 sea water + 1 mM nanosilica with 45 °C pre-treatment, S1N2: 1/12 sea water +0.075 mg/l nanoselenium, S2: 1/6 sea water, S2N1: 1/6 sea water +1 mM nanosilica, and S2N2P: 1/6 sea water +0.075 mg/l nanoselenium with 45 °C pre-treatment. The expression was determined as changes in the transcript amount using qRT-PCR. Relative expression (RQ) was calculated as $2^{-\Delta\Delta ct}$ calibrated with actin gene (accession no. BW652479) as reference gene and control treatment as endogenous control. Different letters indicate significant differences at $p \leq 0.05$ according to one-way ANOVA followed by Duncan test. Data are given as means ± SE of relative expression for three biological replicates for each sample.

concentrations increases the percentage of CAs in root meristem of *Hordeum vulgare*, *Centaurea ragusina*, *Allium cepa* [40–42].

Let us turn to the effect of nanosilica (N1) and nanoselenium (N2) on mitotic activity and the state of chromosomes under salinity. As can be seen from Table 3, both types of nanoparticles have an ameliorative effect on MI of root apical meritem at both levels of salinity, but nanoselenium (N2) has a more valuable effect on this index compared to nanosilica (N1). However, these nanoparticles have differences in the mechanisms for implementing these ameliorating effects. Nanosilica primarily promotes the activation of the transition from G2 to mitosis (as seen from the increase in the proportion of prophases) and releases cells arrested in metaphase; nanoselenium also releases metaphase arrested cells and promotes mitosis progression towards anaphase and telophase (Table 3). Note that CAs were reduced by both nanoparticles' treatments (N1 and N2) used in this study (Table 4). It was recorded also that nanoselenium has a recovery effects against the toxic herbicide (Atrazine) in *Vicia faba* plants at low concentration (10 ppm) in regard to the MI and CAs [19].

3.5. Analysis of gene expression

Analysis of gene expression was performed using qRT-PCR for selected salt-stress responsive and growth-related marker genes (listed in Table 1). Gene expression analysis was performed in soybean genotypes Giza 35 (Fig. 5) and Giza 111 (Fig. 6).

It has been well documented that salinity-induced stresses lead to formation of reactive oxygen species (ROS), which in turn requires number of detoxification systems such as protective antioxidant enzymes such as catalase (*CAT*) and ascorbate peroxidase (*APX*); and ROS scavengers and osmo-protectant proteins such as dehydrins (*DHN2*), [21,47–51].

Therefore, any factors influencing expression profiles of salt stress-responsive marker genes such as *DHN2*, *CAT* and *APX* are of great importance for salt stress tolerance in plants. In this study, the expression profiles were studied for selected salt-stress marker genes for Giza 35 and Giza 111 soybean genotypes (Figs. 5 and 6; B, C and D).

It was observed that under given experimental conditions, the *DHN2* was significantly down regulated in both studied genotypes for both tested salinity concentrations (S1 and S2) compared to the control treatment (Figs. 5 and 6; c). Using the lower salt concentration (S1) on Giza 35 genotype resulted in non-significant increase in *CAT* gene expression compared to the higher salt concentration (S2) and the control treatment (Fig. 5b). Similarly, it was observed that the lower salt concentration (S1) resulted in higher *APX* gene expression compared to the control and the higher salt concentration (S2) (Figs. 5 and 6; d). While in Giza 111 genotype, both salt concentrations (S1 and S2) resulted in significant increase in *CAT* gene expression compared to the control treatment (Fig. 6C).

Soybean's stress-sensitive nature emphasizes the importance and roles of DHNs genes as significant contributors to protect plants against environmental stresses [52]. It was reported that DHNs play an important role in protecting plants against different stresses such as salinity. The DHNs also act as ROS scavenger [53]. It has also been reported that DHNs also act as transcription co-regulators for abiotic stress signals as well as their role as molecular chaperons that prevent proteins misfolding under different stresses [54].

Moreover, it has been reported that dehydrins are expressed in abundance in tolerant soybean varieties than in both moderate and sensitive varieties under drought stress conditions [55]. On other hand, the 45 °C heat pre-treatment of soybean seeds had a positive effect, as it significantly up-regulated the expression of *DHN2* and *APX* gene while it affected negatively on the transcript amount of *CAT* gene in Giza 111 genotype (Fig. 6; B, C and D).

Similarly, the down regulation of stress-tolerance responsive marker genes in both genotypes under salt concentrations in this study was recovered following the nanoparticles treatment (N1 and N2). Such result suggests that plant response to protective treatments is genotype dependent.

Previous reports suggested that changes in the expressions profiles of antioxidant encoding genes in soybean seemed to be genotype dependent. El-Esawi et al., reported that inducing of the antioxidant enzymes encoding genes (*APX* and *CAT*) occurred under high level of salinity in soybean plants [48]. The expression profile of *APX* and *CAT* genes varied in different cultivars at different salinity concentrations, where the high *CAT* and *APX* genes expression was observed in tomato cultivar PS-10 under salinity stress, while Roma cultivar displayed low expression for the same genes [56]. Similarly, increased activities of *CAT*, and *APX* in soybean cultivars PI31, PI37, PI90, and PI5A under salinity treatments were reported [36].

The expression profile of *CAB3* gene whose product is localized at the chloroplast membrane and play a role in regulating the plant photosynthesis activity is shown in (Figs. 5A and 6A). Treatment of both studied soybean genotype with the two different salinity concentrations (S1 and S2) induced significant gradual down regulation in *CAB3* gene expression. In agreement with our finding, it was previously reported that salinity stress down regulate *CAB3* in tomato plants [57].

On the other hand, we observed that the combine application of the 45 °C heat pre-treatment and nanosilica on the soybean plants had a positive effect on the expression profile of *CAB3* gene. The observed down regulation of *CAB3* in (S1 and S2) salt-stress was recovered by both nanosilica and nanoselenium treatments (S1N2 and S2N1).

The protective effects of silicon and selenium on photosynthetic processes against salinity-induced damage were reported in rice plants as well as other crops [58,59]. From our results this protection role could be explained as a result of stimulation effect of these elements on *CAB3* genes which play an important role in photosynthesis process. Recovery effects of silicon nanoparticles treatment on salt-stressed strawberry plants were also well-documented improving salinity tolerance by decreasing ROS content and maintaining the expression level of *APX* gene [20].

Under salinity stress, water uptake is greatly altered making it difficult for plants to absorb or utilize available nutrients [60,61].

Salinity stress also affects the movement of water across the plasma membrane. Plasma membrane intrinsic proteins (PIPs) are localized to the plasma membrane and regulate the water and solutes flow. Given their importance, the changes in the expression levels of *GmPIPL6* gene were analyzed as important salt-stress responsive marker gene [62]. Similarly, *Glycine* max salt tolerance-associated gene on chromosome 3 (*GmSALT3*) expression profiles were also studied as it was described as one of the dominant genes associated

with salt tolerance in soybean [63]. This gene confers net shoot exclusion for both Na^+ and Cl^- and improves salt tolerance of soybean plants [64]. In this study, treating selected soybean genotypes with the two levels of salinity (S1 and S2) induced a gradual significant increase in the transcript amount of *GmPIPL6* in Giza35 genotype (Fig. 5E). Similarly, expression levels for *GmSALT3* were gradually and significantly increased in response to the two levels of salinity in both soybean genotypes (Figs. 5E and 6E). It was also observed that gene expression profiles were different in both studied soybean genotypes in response to the 45 °C heat pretreatment. It was observed that while this treatment had either no effect on *GmPIP6* expression (Fig. 5E) or decreased *GmSALT3* expression in Giza 35 genotype (Fig. 5F), the other studied genotype (Giza 111) responded differently by up-regulating the expression of the same genes (Fig. 6; E, F).

The transcription of *GmPIPL6* and *GmSALT3* was up-regulated under both tested levels of salinity treated with both nanoparticles compared to the non-treated plants (S1 and S2) (Figs. 5 and 6; E,F).

Liu et al. [64], evaluated the impact of *GmSALT3* on soybean performance under saline or non-saline conditions. *GmSALT3*'s function was investigated by Qu et al. [65], in heterologous systems and near isogenic lines that contained the full-length GmSALT3 (NIL-T; salt-tolerant) or a truncated transcript *GmSALT3* (NIL-S; salt-sensitive) in soybean. The findings provide new insights on *GmSALT3*'s impact on salinity tolerance and reveal a novel mechanism for shoot Cl^- exclusion in plants.

Considering previously reported findings as well as our results, we report/confirm the protective potential of tested nanoparticles and/or seeds heat pretreatment on soybean salt-stressed plants. Recovery effects of applied treatments for membrane properties and MDA content pointed to the role of nanosilica and nanoselenium in reducing the oxidative load induced by salinity stress. Observed changes in gene expression profiles for the studied salt stress-related genes (*CAT, APX* and *DHN2*) confirmed the protective role of these treatments in the stimulation of ROS scavenging and preventing proteins miss folding leading to alleviation of the negative effects of salinity stress. Reporting the stimulation effects of nanosilica and nanoselenium for the expression of *GmPIPL6* and *GmSALT3* genes specialize their role in improving water uptake and Cl^- exclusion under salinity stress.

4. Conclusion

In summary, our results demonstrated the negative effects of two levels of salinity in irrigation water on three Egyptian commercial soybean genotypes (Giza 35, Carford and Giza 111). Irrigation with saline water resulted in significant reduction of germination rates and significant increase in MDA values and the rate of electrolytes leakage indicating structural or functional membranes' damage. DNA fragmentation was analyzed through comet assay analysis and demonstrated the severe negative burden of salinity on soybean plants. Moreover, irrigation with two different concentrations of saline water resulted in significant reduction in mitotic index, arrest in metaphase and to an increase in chromosomal abnormalities as well as reduction in gene expression of some studied salt-stress related genes. The obtained results suggest that the 45 °C pre-treatment for soybean seeds appeared as a positive treatment to improve plant growth of unstressed and stressed plants. Application of both tested nanoparticle (nanosilica and nanoselenium) have proven to be very effective against salinity negative effects on soybean plants. Associated improvement in membrane stability as well as upregulation the expression of some studied genes pointed to the stimulation role of nanosilica, nanoselenium and the 45 °C pretreatment for salt tolerance in stressed plants. The protective functions of studied treatments through recovering the adverse effects of salinity on soybean plants provide a promising strategy for alleviating salt stress in soybean genotypes. Our results pointed to the role of nanoparticles in modulating the expression pattern of some stress related genes which could help in understanding their protection mechanisms for plants under oxidative conditions.

Data availability statement

Data will be made available on request.

CRediT authorship contribution statement

Samar Omar: Formal analysis, Data curation, Conceptualization. **Hagar Salim:** Software, Methodology, Formal analysis. **Medhat Eldenary:** Writing – original draft, Methodology, Investigation, Formal analysis, Data curation. **Alexander V. Nosov:** Writing – original draft, Visualization, Software, Investigation, Data curation. **Suleyman I. Allakhverdiev:** Writing – review & editing, Visualization, Project administration, Funding acquisition. **Alsayed Alfiky:** Resources, Methodology, Investigation.

Declaration of competing interest

The authors declare no competing financial interests.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2023.e21446.

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