Heliyon 10 (2024) e30934

Contents lists available at ScienceDirect

Heliyon

journal homepage: www.cell.com/heliyon

Research article

Exogenous application of 5-azacitidin, royal jelly and folic acid regulate plant redox state, expression level of DNA methyltransferases and alleviate adverse effects of salinity stress on *Vicia faba* L. plants

Samar A. Omar[a,**], Yingming Feng[b], Min Yu[b], Samar A. Gamal. Eldin[a], Medhat E. Eldenary[a], Sergey Shabala[b,c,***], Suleyman I. Allakhverdiev[d,e,*], Mohamed H. Abdelfattah[a]

[a] Department of Genetics, Tanta University, Egypt
[b] International Research Centre for Environmental Membrane Biology & Department of Horticulture, Foshan University, Foshan, China
[c] School of Biological Science, University of Western Australia, Crawley, Australia
[d] K.A. Timiryazev Institute of Plant Physiology, Russian Academy of Sciences, Botanicheskaya Street 35, Moscow, 127276, Russia
[e] Bahcesehir University, Istanbul, 34353, Turkey

ARTICLE INFO

Keywords:
Antioxidant enzymes
Chromosomal abnormalities
DNA methyltransferases
Gene expression
GPRP
HSP-17.9
Mitotic index

ABSTRACT

DNA methylation is one of induced changes under salinity stress causing reduction in the expression of several crucial genes required for normal plant's operation. Potential use of royal jelly (RJ), folic acid (FA) and 5-azacitidine (5-AZA) on two Egyptian faba bean varieties (Sakha-3 and Giza-716) grown under saline conditions was investigated. Salinity stress affects negatively on seeds germination (G %), mitotic index, membrane stability and induced a significant increase in chromosomal abnormalities (CAs). DNA methyltransferases genes (*MT1* and *MT2*) were highly up-regulated (~23 and 8 folds for *MT1* and *MT2* in shoots of Giza-716 stressed plants). On the other hand, down regulation of other studied stress related genes: superoxide dismutase (*SOD*), catalase (*CAT*), glutathione reductase (*GR*), heat shock protein (*HSP-17.9*) and proline-rich protein (*GPRP*) were detected in stressed plants of both studied varieties. Treating plants with RJ and FA increase G%, chlorophyll content, improves membrane properties and reduces CAs compared to non-treated stressed plants. Exogenous application of 5-AZA, RJ and FA on salinity stressed plants was associated with a significant reduction in the transcription of *MT1* and *MT2* which was associated with significant up regulation in the expression of *Cu/Zn-SOD*, *CAT*, *GR*, *GPRP* and *HSP-17.9* encoding genes. The Lowest expression of *MT1* and *MT2* were induced with 5-AZA treatment in both studied varieties. Exogenous application of the FA, RJ and 5-AZA modified the methylation state of stressed plants by regulation the expression of DNA

* Corresponding author. K.A. Timiryazev Institute of Plant Physiology, Russian Academy of Sciences, Botanicheskaya Street 35, Moscow, 127276, Russia.
** Corresponding author.
*** Corresponding author. International Research Centre for Environmental Membrane Biology & Department of Horticulture, Foshan University, Foshan, China.
E-mail addresses: samar_omar5@agr.tanta.edu.eg (S.A. Omar), suleyman.allakhverdiev@gmail.com (S. Shabala), Sergey.Shabala@uwa.edu.au (S.I. Allakhverdiev).

https://doi.org/10.1016/j.heliyon.2024.e30934
Received 12 January 2024; Received in revised form 5 May 2024; Accepted 8 May 2024
Available online 11 May 2024

methyltransferases, subsequently, modulated the expression of studied genes and could be proposed as a promising treatment to ameliorate hazardous effects of salt stress on different plants.

1. Introduction

Salinity stress is one of main abiotic stresses that limit crop production and yield quality [1,2]. About 20 % of global cultivated land is affected by salinity [3], which effect negatively on food security. Elevated salt levels in the soil have toxic effects on plants and lead to various metabolic alterations affecting chloroplast activity, decreasing photosynthetic rate and increasing photorespiration rate. This leads to proliferation the oxidative stress and magnification the production of reactive oxygen species (ROS) causing a subsequent loss of membrane permeability, proteins integrity [4,5] and effect negatively on plant performance. Stimulation ROS scavenging and antioxidant enzyme activities such as superoxide dismutase (SOD), catalase (CAT), glutathione reductase (GR) could support plants to combat the hazardous effects induced under salinity condition [2,6]. *Vicia faba* is recognized worldwide as main source of protein and essential nutrients in the majority of developing countries and considered as an important crop in the animal feed market [7]. Salinity stress effects negatively on *V. faba* plants where it causing a substantial reduction in germination rate, growth, nodule formation, nitrogen fixation capabilities and the final yield [8–10].

Hazardous effects of salinity as well as other oxidative stresses are often associated with reduction or even suppression the expression of several key genes essential for growth and stress responses [2,6,11]. DNA methylation process protect genomes against sever factors and their level is affected by changes the biotic and abiotic conditions [12] Although changes in plant transcriptome induced by changes in DNA methylation, mediated by stress, could help plants to survive under stress, it comes with yield penalties, where it causes silencing of several genes involved in different metabolic activities [7,13,14]. Methyltransferases, a group of enzymes which are responsible for the handover of a methyl group (CH_3) from different methyl donor to cytosine residues in DNA, are the main tool to induce DNA methylation [12]. A moderate activity of methyltransferases is required for better adaptation of different stresses [15,16]. Studies on reduction of methylated signs induced in stressed plants showed a significant recovery for plants under oxidative stresses [16].

Recently, DNA methylation inhibitors, 5-azacitidine (5-AZA), as well as some plant regulators such as folic acid (FA) and Royal jelly (RJ), were used to overcome detrimental effects of oxidative stresses on several plant species [17–19]. Although the beneficial role of consumption of FA, RJ and 5-AZA effect of folic have been widely studied in humans and animals [20–23], their effects are rarely demonstrated on most plant species under numerous stress conditions. Their association role with regulation of methyltransferases and other growth and protection related genes have received very little attention.5-azacytidine is analogous to cytosine that is used as popular tool for research in the function of DNA methylation as well as their exogenous application for recovering hazardous effects of oxidative stress [20,24]. 5-AZA treatment activates some transcription factors and structural genes elaborated in licochalcone A biosynthesis in *Glycyrrhiza inflata* seedlings [24,25]. Folic acid is a necessary vitamin that catalyzes essential biochemical reactions such as metabolism of nucleotides for DNA synthesis and methylation processes with antioxidant properties [26]. Significant increase in growth parameters and an alternation in the expression of stress associated genes were recorded in snap bean, *Pisum sativum*, *Hordeum vulgare* and flax Plants treated with FA [27–29]. Royal jelly which is released from glands on the worker bees' heads as queens' diet, has high nutritional and antioxidant properties [30] and play the main role in differential variations between queen and workers in bees (*Apis millifera*). These differential variations are accomplished by epigenetic regulation as DNA methylation of different diet [22].

Application of such novel, promising treatments can be supported by being aware of the processes underlying plant response to the oxidative effects of salinity. Accordingly, this study aimed to investigate effects of exogenous application of RJ, FA and 5-AZA on *V. faba* L. plants as a promising strategy to eliminate hazardous effects of salinity. This investigation demonstrated the cytological and molecular responses as well as changes in the transcription levels of two DNA methyltransferases encoding genes (*MT1, MT2*), antioxidant enzyme encoding genes (*SOD, CAT, GR*) and some stress and growth associated genes [Heat shock protein (*HSP-19.7*) and Glycine-proline rich protein (*GPRP*)] associated with exogenous application of RJ, FA and 5-AZA on *V. faba* L. plants grown under salinity conditions.

2. Materials and methods

2.1. Plant materials

Seeds of Egyptian commercial *V. faba* L. varieties (Sakha-3 and Giza-716) were got from the Agricultural Research Centre, Kafr El-sheikh, Egypt. The experiment was carried out at Tanta University's Faculty of Agriculture farm during the winter season of 2020/2021 in plastic pots (20 cm radius × 30 cm height) filled with 5 Kg soil. About 10 seeds of tested varieties were placed in pots with 3 replicates for each treatment under field condition. Average day light was 10h and 22min, average high temperature was 19 °C, average low temperature was10 °C and average humidity was 8 %.

2.2. Salinity stress

Salinity stress was induced by overnight soaking of seeds in 100 mM NaCl before planting the seed in pots then regular irrigation was carried out with 100 mM NaCl solution when required.

2.3. Treatments

Three different materials, Royal jelly (RJ), Folic acid (FA) and 5-azacytidine (5-AZA) were examined for their ameliorative role against salinity stress on *V. faba* L. plants. Commercial natural fresh RJ (IMTENAN, Egypt) was used at concentration of 100 mg/L. Folic acid (EIPICO, $10^{th}$of Ramadan, Egypt) was applied in concentration of 25 mg/L. Also, 5-azacytidine (Sigma: A2385, Canda) treatment was carried out at concentration of 50 mM. Prepared solution of each material was used for overnight soaking of seeds before planting as well as regular irrigation separately or in combination with salinity treatment. Tap water was used as control treatment. The experiment was divided into eight groups of three replicates each, as shown in Table 1. Samples were collected 30 days after planting and stored at −80 °C for further measurements.

2.4. Rate of seed germination

Germination percentages of seeds were examined in Petri dishes experiment. Seeds from tested varieties were pre-soaked with distilled water in dark for 12h at room temperature before soaking with different experimental treatments. Soaked seeds were kept at 25 °C in the incubator (POL-EKO-APARATURA SP.J, Wodzisław Śląsk, Poland). Seeds with emerged radicles of more than 2 mm long were counted as germinated.

2.5. Membrane stability

Lipid peroxidation and membrane leakage rates were used to assess the membrane stability. Lipid peroxidation were detected as the concentration of thiobarbituric acid (TBA) reactive products equated with malondialdehyde (MDA) as described in Anjum et al., method [31] with slight modification. Leaf tissue (about 0.5 g) was ground in five mL of 5 % (w/v) trichloroacetic acid (TCA) and centrifuged for 10 min at 4000 rpm and 4 °C. To create the chromogen, two mL of supernatant was combined with three mL of reaction mixture that contained 0.5 % (w/v) 2-thiobarbituric acid (TBA) and 20 % (w/v) TCA. The mixture was quickly cooled in an ice –water bath after being heated to 100 °C for 15 min. The mixture was centrifuged for 10 min at 4000 rpm and 4 °C [6]. A spectrophotometer (UV1901PC) was used to detect MDA content and the following equation was applied calculating.

$$MDA = [(Abs\ 532 - Abs\ 600) - 0.0571 \times (Abs\ 450 - Abs\ 600)]/0.155.$$

The electrolyte leakage (EL) rate of leaves was determined by dividing net electrical conductivity of the solution with leaf discs soaked for 1h by the total electrical conductivity after boiling as reported by Ref. [2] and the results was given in in $\mu S\ cm^{-1}\ mg^{-1}$ FW h^{-1}.

2.6. Total chlorophyll and carotenoids contents

Photosynthetic pigments (total chlorophyll and carotenoids) were extracted from 0.1 g of fresh leaves immerged in 2 mL of 80 % acetone for 72 h at 4C° then determined by spectrophotometer (UV1901PC, Shanghai, China) to detect their absorbance at 645, 663, and 470 nm [32].

2.7. Total soluble proteins

Half g of leaf tissues was ground using liquid nitrogen and suspended 1.5 mL of extraction buffer to extract total soluble proteins according to Ref. [33]. Bradford analysis was used to detect protein concentration using Bovine serum albumin (BSA) as standard protein [34]. Protein profile alterations were investigated using SDS-polyacrylamide gel electrophoreses [35]. Depending on protein concentration, the amount of protein in the samples was standardized (15 µg of proteins were added/lane). Separated bands were detected using pre-stained protein Ladder (GeneDirex, Cat.No. PM008-0500). Banding patterns were analyzed using gel analysis software (GelAnalyzer2010a, available at www.gelanalyzer.com).

Table 1
Experimental treatments.

Group	Code	Solution of Treatment
1	Control	tap water without NaCl
2	FA	25 mg/L FA
3	RJ	100 mg/L of RJ
4	5-AZA	50 mM 5-AZA
5	100 mM NaCl	100 mM NaCl
6	FA+100 mM NaCl	25 mg/L FA+ 100 mM NaCl
7	RJ+100 mM NaCl	100 mg/L of RJ+100 mM NaCl
8	5-AZA+100 mM NaCl	50 mM 5-AZA +100 mM NaCl

2.8. Cytological analysis

For cytological investigation, root tips (1–1.5 cm) of 3 days-old, germinated seeds in solutions of various treatments were utilized. After being fixed for 24 h in Carnoy's solution, root tips were kept at 4 °C in 70 % ethyl alcohol until slide processing. Slide staining was done using aceto-carmineaccording Kihlman method [36]. Light microscope (PT/Slope, Pearl, England) was used for examining prepared slides. Chromosomal aberrations (CAs) and all mitotic phases have been recorded in at least 3000 assessed cells/treatment. Following formulas were used in calculation of chromosomal abnormalities percentage and mitotic index (MI) based on obtained results.

$$\text{Mitotic index (MI)} = \frac{\text{Total dividing cells}}{\text{Total dividing and non dividing cells}} \times 100$$

$$\text{Percentage of abnormal cells} = \frac{\text{Total abnormal cells}}{\text{Total dividing cells}} \times 100$$

2.9. Analysis of gene expression

Changes in level of gene expression were determined using real-time PCR (RT-qPCR) analysis. Frozen leaves stored at −80 °C were used for total RNA extraction from seedlings under control and other selected experimental treatments using RNA Mini-Preps Kit (EZ-10 Spin Column -BIO BASIC CANADA INC, Ontario, Canada). Agarose gel electrophoresis (1.0 %) was used to check the integrity of extracted RNA. RNA purity and concentration were measured using a Nano drop spectrophotometer (BioDrop ULite, Wales, and England). Samples with purity of 1.8 or more were considered acceptable for gene expression analysis. cDNA was synthesis of 1 μg DNase free total RNA in 20 μL reaction mix using HiSenScript^{TM}RH cDNA synthesis Kit (iNTRON Biotechnology, Cat. No: 25087) using Oligo (dT^{18}) primer. Quantitative evaluation of gene expression was performed using low rox SYBR Green stain (5xHOT FIREPoL ®EvaGreen® qPCRSuperix) in a reaction volume of 20 μL. The Step One Plus™ Real Time PCR system (Applied Biosystem™ 4376600) was used to conduct all reactions. Transcript level of *Actin* gene (AC: JX444700.1) was employed as an internal control. Variflex option was applied to provide different annealing temperature on the same plate for different genes. The reaction was carried out using 3 biological replicates for each treatment with specific primers designed for all studied genes (Table S1). Relative gene expression (RQ) was represented after calibration with values of reference gene and control treatment as $2^{-\Delta\Delta ct}$ [37].

2.10. Statistical analysis

Throughout the investigation, the experimental architecture was a full factorial split-plot design with three biological replicates per treatment, arranged in randomized complete blocks. The analysis of variance (ANOVA) was used to statistically assess all the data, and Tukey's honestly significant difference (HSD) test was used as a post-hoc analysis to compare the means ($p \leq 0.05$). Using JMP Data

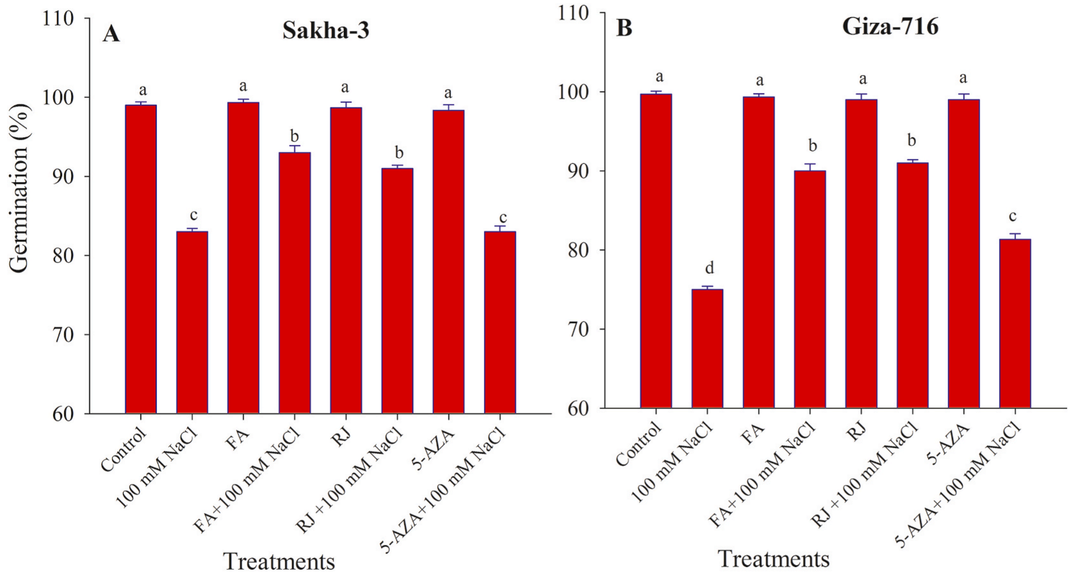

Fig. 1. Changes in germination % of two studied varieties (Sakha-3 and Giza-716) under all studied treatments. Control: tap water; FA: 25 mg/L folic acid, RJ: 100 mg/L Royal Jelly, 5-AZA: 50 mM 5-azacytidine, 100 mM: 100 mM NaCl, FA+100: 25 mg/L folic acid+100 mM NaCl, RJ+100: 100 mg/L royal jelly+100 mM NaCl, 5-AZA+100 mM: 50 mM 5-azacytidine+ 100 mM NaCl. Values that have distinct lettering are statistically significant at $p < 0.05$ when subjected to a two-way ANOVA and Duncan test analysis.

analysis software Version 15, an ANOVA and Tukey's test were performed.

3. Results and discussion

3.1. Germination (%)

Salinity stress induced a significant reduction in plant growth (Fig. 1S) in both studied variety and it also affects germination percentage (G %) in both studied varieties (Fig. 1). Germination % of Giza-716 (Fig. 1A) was more affected than Sakha-3's (Fig. 1B). Osmotic stress brought on by the presence of ions could explain salt's ability to prevent or slowing down seed germination [38] due to less water moving into the seeds during imbibition [39]. Also, the excessive levels of NaCl induces nutritional imbalances, reduction in germination rate and plant development because of their role in disturbing ionic balance of the cells [40,41]. Treatments with FA, RJ and 5-AZA did not induce any changes in germination % of unstressed plants. Under salinity stress, all tested chemicals (FA, RJ and 5-AZA) induced a significant increase in G % for both studied varieties except for Sakha-3 treated with 5-AZA where induced changes being non-significant (Fig. 1A).

3.2. Photosynthetic pigments and membrane stability

Salinity stress caused a significant decrease in the total chlorophyll content. This decrease was non-significant for carotenoids contents in both studied varieties (Fig. 2: A, B). Salt stress inhibits photosynthesis by lowering the amount of leaf photosynthetic

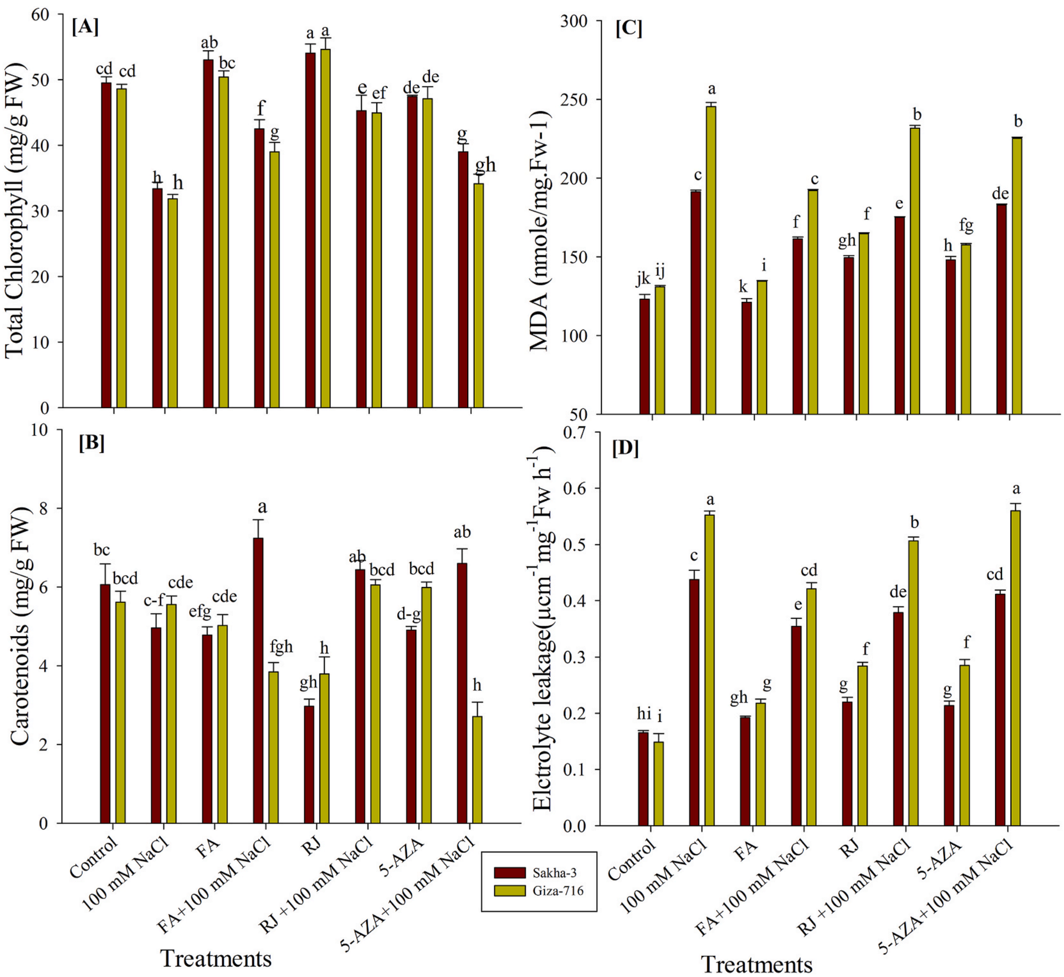

Fig. 2. Changes in photosynthetic pigments and membrane stability. Changes in total chlorophyll [A], carotenoid [B], leakage rate [C] and MDA content [D] of two studied varieties (Sakha-3 and Giza-716) under all experimental conditions. Control: tap water; FA: 25 mg/L folic acid, RJ: 100 mg/L Royal Jelly, 5-AZA: 50 mM 5-azacytidine, 100 mM: 100 mM NaCl, FA+100: 25 mg/L folic acid+100 mM NaCl, RJ+100: 100 mg/L royal jelly+100 mM NaCl, 5-AZA+100 mM: 50 mM 5-azacytidine+100 mM NaCl. Values that have distinct lettering are statistically significant at $p < 0.05$ when subjected to a two-way ANOVA and Duncan test analysis.

pigments, where chloroplasts exposed to high excitation energies due to salt stress, subsequently boosts the generation of ROS [42]. A significant increase in total chlorophyll of both studied varieties was recorded in unstressed and stressed plants treated with FA and RJ (Fig. 2A). These increases in chlorophyll contents were associated with reduction in carotenoids contents in unstressed and stressed plants (Fig. 2B) except for FA and RJ treatments with stressed Sakha-3 variety. Treatments with 5-AZA showed non-significant reduction in total chlorophyll in treated plants compared to control. Under salinity condition treatment with 5-AZA showed non-significant decrease in chlorophyll content under control and stress condition except for Sakha-3 plants under stress (Fig. 2A).

Effect of RJ and 5-AZA on carotenoids content was variety-dependent. A significant increase in chlorophyll content in stressed Giza-716 plants under RJ and FA treatment was accompanied with a significant decrease in carotenoids content. This correlation was previously documented in several plants [43]. Protective effects of tested treatment on either chlorophyll or carotenoids are consistent with mitigating role of these materials against oxidative stress. It is well recognized that carotenoids function as antioxidants in plants [44,45].

Effect of different experimental condition on membrane stability was determined based on induced modification in values of both EL and MDA. Salinity stress induced significant increases in EL (Fig. 2C) and MDA (Fig. 2D) in both studied varieties. Giza-716 variety showed higher values of El and MDA than Sakha-3 which indicate to its sensitivity to salinity stress. Plasma membranes are the main location of ion-specific salt damage [46]. Therefore, leakage rate of plasma membranes is considered as crucial selection criteria in recognizing salt-tolerant plants [47,48]. Combined treatment of FA, RJ and 5-AZA with salinity caused a reduction in EL and MDA

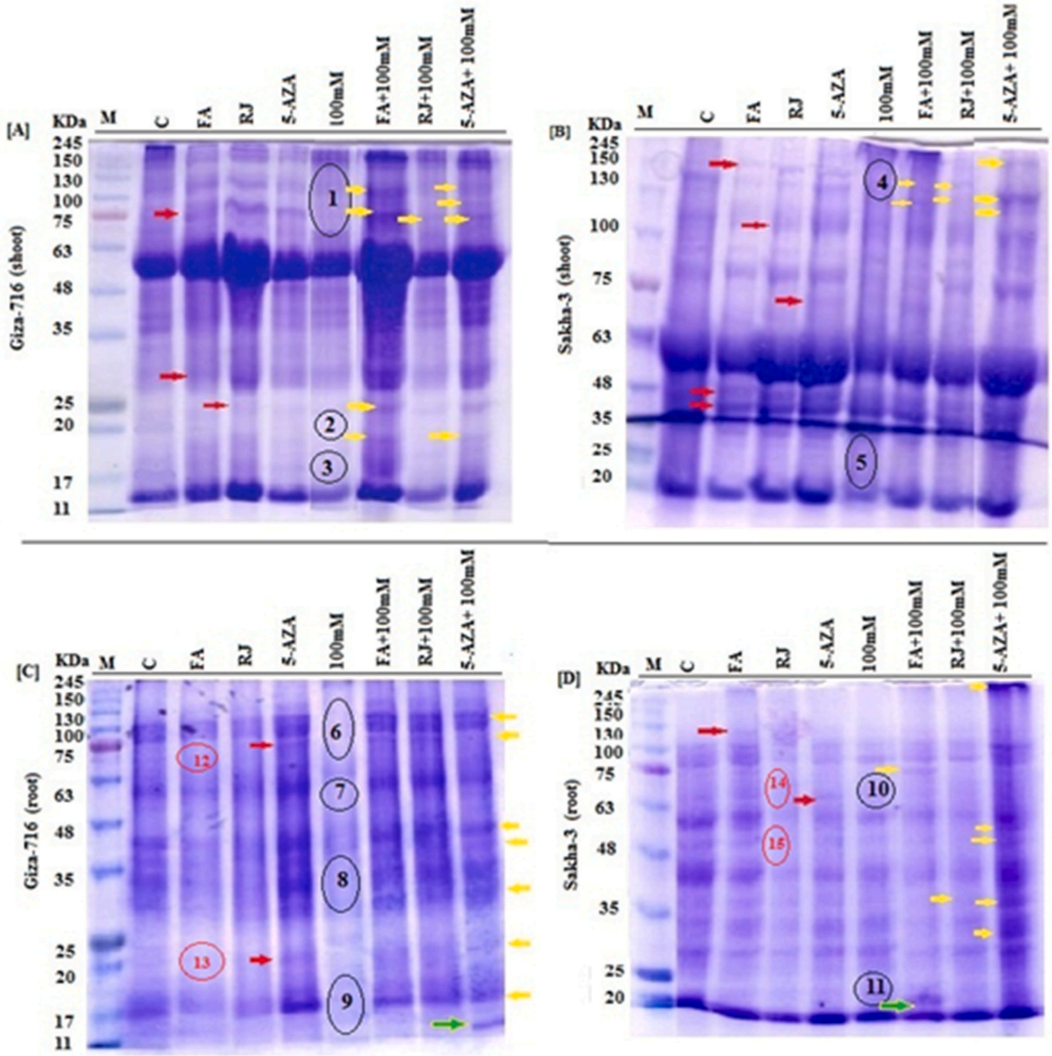

Fig. 3. SDS–PAGE of total soluble proteins separated bands extracted from shoots [A] and roots [C] of Giza-716, as well as shoots [B] and roots [D] of Sakha-3. Circled zones indicate to the lost bands. Red arrows indicate to the induced bands. Yellow arrows indicate bands that were recovered in stressed plants. Green arrows indicate uniquely induced bands. C: control irrigated with (tap water); FA (folic acid: 50 mM); RJ (Royal Jelly: 100 mg/L); 5-AZA (5-azacytidine 50 mM); 100 mM: 100 mM NaCl; FA+100 (25 mg/L folic acid+100 mM NaCl); RJ+100 (100 mg/L royal jelly+100 mM NaCl), 5-AZA+100 mM (50 mM 5-azacytidine+ 100 mM NaCl).

values comparing with untreated plants except for value of EL for Giza-716 treated with 5-AZA under stress. The recovery effect (Fig. 2S) of RJ could be explained due to the potential role of its components, such as flavonoids and phenolic, as a radical scavenger [49,50]. Besides the antioxidant properties of RJ, it may also play a role in suppressing the enzymes that initiate the peroxidation of endogenous lipids and the expression of cytochrome *P450* gene which consider as intracellular source of some free radicals such as H_2O_2, $O_2^{\bullet}$ and $HO^{\bullet}$ radicals [51]. Recovery effect of folic acid on stressed plants could be explained by increased activity of CAT and APX H_2O_2 scavenging enzymes [52]. Inhibition of DNA-methylation by 5-AZA increased plant viability and adaptation to several abiotic stresses [52].

3.3. Analysis of total soluble proteins

Electrophoretic analysis using SDS-PAGE of total soluble proteins fraction extracted from shoots and roots revealed that under salt treatment some of protein bands were disappeared (circled zones no. 1 to 11) in both shoots (Fig. 3; A, B) and roots (Fig. 3; C,D), regardless of molecular weight. Changes in protein patterns under salinity stress could be explained as a result of the suppression or alteration the expression of some genes in plants during acclimation process [53,54]. Treatment with FA, RJ and 5-AZA induced expression of some new separated bands in both varieties, indicated with red arrows, (Fig. 3; A, B). It also caused loosing for some bands where, bands in circle no. 12 and 13 were disappeared in Giza-716 roots under FA treatment (Fig. 3C). Also, bands in circles no 14 and 15 were disappeared in roots of Sakha-3 treated with RJ (Fig. 3 D). Treatment of stressed plants with FA, RJ and 5-AZA caused a recovery for most of lost bands under salinity treatment, indicated with yellow arrows, (Fig. 3; A, B, C, D). It also induced some new bands, where treatment stressed plants of Giza 716 with 5-AZA induced expression of new band in roots with molecular weight about 12 KDa (indicated with a green arrow) (Fig. 3C). Also, another band with molecular weight about 15 KDa (indicated with a green arrow) was induced in roots of stressed Sakha-3 plants treated with FA (Fig. 3 D). Considering the improvement of plant performance under these treatments, these newly induced bands could be associated with acquisition of salinity tolerance and could be used for further analysis to detect their encoding genes.

Table 2 summarizes the changes in patterns of separates bands and the percentage of lost and induced bands under different treatment compared with control treatment. In Sakha-3 shoots', total number of separated bands was increased by 12.5 % under salinity stress. In Sakha-3′ roots, the total number of separated bands decreased by 9 % comparing with control treatment. In Giza-716, about 40 % and 57.2 % of total separated bands in shoots and roots respectively, were lost under salt treatment compared to control. These results could explain the higher sensitivity of Giza-716 variety to salt comparing with Sakha-3 as it lost higher number of protein bands under salinity stress. Percentage of new induced bands under recovery treatment for stressed plants ranged from 9 % (with RJ treatment to Sakha-3 root) to 42 % (with 5-AZA treatment to Giza-716 roots).

Application of FA exogenously improves plant growth and metabolism [55], where it is considered as a cofactor for many enzymes, especially those elaborated in synthesis of cell wall and proline residues hydroxylation [56,57]. Treatment of potato with FA induced a significant improvement in both of total soluble proteins and carbohydrates [58]. As genetic programs of plants could be altered by treatments with 5-AZA which led to inhibition of DNA methylation and control of gene expression [13], they could induce expression of new protein bands [59].

3.4. Cytological analyses

Effect of salinity and different experimental treatments on chromosomal abnormalities (CA) and mitotic index (MI) in *V. faba* was performed using cytological investigation (Table 3). Salinity stress caused a significant inhibition for mitotic division. The MI was reduced in Sakha-3 from 22 % under control treatment to 13 % at 100 mM NaCl treatment and reduced in Giza-716 from 24.8 under control treatment to 12 % under 100 mM NaCl treatment. High level of salinity induced a significant reduction in the MI of *Hordeum*

Table 2

Changes (% of control) in total numbers of separated bands for soluble proteins.

Variety Tissue		Control	FA	RJ	5-AZA	100 mM NaCl	FA+100 mM NaCl	RJ+100 mM NaCl	5-AZA+100 mM NaCl
Sakha-3 Shoot	Total number of bands	8	12	12	13	9	13	12	13
	% of changes	–	+50	+50	+62.5	+12.5	+62.5	+50	+62.5
Sakha-3 Root	Total number of bands	11	12	9	12	10	12	10	12
	% of changes	–	9	−18	27	−9	+9	−9	+9
Giza-716 Shoot	Total number of bands	10	12	13	13	8	13	13	14
	% of changes	–	+20	+30	+30	−40	+30	+30	+40
Giza-716 Root	Total number of bands	7	7	7	9	3	9	9	10
	% of changes	–	–	–	+28	−57.2	+28.5	+28.5	+42.8

- Control: tap water; FA: 25 mg/L folic acid, RJ: 100 mg/L Royal Jelly, 5-AZA: 50 mM 5-azacytidine, 100 mM: 100 mM NaCl, FA+100: 25 mg/L folic acid+100 mM NaCl, RJ+100: 100 mg/L royal jelly+100 mM NaCl, 5-AZA+100 mM: 50 mM 5-azacytidine+ 100 mM NaCl. (−/+) indicates a loss or increase in band numbers.

vulgare [60]. High salt concentrations caused total totally suppression of mitotic division of *in vitro* cultured cells of *Centaurea ragusina* [61]. reduction in DNA, RNA and protein synthesis under salinity condition as well as increased lipid peroxidation and cellular membrane damage could induce inhibition for cell division [62]. A disruption in the progression from the G2 phase of the mitotic cycle to mitosis is shown by a decrease in the prophase percentage under salt stress [63]. Under salt stress, metaphase arrest was rarely documented in root apical meristem cells [6].

Treatment of non-stressed plants with 5-AZA recorded a highest MI value followed by RJ treatment with significant increase comparing to control treatment for both studied varieties. At the same time, FA treatment showed a significant reduction in mitotic index compared with control for Giza-716 and a non-significant decrease for Sakha-3 (Table 3). Under salinity stress, RJ treatments showed significant increase in MI for both varieties compared to non-treated plants, while 5-AZA treatment showed non-significant increase in MI for both varieties. However, when compared to untreated plants, FA treatment resulted in a non-significant rise in MI for Sakha-3 and a substantial increase for Giza-716 (Table 3).

Different types of CAs were observed in dividing cells of root apical meristem for each treatment (Fig. 4; A-R), total number and percentage of every type of abnormalities were recorded (Table 4). The number of induced CAs differed under different experimental conditions. The majority of CAs was caused by salinity stress where the most recorded CAs were bridges, C-metaphase and laggard chromosomes (Fig. 4; B, H, M). The induced aberrations under salinity were similar to colchicine type action. C-metaphase abnormality caused by inhibition of spindle fiber formation and causes a metaphase arrest, which has an effect similar to colchicine [45]. The disruption of the spindle apparatus may be responsible for the irregular spreading of chromosomes [64]. Laggards could be caused due to abnormal organization of spindle fiber [65]. Chromosome segment inversions could create the chromosome bridges during anaphase and telophase, or it may be caused by chromosomal stickiness and inability of free anaphase separation due to unequal translocation [66]. Micronuclei most eventually create from stray chromosomes and fragments [66]. The percentage of CA under salinity treatment in Giza 716 (29.26 %) was higher than its value in Sakha-3 (18.30) (Table 4).

Treatment with FA, RJ or 5-AZA on stressed plants alleviated salinity stress damage and caused significant reduction in CA % comparing with untreated stressed plants for both varieties, except for Sakha-3 with RJ treatment which showed non-significant reduction in CA % comparing with untreated stressed plants (Table 4). Increasing CA% under RJ treatment was reported as a result of its stimulation effect and it was directly proportional to the increase in MI% (Table 3). Using of convenient doses of RJ may assist in reducing the hazardous effects of salinity stress [30]. Considering properties of RJ components which provide stabilization of cell membranes and raise antioxidant activities could elucidate the increase in cell division, growth, nucleic acid and protein synthesis and support its recovery role and reduction of CAs on stressed plants [49,67].

Obtained results demonstrated that FA has the protective role against salt damage during mitosis division by decreasing the inhibitive effect of salt stress on MI. Folic acid treatment reduced detrimental effects of salinity stress by reducing bridges aberration (partially in Sakha-3, and almost completely in Giza-716 variety). Folic acid binding to essential elements and increasing their absorption which stimulates cell division and increases growth and differentiation during irrigation with saline water [68]. It also has the potential to function as natural antioxidants and growth regulators where it was reported that applying of FA at concentration of 50 mM was very operative in eliminate the harmful effects of salinity (100 mM) in relation to CAs [69]. Treatment of 5-AZA also induced a reduction in laggard and bridge aberrations in both varieties, being most in Giza-716 variety (Table 4).

Table (3)
Changes in the mitotic index in two varieties of faba bean under all experimental conditions.

Variety	Treatment	Total No. of examined cells	No. of dividing cells	Mitotic phase (%)				Mitotic index (%)
				Prophase	Metaphase	Anaphase	Telophase	
Sakha-3	control	3277	722	50.6	8.29	15.73	24.43	22.00 ± 1.80^{bcd}
	FA	3460	692	59.89	8.39	11.64	20.07	19.99 ± 0.32^{cde}
	RJ	3321	1090	64.09	6.88	11.46	17.57	32.84 ± 2.04^{a}
	5-AZA	3249	1188	80.31	4.04	7.35	8.2	36.57 ± 1.11^{a}
	100 mM	3297	440	47.73	11.54	13.83	26.91	13.33 ± 1.67^{fg}
	FA+100	3401	566	65.23	6.29	9.16	19.31	$16.65 \pm 1.01^{d\text{-}g}$
	RJ + 100	3172	619	55.21	7.8	10.52	26.47	19.52 ± 0.53^{cde}
	5-AZA+100	3353	557	53.52	5.73	8.18	32.57	16.63 ± 0.47^{efg}
Giza-716	Control	3402	845	66.44	5.88	11.25	16.44	24.81 ± 1.77^{b}
	FA	3290	658	55.86	8.23	9.19	26.38	20.02 ± 1.19^{cde}
	RJ	3279	1087	70.34	7.95	12.07	9.25	33.13 ± 1.57^{a}
	5-AZA	3300	1105	75.5	4.08	7.91	12.51	33.50 ± 1.94^{a}
	100 mM	3353	419	59.38	4.87	8.83	26.5	12.48 ± 0.69^{g}
	FA+100	3295	621	60.97	6.17	8.75	24.11	18.84 ± 0.24^{def}
	RJ + 100	3198	795	65.84	6.17	14.08	16	24.94 ± 1.98^{bc}
	5-AZA+100	3262	562	52.88	3.58	7.96	35.58	$17.23 \pm 0.74^{d\text{-}g}$

- Numbers carrying different letters are significantly different at $p \leq 0.05$ Control: tap water; FA: 25 mg/L folic acid, RJ: 100 mg/L Royal Jelly, 5-AZA: 50 mM 5-azacytidine, 100 mM: 100 mM NaCl, FA+100: 25 mg/L folic acid+100 mM NaCl, RJ+100: 100 mg/L royal jelly+100 mM NaCl, 5-AZA+100 mM: 50 mM 5-azacytidine+ 100 mM NaCl. Values that have distinct lettering are statistically significant at $p \leq 0.05$ when subjected to a two-way ANOVA and Duncan test analysis.

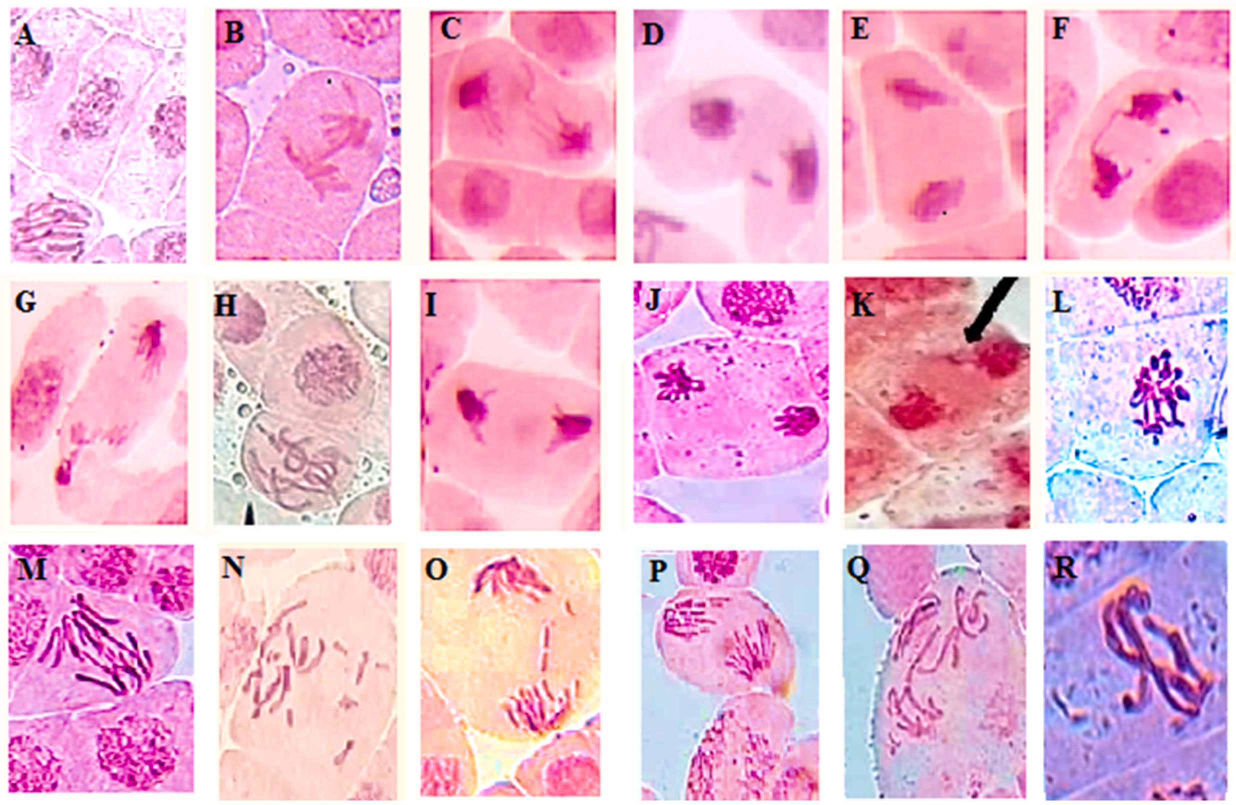

Fig. 4. All types of chromosomal abnormalities scored during cytological examination of root tips from two studied varieties of *V. faba* under all experimental conditions. Scored abnormalities included A: micronuclei; B: Bridge; C, D: chromosome loss; E: compacted telophase; F: telophase bridge; G: centromere disturbance; H: C-metaphase; I: lagging chromosome; J: fault polarization; K: vagrant chromosome; L: sticky chromosomes; M: multiple bridges; N, O, P: breaks and fragments; Q, R: uncoiling chromosomes.

3.5. Quantitative analysis of gene expression

Transcript amount of all studied genes in shoots and roots of Sakha-3 and Giza-716 varieties were determined under control, salinity stress (100 mM NaCl) and salinity treatment combined with FA, RJ or 5-AZA exogenous applications. Studied genes included antioxidant enzyme encoding genes: superoxide dismutase (*cu/zn-SOD*), glutathion reductase (*GR*) and catalase (*CAT*) and genes encodes for some protective proteins: heat shock proteins (*HSP-17.9*) as well as DNA methyltransferase encoding genes: *MT1* and *MT2.* In general, expression levesl of studied genes were higher in shoots than roots in both studied varieties, except for *Cu/Zn-SOD* and *CAT* in Giza-716 under 5-AZA treatment (Fig. 5AB,6AB) where their expression was higher in the root than shoots. Obtained results showed that salinity treatment induced decrease in the transcription level of all considered genes in Giza-716 variety and most of studied genes in Sakha-3 variety, except for *MT1* and *MT2* where it showed a significant rise in their transcript amount under salinity condition in both studied varieties (Fig. 5: F, G; 6: F, G).

The activity of ROS scavenging enzymes is usually improved in responses to many stresses [64]. Increasing the transcription level of both *Cu/Zn-SOD* and *CAT* under salinity treatment in shoots of Sakha3 could explain their salinity adaptation comparing with Giza-716 (Fig. 5: A, B; 6:A, B), where SOD and CAT enzymes constitute a very important antioxidant defense against ROS produced with salinity stress. On another hand, expression levels of *Cu/Zn-SOD*, *CAT* and *GR* (Fig. 6: A, B, C), in both shoots and roots of Giza-716 were down-regulated significantly under salinity treatment.

Treatment with FA, RJ and 5-AZA for stressed plants caused a significant increase in the transcription level of all considered genes, except for methyltransferases encoding genes (*MT1* and *MT2*) (Figs. 5 and 6: F, G). The highest level of *Cu/Zn-SOD* expression (~23 folds) was recorded at treatment with FA in Sakha-3 (Fig. 5A) while the lowest level (~3.5 folds) was at treatment with 5-AZA in Giza-176 (Fig. 6 A). Treatment with RJ induced a massive increase (~30 folds) in the expression level of *CAT* at Sakha-3 (Fig. 5 B). For *GR* encoding gene, the highest expression levels (~4 folds) were recorded with RJ treatment at both studied varieties (Figs. 5 and 6: C).

When plants suffer from stress, the antioxidant defense system is boosted to maintain ROS content at the levels require for a normal metabolism. In this context, exogenous application of FA, RJ and 5-AZA improved the defense system by inducing the expression of antioxidant encoding genes (Figs. 5 and 6: A, B, C). Up-regulation the expression of cu/zn-SOD, CAT and GR associated with FA, RJ and 5-AZA treatments helps in directly scavenge a free radical induced under salinity stress. Depending on where the SOD enzyme is located, these proteins shield intracellular and extracellular components from oxidation and damage, [70]. Additionally, by efficiently

Table (4)
Number of different chromosomal abnormalities scored under control and all experimental condition in Sakha-3 and Giza-716 varieties.

Variety	Treatment	Types of aberration (%)												Abnormality %
		Micronucleus	C-metaphase	Laggard	bridges	vagrant chromosome	chromosome loss	fault polarization in telophase	irregular prophase	uncoiling chromosomes	chromosome break	spindle disturbance	Multiple nuclear	
Sakha-3	control	0	4	6	7	2	1	0	3	1	2	0	0	3.65 ± 0.85^{g}
	100 mM	7	13	12	18	4	8	4	2	3	9	0	0	18.30 ± 2.75^{b}
	FA	5	14	12	19	3	11	7	6	5	6	0	0	12.71 ± 1.37^{bcd}
	FA+100	5	11	14	15	5	5	3	0	0	3	0	0	10.79 ± 0.60^{cde}
	RJ	4	19	12	14	6	7	5	5	3	7	3	0	$7.85 \pm 0.86^{d-g}$
	RJ + 100	10	18	3	13	4	9	4	11	3	12	0	0	14.06 ± 0.66^{bc}
	5-AZA	0	19	1	13	0	6	0	3	0	16	1	2	5.13 ± 0.17^{efg}
	5-AZA+100	4	13	2	5	7	4	0	0	0	15	0	0	$8.94 \pm 1.09^{c-g}$
Giza-716	control	0	8	4	9	0	2	1	5	0	5	1	1	4.31 ± 0.65^{fg}
	100 mM	12	20	17	21	8	14	8	8	3	11	0	0	29.26 ± 3.59^{a}
	FA	4	15	8	15	9	8	6	0	4	3	0	0	10.96 ± 0.96^{cde}
	FA+100	5	13	12	15	0	9	0	0	3	4	0	0	$9.87 \pm 1.14^{c-f}$
	RJ	0	20	12	15	5	4	7	12	0	0	6	0	$7.42 \pm 0.61^{d-g}$
	RJ + 100	7	11	7	12	2	4	5	6	3	0	0	0	$7.23 \pm 0.93^{d-g}$
	5-AZA	8	18	3	13	2	2	3	0	0	13	0	0	5.62 ± 0.63^{efg}
	5-AZA+100	10	11	4	3	0	6	0	0	0	21	0	4	10.52 ± 0.51^{cde}

- Control: tap water; FA: 25 mg/L folic acid, RJ: 100 mg/L Royal Jelly, 5-AZA: 50 mM 5-azacytidine, 100 mM: 100 mM NaCl, FA+100: 25 mg/L folic acid+100 mM NaCl, RJ+100: 100 mg/L royal jelly+100 mM NaCl, 5-AZA+100 mM: 50 mM 5-azacytidine+ 100 mM NaCl.
Values that have distinct lettering are statistically significant at $p \leq 0.05$ when subjected to a two-way ANOVA and Duncan test analysis.

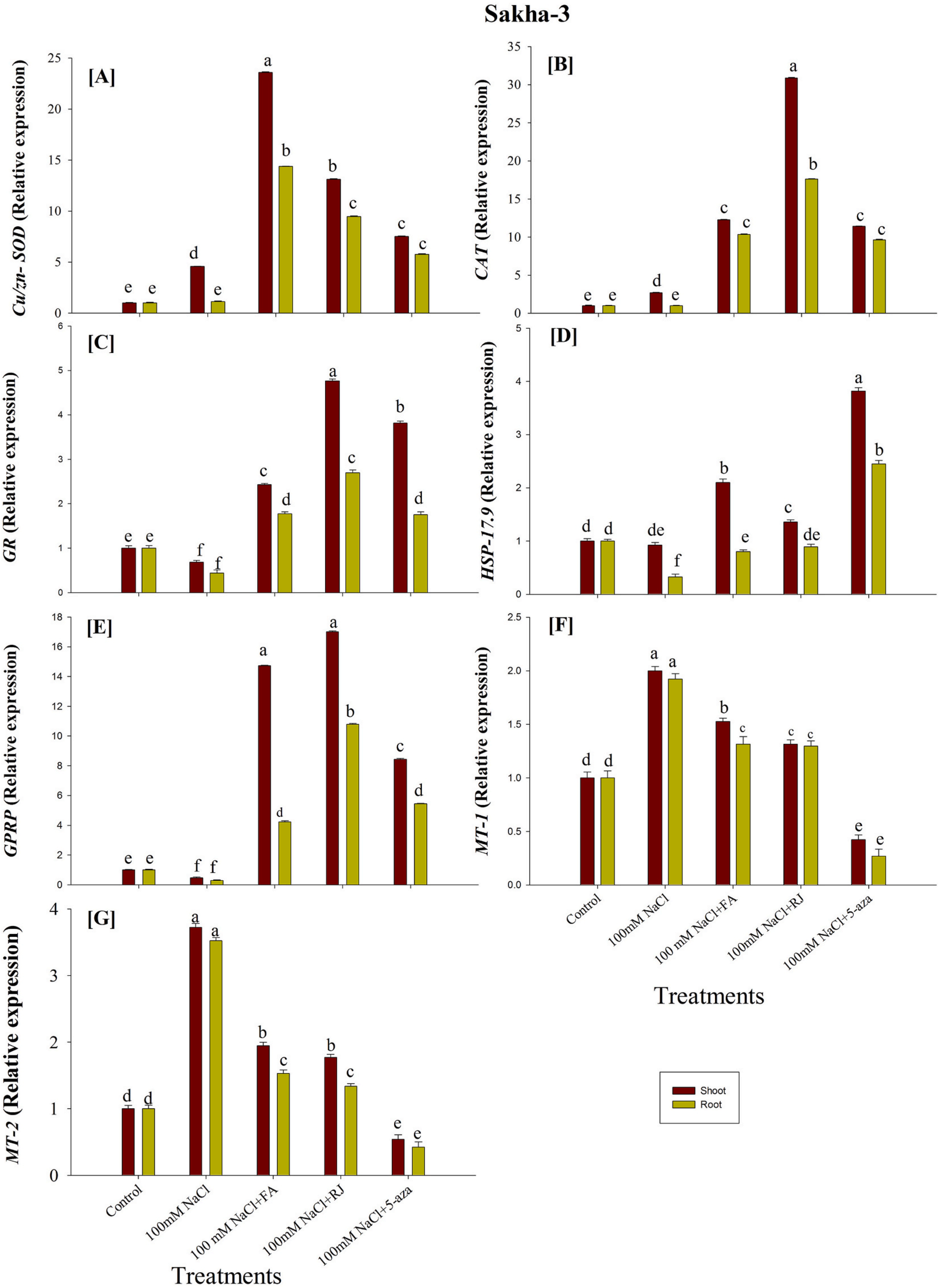

Fig. 5. Changes in the expression level of some particular genes of Sakha-3 variety seedling under control and other experimental treatments. The transcription level was detected as changes in the transcript amount using qRT−PCR. Relative expression (RQ) was calculated as $2^{-\Delta\Delta ct}$ calibrated with *actin* gene (accession no. JX444700.1) and control treatment as internal control. For every cDNA sample, the data are shown as the means ± SEs of relative expression for three biological replicates. Values that have distinct lettering are statistically significant at $p \leq 0.05$ when subjected to a two-way ANOVA and Duncan test analysis.

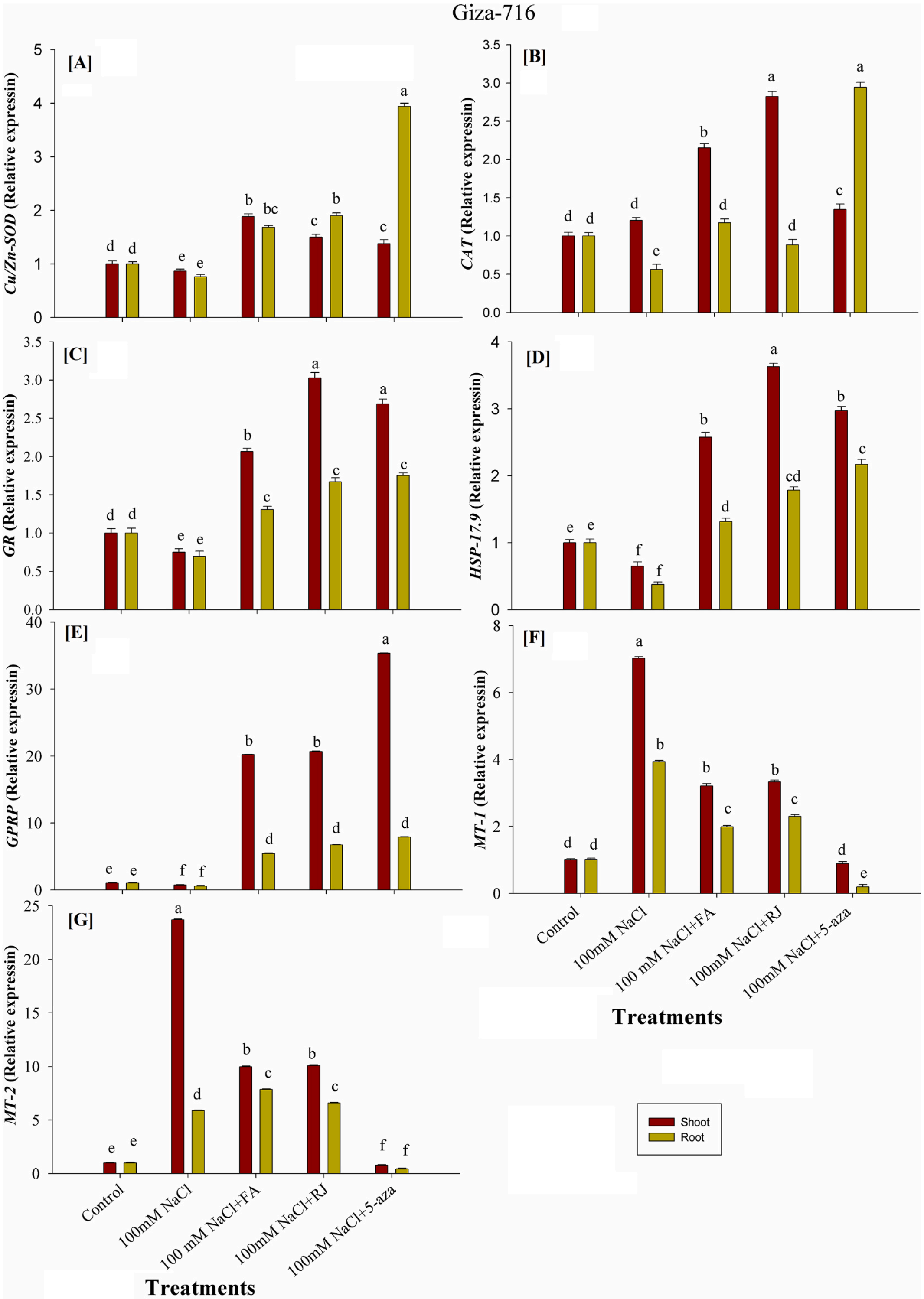

Fig. 6. Changes in the expression level of some particular genes of Giza-716 variety seedling under control and other experimental treatments. The transcription level was detected as changes in the transcript amount using qRT−PCR. Relative expression (RQ) was calculated as $2^{-\Delta\Delta ct}$ calibrated with *actin* gene (accession no. JX444700.1) and control treatment as internal control. For every cDNA sample, the data are shown as the means ± SEs of relative expression for three biological replicates. Values that have distinct lettering are statistically significant at $p \leq 0.05$ when subjected to a two-way ANOVA and Duncan test analysis.

breaking down hydrogen peroxide (H_2O_2) into water and oxygen, CAT plays a critical role in preventing cellular oxidative damage [54], while GR activity is essential in speeding scavenging process of H_2O_2 under stress [71]. This may explain reduced leakage rate and MDA content reported for FA, RJ and 5-AZA treatments (Fig. 2: A, B).

HSP17.9 encoding gene was involved as member of conserved proteins which known to be produced in response to a sudden rise in temperature and under a variety of abiotic stresses for instance salinity [2,6,72]. Expression levels of *HSP17.9* with salinity treatment were significantly down regulated in shoots and roots in both varieties except for shoots of Sakha-3 where the reduction in their expression was non-significant. Treatment with all of FA, RJ and 5-AZA induced a significant up-regulation in *HSP17.9* expressions in both varieties (Figs. 5 and 6: D). The highest expression level of *HSP17.9* transcript (~4 folds) was induced at treatment with 5-AZA in Sakha-3 (Fig. 5 D) and with RJ in Giza-716 shoots (Fig. 6 D). Small heat shock proteins serve as chaperones for other proteins inside cells. They are crucial for the folding of denatured proteins, de novo folding and the prevention of unintended protein aggregation, among other protein-protein interactions. As well as their role within the cells in proteins transferring across membranes [73].

Alteration in the expression level of the glycine and proline-rich protein encoding gene (*GPRP*) has been suggested to be crucial for plant growth and development as well as environmental adaptation [74]. Expression level of *GPRP* was inhibited significantly under salinity stress at both studied varieties (Figs. 5E and 6E). Expression levels of *GPRP* were significantly up-regulated under treatment with all of FA, RJ and 5-AZA to reach their highest expression level (~35 folds) in Giza-716 shoots for treatment with 5-AZA (Fig. 6 E) and about 17 folds in Sakha-3 shoots for treatment with RJ (Fig. 5E). It was recorded that GPRPs interact with catalases and control their activity under conditions of biotic and abiotic stressors. This reflect their role in effective removal of excessive H_2O_2 produced under these conditions [75,76]. Overexpression of proline-rich proteins in plants or yeast usually results in multiple abiotic stress tolerance [77]. Folic acid is involved in synthesis pathways of a wide range of amino acids, including methionine, glycine, valine, tryptophan and proline [27]. Royal jelly's beneficial effects on growth are due to its great content of lipids, amino acids, protein, vitamins, carbohydrates, minerals as well as other antioxidants [78]**.** Then, the up-regulation of *GPRP* in salinity stressed plants under different tested treatments could explain the associated improvement in plant growth under salinity treatment.

The transcription levels of *MT1* and *MT2* encoding genes (Fig. 5: F, G; 6: F, G) were significantly up regulated under salinity treatment comparing to control in both studied varieties. Boosting global DNA methylation will decrease global transcription, which will lower the energy required by the cell to fend off pathogens or endure other stressors linked to environmental difficulties [13]**.** The role of these enzymes in DNA methylation resulted in gene silencing subsequently reducing the expression of different genes which lead to plant developmental abnormalities [25,79]. Thus, the detrimental effect of salinity stress on Giza-716 may result from over expression of methyltransferases enzyme encoding genes (*MT1* about 7 folds*, MT2* about 25 folds) (Fig. 6: F, G). On another hand, the expression of *MT1* and *MT2* in shoots of Sakha-3 (more tolerant variety) were only 2–3 folds (Fig. 5: F, G), explaining observed genotypic difference.

Treatment of stressed plants with FA, RJ and 5-AZA showed a significant down regulation for *MT1* and *MT2* transcription level comparing with non-treated stressed plants (Fig. 5: F, G; 6:F, G).

It is known that FA can reduce the cytotoxic effects of salinity stress by minimizing the oxidative damage brought by osmotic condition and improving the glutathione-ascorbate cycle activity to increase plant antioxidant capacity under various stress conditions [80]. Folic acid plays a crucial effect in boosting plant tolerance and lowering oxidative damage induced by salinity-because FA-treated plants under saline condition increase the activities of CAT and SOD enzymes [28]. This will subsequently reduce the oxidative load and, hence, the extent of methylation. Also, RJ is considered as a scavenger of free radicals due to some of its components with antioxidant activity [49]. Some components such as flavonoids and phenolic were ingredients with strong antioxidant properties of hydroxyl radical scavenging [50]. Royal jelly was able to reduce the inhibitory effects of salinity as it was associated with a significant decrease in *MT1* and *MT2* expression at both studied varieties comparing with untreated stressed plants. Using of RJ at convenient doses may assist in decreasing the harmful effects of salinity stress [30]**.** It is clear that RJ has a major epigenetic impact in the differentiation of the larvae into workers and queens [81]. With the exception of methionine, which is reported to be marginally higher in worker jelly (WJ) than in RJ, the levels of most EAAs were higher in WJ than in RJ [82]. Since DNA methylation levels target a group of genes rather than a single gene, hypomethylation of a resistance gene would increase its expression and enable the cell to overcome the temporary challenge [13,25]. DNA hypomethylation and dormancy acceleration was induced in buds of tree peony treated with 5-AZA [83]. Changing DNA methylation pattern using 5-AZA affected sex differentiation in *Sphaeropteris lepifera* tree [84].

Obtained results indicate to the role of studied material in modifying the methylation state of stressed plants by regulation the expression level DNA methyltransferases encoding genes. Thus subsequently modulate the transcription level of numerous stress related genes which could explain the recovery effect of studied material on different stressed plants in various previous studies.

4. Conclusion

Presented results verified the negative effects of salt stress on two Egyptian commercial *V. faba* L. varieties. Irrigation with saline water affected negatively seed germination %, mitotic index, membrane stability and caused a significant increase in chromosomal abnormalities (CA %). DNA methyltransferases genes (*MT1* and *MT2*) were highly up-regulated under salinity treatment. Other studied stress related genes; *Cu/Zn-SOD*, *CAT*, *GR*, *HSP-17.9* and *GPRP* showed a significant down regulation under salinity treatment. Treatments with royal jelly (RJ), folic acid (FA) and 5-azacitidene (5-AZA) led to salt-tolerant phenotypes improving chlorophyll content, membrane stability, and reducing the percentage of CAs. These beneficial effects were accompanied with a significant reduction in the expression level of two studied DNA methyltransferases (*MT1* and *MT2*) and significant up regulation of *SOD*, *CAT*, *GR*, *GPRP* and *HSP-17.9* encoding genes. Thus, exogenous application of the FA, RJ and 5-AZA regulate the expression of two studied DNA methyltransferase (*MT1, MT2*) induced under salinity which resulted in restoring the expression of antioxidant enzymes. Changes in the expression of antioxidant enzymes as well as *GPRP* and *HSP-17.9* encoding genes improved plant redox state and alleviate oxidative and detrimental effects of salinity on *V. faba* plant. Exogenous application of the FA, RJ and 5-AZA could be recommended as a promising treatment to ameliorate hazardous effects of salinity stress on faba bean plants. Additional investigations are required to explore the effect of different concentration of studied material considering their effects on the expression of other DNA methyltransferases as well as DNA demethylases encoding genes.

CRediT authorship contribution statement

Samar A. Omar: Data curation, Conceptualization. **Yingming Feng:** Investigation, Formal analysis, Data curation. **Min Yu:** Methodology, Investigation, Data curation. **Samar A. Gamal. Eldin:** Investigation, Conceptualization. **Medhat E. Eldenary:** Software, Methodology, Investigation, Data curation. **Sergey Shabala:** Writing – review & editing, Writing – original draft, Supervision, Project administration. **Suleyman I. Allakhverdiev:** Writing – review & editing, Writing – original draft, Supervision, Project administration. **Mohamed H. Abdelfattah:** Supervision, Project administration.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Samar Omar reports a relationship with Tanta University that includes: employment. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgment

This study is supported by Tanta University Competitive ProjectUnit (TU-03-13-06) granted to Prof. Nabil Elsheery, Egypt. Table 4 were supported under the state assignment of Ministry of Science and Higher Education of the Russian Federation (theme No. 122050400128-1).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e30934.

References

[1] J. Krasensky, C. Jonak, Drought, salt, and temperature stress-induced metabolic rearrangements and regulatory networks, J. Exp. Bot. 63 (2012) 1593–1608, https://doi.org/10.1093/jxb/err460.
[2] S.A. Omar, N.I. Elsheery, P. Pashkovskiy, V. Kuznetsov, S.I. Allakhverdiev, A.M. Zedan, Impact of titanium oxide nanoparticles on growth, pigment content, membrane stability, DNA damage, and stress-related gene expression in *Vicia faba* under saline conditions, Horticulturae 9 (2023) 1030, https://doi.org/10.3390/horticulturae9091030.
[3] G.L. Petretto, P.P. Urgeghe, D. Massa, S. Melito, Effect of salinity (NaCl) on plant growth, nutrient content, and glucosinolate hydrolysis products trends in rocket genotypes, Plant Physiol. Biochem. 141 (2019) 30–39, https://doi.org/10.1016/j.plaphy.2019.05.012.
[4] A.K. Parida, A.B. Das, Salt tolerance and salinity effects on plants: a review, Ecotoxicology and environmental safety 60 (2005) 324–349, https://doi.org/10.1016/j.ecoenv.2004.06.010.
[5] S.A. Omar, N.A. Fetyan, M.E. Eldenary, M.H. Abdelfattah, H.M. Abd-Elhalim, J. Wrobel, H.M. Kalaji, Alteration in expression level of some growth and stress-related genes after rhizobacteria inoculation to alleviate drought tolerance in sensitive rice genotype, Chemical and Biological Technologies in Agriculture 8 (2021) 1–19, https://doi.org/10.1186/s40538-021-00237-4.
[6] S. Omar, H. Salim, M. Eldenary, A.V. Nosov, S.I. Allakhverdiev, A. Alfiky, Ameliorating effect of nanoparticles and seeds' heat pre-treatment on soybean plants exposed to sea water salinity, Heliyon 9 (2023), https://doi.org/10.1016/j.heliyon.2023.e21446.
[7] M.W. Yaish, J. Colasanti, S.J. Rothstein, The role of epigenetic processes in controlling flowering time in plants exposed to stress, J. Exp. Bot. 62 (2011) 3727–3735, https://doi.org/10.1093/jxb/err177.
[8] M. Farooq, N. Gogoi, M. Hussain, S. Barthakur, S. Paul, N. Bharadwaj, H.M. Migdadi, S.S. Alghamdi, K.H. Siddique, Effects, tolerance mechanisms and management of salt stress in grain legumes, Plant Physiol. Biochem. 118 (2017) 199–217, https://doi.org/10.1016/j.plaphy.2017.06.020.
[9] J. Flexas, J. Bota, F. Loreto, G. Cornic, T. Sharkey, Diffusive and metabolic limitations to photosynthesis under drought and salinity in C3 plants, Plant Biol. 6 (3) (2004) 269–279, https://doi.org/10.1055/s-2004-820867.
[10] V. Mishra, R.K. Mishra, A. Dikshit, A.C. Pandey, Interactions of nanoparticles with plants: an emerging prospective in the agriculture industry, in: Emerging Technologies and Management of Crop Stress Tolerance, Elsevier, 2014, pp. 159–180, https://doi.org/10.1016/B978-0-12-800876-8.00008-4.
[11] N. Ma, C. Hu, L. Wan, Q. Hu, J. Xiong, C. Zhang, Strigolactones improve plant growth, photosynthesis, and alleviate oxidative stress under salinity in rapeseed (*Brassica napus* L.) by regulating gene expression, Frontiers in plant science 8 (2017) 1671, https://doi.org/10.3389/fpls.2017.01671.
[12] B. Jin, K.D. Robertson, DNA methyltransferases, DNA damage repair, and cancer, Epigenetic alterations in oncogenesis (2012) 3–29, https://doi.org/10.1007/s10565-020-09522-8.
[13] M.W. Yaish, DNA methylation-associated epigenetic changes in stress tolerance of plants, in: Molecular Stress Physiology of Plants, Springer, 2013, pp. 427–440, https://doi.org/10.1038/srep14922.
[14] R. Garg, V. Narayana Chevala, R. Shankar, M. Jain, Divergent DNA methylation patterns associated with gene expression in rice cultivars with contrasting drought and salinity stress response, Sci. Rep. 5 (2015) 14922, https://doi.org/10.1038/srep14922.
[15] D. Baek, J. Jiang, J.-S. Chung, B. Wang, J. Chen, Z. Xin, H. Shi, Regulated AtHKT1 gene expression by a distal enhancer element and DNA methylation in the promoter plays an important role in salt tolerance, Plant Cell Physiol. 52 (2011) 149–161, https://doi.org/10.1093/pcp/pcq182.

[16] Z. Li, Y. Hu, M. Chang, M.H. Kashif, M. Tang, D. Luo, S. Cao, H. Lu, W. Zhang, Z. Huang, 5-azacytidine pre-treatment alters DNA methylation levels and induces genes responsive to salt stress in kenaf (*Hibiscus cannabinus* L.), Chemosphere 271 (2021) 129562, https://doi.org/10.1093/pcp/pcq182.

[17] H. Shi, W. Liu, Y. Yao, Y. Wei, Z. Chan, Alcohol dehydrogenase 1 (ADH1) confers both abiotic and biotic stress resistance in Arabidopsis, Plant Sci. 262 (2017) 24–31, https://doi.org/10.1093/pcp/pcq182.

[18] H. Zhang, Z. Lang, J.-K. Zhu, Dynamics and function of DNA methylation in plants, Nat. Rev. Mol. Cell Biol. 19 (2018) 489–506, https://doi.org/10.1038/s41580-018-0016-z.

[19] M.H. Abdelfattah, S.A. Gamal Eldin, M.E. E. Eldenary, S.A. Omar, Assessment of physiological and cytological responses of salinity stressed *Vicia faba* plants to royal jelly treatment, Journal of Sustainable Agricultural and Environmental Sciences 2 (1) (2023) 209–220.

[20] C. Stresemann, F. Lyko, Modes of action of the DNA methyltransferase inhibitors azacytidine and decitabine, Int. J. Cancer 123 (2008) 8–13, https://doi.org/10.1038/s41580-018-0016-z.

[21] M.G. Hertog, E.J. Feskens, D. Kromhout, P. Hollman, M. Katan, Dietary antioxidant flavonoids and risk of coronary heart disease: the Zutphen Elderly Study, The lancet 342 (8878) (1993) 1007–1011, https://doi.org/10.1016/0140-6736(93)92876-U.

[22] H. Kunugi, A. Mohammed Ali, Royal jelly and its components promote healthy aging and longevity: from animal models to humans, Int. J. Mol. Sci. 20 (2019) 4662, https://doi.org/10.3390/ijms2019466.

[23] S. Landman, C. van der Horst, P.E. van Erp, I. Joosten, R. de Vries, H.J. Koenen, Immune responses to azacytidine in animal models of inflammatory disorders: a systematic review, J. Transl. Med. 19 (2021) 1–17, https://doi.org/10.1186/s12967-020-02615-2.

[24] A. Al-Lawati, S. Al-Bahry, R. Victor, A. Al-Lawati, M. Yaish, Salt stress alters DNA methylation levels in alfalfa (*Medicago spp*), Genet. Mol. Res. 15 (2016) 15018299, https://doi.org/10.4238/gmr.15018299.

[25] X. Ma, N. Jiang, J. Fu, Y. Li, L. Zhou, L. Yuan, Y. Wang, Y. Li, A cytosine analogue 5-azacitidine improves the accumulation of licochalcone A in licorice *Glycyrrhiza inflata*, J. Plant Physiol. 292 (2024) 154145, https://doi.org/10.1016/j.jplph.2023.154145.

[26] S.J. Duthie, S. Narayanan, G.M. Brand, L. Pirie, G. Grant, Impact of folate deficiency on DNA stability, The Journal of nutrition 132 (2002) 2444S–2449S, https://doi.org/10.1016/j.gene.2013.09.063.

[27] L. Stakhova, L. Stakhov, V. Ladygin, Effects of exogenous folic acid on the yield and amino acid content of the seed of *Pisum sativum* L. and *Hordeum vulgare* L, Appl. Biochem. Microbiol. 36 (2000) 85–89, https://doi.org/10.1007/BF02738142.

[28] H. Alsamadany, H. Mansour, A. Elkelish, M.F. Ibrahim, Folic acid confers tolerance against salt stress-induced oxidative damages in snap beans through regulation growth, metabolites, antioxidant machinery and gene expression, Plants 11 (2022) 1459, https://doi.org/10.3390/plants11111459.

[29] N.M. Hassan, Z.M. El-bastawisy, E.G. Badran, E.M. Hamady, Role of stigmasterol and folic acid in improving the growth and yield of flax under drough, Scientific Journal for Damietta Faculty of Science 6 (2016) 40–48, https://doi.org/10.21608/SJDFS.2016.194531.

[30] D. Çavuşoğlu, S. Tabur, K. Çavuşoğlu, Physiological and cytogenetical effects of royal jelly (honey bee) in *Allium cepa* L. seeds exposed to salinity, Cytologia 82 (2017) 115–121, https://doi.org/10.1508/cytologia.82.115.

[31] N.A. Anjum, I. Ahmad, I. Mohmood, M. Pacheco, A.C. Duarte, E. Pereira, S. Umar, A. Ahmad, N.A. Khan, M. Iqbal, Modulation of glutathione and its related enzymes in plants' responses to toxic metals and metalloids—a review, Environ. Exp. Bot. 75 (2012) 307–324, https://doi.org/10.1016/j.envexpbot.2011.07.002.

[32] D.I. Arnon, Copper enzymes in isolated chloroplasts. Polyphenoloxidase in *Beta vulgaris*, Plant physiology 24 (1949) 1, https://doi.org/10.1104/pp.24.1.1.

[33] A. Karami, A. Sepehri, Beneficial role of MWCNTs and SNP on growth, physiological and photosynthesis performance of barley under NaCl stress, J. Soil Sci. Plant Nutr. 18 (2018) 752–771, https://doi.org/10.4067/S0718-95162018005002202.

[34] M.M. Bradford, A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding, Anal. Biochem. 72 (1976) 248–254, https://doi.org/10.1016/0003-2697(76)90527-3.

[35] U.K. Laemmli, Cleavage of structural proteins during the assembly of the head of bacteriophage T4, nature 227 (1970) 680–685, https://doi.org/10.1038/227680a0.

[36] B. Kihlman, Root tips of *Vicia faba* for the study of the induction of chromosomal aberrations, Mutat. Res. Environ. Mutagen Relat. Subj. 31 (1975) 401–412, https://doi.org/10.1016/0165-1161(75)90050-3.

[37] K.J. Livak, T.D. Schmittgen, Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ method, methods 25 (2001) 402–408, https://doi.org/10.1006/meth.2001.1262.

[38] W. Wang, B. Vinocur, A. Altman, Plant responses to drought, salinity and extreme temperatures: towards genetic engineering for stress tolerance, Planta 218 (2003) 1–14, https://doi.org/10.1007/s00425-003-1105-5.

[39] A. Hadas, Water uptake and germination of leguminous seeds in soils of changing matric and osmotic water potential, J. Exp. Bot. (1977) 977–985. https://www.jstor.org/stable/23689638.

[40] M. Jamil, K.J. Lee, J.M. Kim, H.-S. Kim, E.S. Rha, Salinity reduced growth PS2 photochemistry and chlorophyll content in radish, Sci. Agric. 64 (2007) 111–118, https://doi.org/10.1590/S0103-90162007000200002.

[41] S. Kilic, H.T. Aca, Role of exogenous folic acid in alleviation of morphological and anatomical inhibition on salinity-induced stress in barley, Ital. J. Agron. 11 (2016) 246–251, https://doi.org/10.4081/ija.2016.777.

[42] T.A. Abd El-Mageed, M.O. Rady, W.M. Semida, A. Shaaban, A.A. Mekdad, Exogenous micronutrients modulate morpho-physiological attributes, yield, and sugar quality in two salt-stressed sugar beet cultivars, J. Soil Sci. Plant Nutr. 21 (2021) 1421–1436, https://doi.org/10.1007/s42729-021-00450-y.

[43] B. Datt, Remote sensing of chlorophyll a, chlorophyll b, chlorophyll a+ b, and total carotenoid content in eucalyptus leaves, Remote Sensing of Environment 66 (1998) 111–121, https://doi.org/10.1016/S0034-4257(98)00046-7.

[44] M. Havaux, Carotenoid oxidation products as stress signals in plants, Plant J. 79 (2014) 597–606, https://doi.org/10.1111/tpj.12386.

[45] F. Ramel, S. Birtic, S. Cuiné, C. Triantaphylidès, J.-L. Ravanat, M. Havaux, Chemical quenching of singlet oxygen by carotenoids in plants, Plant physiology 158 (2012) 1267–1278, https://doi.org/10.1104/pp.111.182394.

[46] M. Ashraf, Q. Ali, Relative membrane permeability and activities of some antioxidant enzymes as the key determinants of salt tolerance in canola (*Brassica napus* L.), Environ. Exp. Bot. 63 (2008) 266–273, https://doi.org/10.1016/j.envexpbot.2007.11.008.

[47] S.A. Omar, Q.-T. Fu, M.-S. Chen, G.-J. Wang, S.-Q. Song, N.I. Elsheery, Z.F. Xu, Identification and expression analysis of two small heat shock protein cDNAs from developing seeds of biodiesel feedstock plant *Jatropha curcas*, Plant science 181 (2011) 632–637, https://doi.org/10.1016/j.plantsci.2011.03.004.

[48] M.M. Khan, R.S. Al-Mas'oudi, F. Al-Said, I. Khan, Salinity effects on growth, electrolyte leakage, chlorophyll content and lipid peroxidation in cucumber (*Cucumis sativus* L.), in: Proceedings of the International Conference on Food and Agricultural Sciences Malaysia, IACSIT Press, 2013, pp. 28–32, https://doi.org/10.7763/IPCBEE.

[49] Z. Nabas, M.S. Haddadin, J. Haddadin, I.K. Nazer, Chemical composition of royal jelly and effects of synbiotic with two different locally isolated probiotic strains on antioxidant activities, Pol. J. Food Nutr. Sci. 64 (2014), https://doi.org/10.2478/pjfns-2013-0015.

[50] J. Kocot, M. Kiełczykowska, D. Luchowska-Kocot, J. Kurzepa, I. Musik, Antioxidant potential of propolis, bee pollen, and royal jelly: possible medical application, Oxid. Med. Cell. Longev. (2018), https://doi.org/10.1155/2018/7074209, 2018.

[51] Y. Nakajima, K. Tsuruma, M. Shimazawa, S. Mishima, H. Hara, Comparison of bee products based on assays of antioxidant capacities, BMC Compl. Alternative Med. 9 (2009) 1–9, https://doi.org/10.1186/1472-6882-9-4.

[52] Z.V. Ogneva, A.R. Suprun, A.S. Dubrovina, K.V. Kiselev, Effect of 5-azacytidine induced DNA demethylation on abiotic stress tolerance in *Arabidopsis thaliana*, Plant Protect. Sci. 55 (2019) 73–80, https://doi.org/10.17221/94/2018-PPS.

[53] A. Matsui, J. Ishida, T. Morosawa, Y. Mochizuki, E. Kaminuma, T.A. Endo, M. Okamoto, E. Nambara, M. Nakajima, M. Kawashima, Arabidopsis transcriptome analysis under drought, cold, high-salinity and ABA treatment conditions using a tiling array, Plant Cell Physiol. 49 (2008) 1135–1149, https://doi.org/10.1093/pcp/pcn101.

[54] A. Bybordi, Effect of salinity and N sources on the activity of antioxidant enzymes in canola (*Brassica napus* L.), J. Food Agric. Environ. 8 (2010) 350–353. http://www.isfae.org/scientificjourna.

[55] M. Al-Said, A. Kamal, Effect of foliar spray with folic acid and some amino acids on flowering, yield and quality of sweet pepper, Journal of Plant Production 33 (2008) 7403–7412, https://doi.org/10.21608/JPP.2008.171240.
[56] A.G. Smith, M.T. Croft, M. Moulin, M.E. Webb, Plants need their vitamins too, Curr. Opin. Plant Biol. 10 (2007) 266–275, https://doi.org/10.1016/j.pbi.2007.04.009.
[57] M. Abo-Hinna, Effect of Organic Manure, tuber weight and ascorbic acid spraying on, Kufa Journal for Agricultural Sciences 4 (2012). https://journal.uokufa.edu.iq/index.php/kjas/article/view/9778.
[58] M. Ibrahim, H. Abd El-Gawad, A. Bondok, Physiological impacts of potassium citrate and folic acid on growth, yield and some viral diseases of potato plants, Middle East J. Agric. Res 4 (2015) 577–589.
[59] S. Kumar, A. Singh, Epigenetic regulation of abiotic stress tolerance in plants, Adv. Plants Agric. Res 5 (2016) 00179, https://doi.org/10.15406/apar.2017.07.00243.
[60] S. Di Bucchianico, F. Cappellini, F. Le Bihanic, Y. Zhang, K. Dreij, H.L. Karlsson, Genotoxicity of TiO_2 nanoparticles assessed by mini-gel comet assay and micronucleus scoring with flow cytometry, Mutagenesis 32 (2017) 127–137, https://doi.org/10.1093/mutage/gew030.
[61] H.M. Abdelmigid, M.M. Morsi, N.A. Hussien, A.A. Alyamani, N.A. Alhuthal, S. Albukhaty, Green synthesis of phosphorous-containing hydroxyapatite nanoparticles (nHAP) as a novel nano-fertilizer: preliminary assessment on pomegranate (*Punica granatum* L.), Nanomaterials 12 (2022) 1527, https://doi.org/10.3390/nano12091527.
[62] S. Anuradha, S. Seeta Ram Rao, Effect of brassinosteroids on salinity stress induced inhibition of seed germination and seedling growth of rice (*Oryza sativa* L.), Plant Growth Regul. 33 (2001) 151–153, https://doi.org/10.1023/A:1017590108484.
[63] K. Mahapatra, S. Roy, SOG1 transcription factor promotes the onset of endoreduplication under salinity stress in Arabidopsis, Sci. Rep. 11 (2021) 11659, https://doi.org/10.1038/s41598-021-91293-1.
[64] A.N. Gömürgen, Cytological effect of the potassium metabisulphite and potassium nitrate food preservative on root tips of *Allium cepa* L, Cytologia 70 (2005) 119–128, https://doi.org/10.1508/cytologia.70.119.
[65] B. Gi, A comparative study of MH and EMS in the induction of chromosomal aberrations on lateral root meristem in *Clitoria ternatea* L, Cytologia 57 (1992) 259–264, https://doi.org/10.1508/cytologia.57.259.
[66] C.H. Briand, B.M. Kapoor, The cytogenetic effects of sodium salicylate on the root meristem cells of *Alium sativum* L, Cytologia 54 (1989) 203–209, https://doi.org/10.1508/cytologia.54.203.
[67] S.-i. Inoue, S. Koya-Miyata, S. Ushio, K. Iwaki, M. Ikeda, M. Kurimoto, Royal Jelly prolongs the life span of C3H/HeJ mice: correlation with reduced DNA damage, Exp. Gerontol. 38 (2003) 965–969, https://doi.org/10.1016/S0531-5565(03)00165-7.
[68] Z. Poudineh, Z.G. Moghadam, S. Mirshekari, Effects of humic acid and folic acid on sunflower under drought stress, in: Proceedings of the Biological Forum, 2015, p. 451.
[69] S. Özmen, S. Tabur, Functions of folic acid (vitamin B9) against cytotoxic effects of salt stress in *Hordeum vulgare* L, Pak. J. Bot 52 (30) (2020) 17–22, https://doi.org/10.30848/PJB2020-1.
[70] G.E. Borgstahl, R.E. Oberley-Deegan, Superoxide Dismutases (SODs) and SOD Mimetics, vol. 7, 2018, p. 156, https://doi.org/10.3390/antiox7110156.
[71] C.-H. Pang, B.-S. Wang, Role of ascorbate peroxidase and glutathione reductase in ascorbate–glutathione cycle and stress tolerance in plants, Ascorbate-glutathione pathway and stress tolerance in plants (2010) 91–113, https://doi.org/10.1007/978-90-481-9404-9_3.
[72] T. Mahmood, W. Safdar, B.H. Abbasi, S.S. Naqvi, An overview on the small heat shock proteins, Afr. J. Biotechnol. 9 (2010) 927–939, https://doi.org/10.5897/AJB09.006.
[73] S. Walter, J. Buchner, Molecular chaperones—cellular machines for protein folding, Angew. Chem. Int. Ed. 41 (2002) 1098–1113, https://doi.org/10.1002/1521-3773(20020402)41:7<1098::AID-ANIE1098>3.0.CO;2-9.
[74] J. Li, J. Liu, G. Wang, J.-Y. Cha, G. Li, S. Chen, Z. Li, J. Guo, C. Zhang, Y. Yang, A chaperone function of no catalase activity1 is required to maintain catalase activity and for multiple stress responses in Arabidopsis, Plant Cell 27 (2015) 908–925, https://doi.org/10.1105/tpc.114.135095.
[75] T. Halder, G. Upadhyaya, S. Roy, R. Biswas, A. Das, A. Bagchi, T. Agarwal, S. Ray, Glycine rich proline rich protein from *Sorghum bicolor* serves as an antimicrobial protein implicated in plant defense response, Plant Mol. Biol. 101 (2019) 95–112, https://doi.org/10.1007/s11103-019-00894-y.
[76] X. Liu, X. Wang, X. Yan, S. Li, H. Peng, The glycine-and proline-rich protein AtGPRP3 negatively regulates plant growth in Arabidopsis, Int. J. Mol. Sci. 21 (2020) 6168, https://doi.org/10.3390/ijms21176168.
[77] B. Priyanka, K. Sekhar, V.D. Reddy, K.V. Rao, Expression of pigeonpea hybrid-proline-rich protein encoding gene (CcHyPRP) in yeast and Arabidopsis affords multiple abiotic stress tolerance, Plant Biotechnol. J. 8 (2010) 76–87, https://doi.org/10.1111/j.1467-7652.2009.00467.x.
[78] M. Abada, R. Ahmed-Basma, The beneficial effects of using Royal Jelly, Arginine and tryptophane on fruiting of superior grapevines, Egypt. J. Hort 42 (2015) 345–354, https://doi.org/10.21608/EJOH.2015.1302.
[79] R. Zhang, Q. Miao, C. Wang, R. Zhao, W. Li, C.N. Haile, W. Hao, X.Y. Zhang, Genome-wide DNA methylation analysis in alcohol dependence, Addiction Biol. 18 (2013) 392–403, https://doi.org/10.1111/adb.12037.
[80] M. Ibrahim, H.A. Ibrahim, H. Abd El-Gawad, Folic acid as a protective agent in snap bean plants under water deficit conditions, J. Hortic. Sci. Biotechnol. 96 (2021) 94–109, https://doi.org/10.1080/14620316.2020.1793691.
[81] A.N.K.G. Ramanathan, A.J. Nair, V.S. Sugunan, A review on Royal Jelly proteins and peptides, J. Funct.Foods 44 (2018) 255–264, https://doi.org/10.1016/j.jff.2018.03.008.
[82] W.F. Chen, Y. Wang, W.X. Zhang, Z.G. Liu, B.H. Xu, H.F. Wang, Methionine as a methyl donor regulates caste differentiation in the European honey bee (*Apis mellifera*), Insect Sci. 28 (2021) 746–756, https://doi.org/10.1111/1744-7917.12788.
[83] Y. Zhang, F. Si, Y. Wang, C. Liu, T. Zhang, Y. Yuan, S. Gai, Application of 5-azacytidine induces DNA hypomethylation and accelerates dormancy release in buds of tree peony, Plant Physiol. Biochem. 147 (2020) 91–100, https://doi.org/10.1016/j.plaphy.2019.12.010.
[84] N. Zheng, Y. Zhang, Z. Sun, D. Zhao, B. Liu, N.I. Elsheery, G. Ding, Transcriptome analysis of 5-azacytidine-treated *Sphaeropteris lepifera* gametophytes, Tropical Plant Biology (2023) 1–10, https://doi.org/10.1007/s12042-023-09338-w.