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PHYSIOLOGICAL AND MOLECULAR RESPONSE OF *In vitro* APPLE ROOTSTOCKS CULTIVARS TREATED WITH SILICON NANOPARTICLES UNDER SALINITY CONDITIONS

Nada S. Mohamed[1]; M.D. El-Deeb[1]; H.M.S. Hassan[1] and M.A. El-Hamahmy[2]

1. Dept. Plant Prod., Fac. Environ. Agric. Sci., Arish Univ., Egypt.

2. Dept. Agric. Botany, Fac. Agric., Suez Canal Univ., Ismailia, Egypt.

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ABSTRACT

Response of callus of three apple (*Malus domestica*, Borkh) cultivars (cv.) namely: Balady, MM 106 and MM 111 were investigated under different concentrations of silicon nanoparticles (Si-NPs) (0, 10 and 100 mgl^{-1}) and salt stress, induced by NaCl (0, 2000 and 4000 ppm). At non-salt stressed conditions, the highest values of fresh weight (FW) of callus were obtained with addition of 4 mgl^{-1} of 2,4-D with Kin at 0.4 mgl^{-1} to MS medium for all cvs. FW was increased by more than 80% after application of 10 and 100 mg/l of Si-NPs in Balady and MM111 cvs., respectively under 2000 ppm of NaCl. Anatomically, the maximum polar and equatorial diameters (72 and 53µm, respectively), of callus cells were observed in MM111cv. under high salinity level. Also, 7 amplicons amplified with three different SCoT primers (SCoT 77, 66 and 26) in both salt stressed and unstressed callus in MM111cv. Addition of Si-NPs increased the total antioxidants (%), the activity of peroxidase (POD) and superoxide dismutase (SOD) in salt stressed callus in all cvs. Application of high Si-NPs concentration increased the POD and SOD activity by 6.5 and 5.7 times, respectively in Ballady cv. under high NaCl levels. Also, high concentration of free amino acids, proline, reducing sugars and free phenolics concentration were observed in salt stressed callus with increment the concentration of Si-NPs. Proline and reducing sugars concentrations were increased by 80% in salt-stressed callus of Ballady cv. under 100 mgl- of Si-NPs. It can recommend that, application of Si-NPs at 10 mgl^{-1} will increase the tolerance of apple callus to 2000 ppm NaCl, especially in Balady and MM111 cvs, as a suitable salt tolerant rootstock for apple grafting.

INTRODUCTION

Apple is one of the major recommended fruit crops for new reclaimed soils in Egypt. Salinity is a critical problem facing agriculture, especially in irrigated lands located in semiarid zones. Water salinity considered one of the most important abiotic stresses that limits plant growth and yield of most crops **(Zhu, 2016).** Apple varieties are grown in Egypt, such as the Anna variety, grafted on more tolerant rootstocks to different environmental and biological conditions, such as Balady, MM 111, and MM 106.

Salinity induced oxidative stress by formation different types of reactive oxygen species (ROS), as superoxide radicals ($O_2^{\bullet -}$) and hydrogen peroxide (H_2O_2), which interact with the functional molecules in plants and cause multiple damage including lipid peroxidation, membrane destruction, protein denaturation and DNA mutation **(Hazman *et al.*, 2015)**. To alleviate the deleterious effects of ROS, plants have

*** Corresponding author: E-mail address:** nsayed@agri.aru.edu.eg
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evolved specific antioxidative mechanisms to scavenge ROS. Thus, antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT), peroxidase (POD) and glutathione reductase (GR), the key members of antioxidant protective system, seem to be main important determinants of plant tolerance to salt stress **(Zhan *et al.*, 2019)**.

Tolerance to abiotic stresses such as salinity is an important trait for selection of fruit trees rootstocks, which affect the nutritional status of the scion and appreciably influence scion tolerance to salinity. Producing sustainable and profitable crops under these conditions needs technological and biological approaches, including selection of new and more salt tolerant cultivars of named plants using conventional breeding programs or tissue culture techniques **(Ashraf *et al.*, 2012; Helaly *et al.*, 2016; Elsheery *et al.*, 2020).** Therefore, developing rootstocks that tolerate to different abiotic stresses is one of the major goals of breeding programs of apple rootstocks to insure rootstocks suitable for different environmental conditions**.** Salt stress increased the soluble sugars in plant leaves to maintain turgor pressure and reducing osmotic pressure **(Munns, 2003).** Proline is an amino acid that normally accumulates in large quantities in response to environmental stresses, also proline is a rich source of carbon, nitrogen and neutralizing free radicals. So, proline acts as a protector of the structure of cell membranes and proteins **(Kishor *et al.*, 2005 and Jalili *et al.*, 2009).** Proline can adjust the osmotic pressure of cells under various stresses. Additionally, free proline can remove and detoxify the reactive oxygen species (ROS) generated because of stresses, thus protecting cell membranes against these radicals **(Omar *et al.*, 2012; Saed-Moucheshi *et al.*, 2014 and Elsheery *et al.*, 2020).** Protein degradation may be essential to provide amino acids for synthesis of new proteins which important for plant survive under stress conditions **(Kocisko *et al.*, 1994).**

Silicon (Si) is the most abundant element in the surface of the earth after oxygen. Si is not an essential element, therefore its role in plant biology still understood. Nano-structured silicon dioxide after absorption can form a film at cell walls and can reduce plant transpiration, increase disease resistance, and improve plant growth under stresses such as salinity. Also, nanostructure silicon dioxide can enhance cell wall rigidity **(Mahmoud *et al.*, 2020)**.

Silicon nanoparticles (Si-NPs) had unique properties, therefore it had high potential in agriculture and may work better in alleviating different abiotic stresses than bulk material. Si-NPs had direct effect on plant growth and development, due to it may be used as delivery agents for proteins, nucleotides, and other chemicals in plants. However, Si-NPs may be effectively used in agriculture for increasing the water retention of soil **(Tripathi *et al.*, 2015; 2017; Abdel-Haliem *et al.*, 2017; Cui *et al.*, 2017).**

Using of Start Codon Targeted (SCoT) markers was more efficient tool compared to other markers, because of the longer primer distances and high annealing temperatures. **(Collard and Mackill, 2009)**. SCoT analysis was more effective for differentiation among varieties and treatment than other techniques as inter simple sequence repeat (ISSR) and random amplified polymorphic DNA (RAPD) (**Gorji *et al.*, 2011**).

The main objective of research was investigating the role of Si-NPs on physiological and molecular response of callus of three apple rootstocks (Balady, MM111 and MM106) under salt stress conditions.

MATERIALS AND METHODS

This study was carried out in Prof. Dr. Abd El-Fatah H. Belal Plant Tissue Culture Laboratory, Fac. Environ. Agric. Sci. (FEAS), Arish Univ., North Sinai, Egypt, during 2018 to 2020. Plantlets of three apple rootstocks (Balady, MM111 and MM106) were obtained from Faculty of Agriculture, Mushtohor, Banha University, and its leaves were used as an explant for callus induction.

Explant Sterilization and Callus Induction

Leaf explants were sterilized by Clorox solution (30 %) for 20 min., then soaked in ethanol alcohol (70%) for 1 min., followed by mercuric chloride solution ($HgCl_2$) $1gl^{-1}$ for 5 min. **(Belal *et al.*, 2004).** Sterilized explants were cultured on Murashige and Skoog medium (MS) **Murashige and Skoog (1962)** supplemented with 3% sucrose, 7.50 gl^{-1} agar and pH 5.7- 5.8. For callus induction MS supplemented with 2,4-D at (0.0, 1.0, 2.0 or 4.0 mgl^{-1}) in combination with kinetin (Kin) at (0.0, 0.1, 0.2, 0.4 mgl^{-1}). Cultures were maintained under continuous light at intensity of 2000 Lux using florescent lamps (Phillips, Egypt). All cultures were incubated at 25 ± 2^oC for 40 days, each treatment consisted of five replicates and each replicate consisted of three jars each one containing four explants. After 40 days the following parameters were recorded:

1. Callus formation Percentage = (Explants formatted callus/Total No. explants) × 100
2. Callus color (visually).
3. Callus fresh weight (g /jar).

Si-Nanoparticles Application and Salinity Stress Induction

Nanoparticles (Sigma) with 25 nm size, Si Hydrophilic at 10 and 100 mg/l were used. Si-NPs were photographed using TEM (JEOL, Japan) to ensure its nano-dimensions (Fig. 1).

For salt stress induction sodium chloride (NaCl) were added to the MS with different concentrations (0.0, 2000 or 4000 mgl^{-1}) combined with SiNPs at different levels (0.0, 10 and 100 mgl^{-1}). Similar pieces of calli (0.5 g) were used as explants. After 50 d, calli of different treatments were collected and prepared for determine:

Growth Parameters

1. Callus color (visually).
2. Callus fresh weight (g /jar).

Biochemical parameters

All spectrophotometric analyses were done using UV/VIS spectrophotometer, PG instrument Ltd, USA.

Proline concentration:

Proline was estimated spectrophotometrically at 520 nm in fresh callus with ninhydrin reagent as described by **Bates *et al.* (1973).**

Phytochemical compounds and total antioxidants concentrations

Phytochemicals in fresh callus was extracted with ethanol 70% according to **Abdel-Rahman *et al.* (1975)**. Ethanolic extracts were used to determine free phenolics, reducing sugars, free amino acids, and total antioxidants. Free phenolics were determined using Folin-Ciocalteu method described by **William *et al.* (1965)** at 650nm**.** Free amino acids were estimated using the method of **Rosen (1957)** at 570nm. Reducing sugars were determined by Nelson's method described by **Moore, (2012)** at 540 nm. The total antioxidants were estimated by determine the inhibition % of DPPH (2,2-diphenyle-1-picryllhydrazyl) according to **Hatano *et al.* (1988).**

Activity of Antioxidants Enzymes

Enzymes extract was prepared according to **Urbanek *et al.* (1991).** Peroxidase (POD) activity determined by measuring the oxidation of O-dianisidine at 430 nm according to **Urbanek *et al.* (1991).** Superoxide dismutase activity was assayed

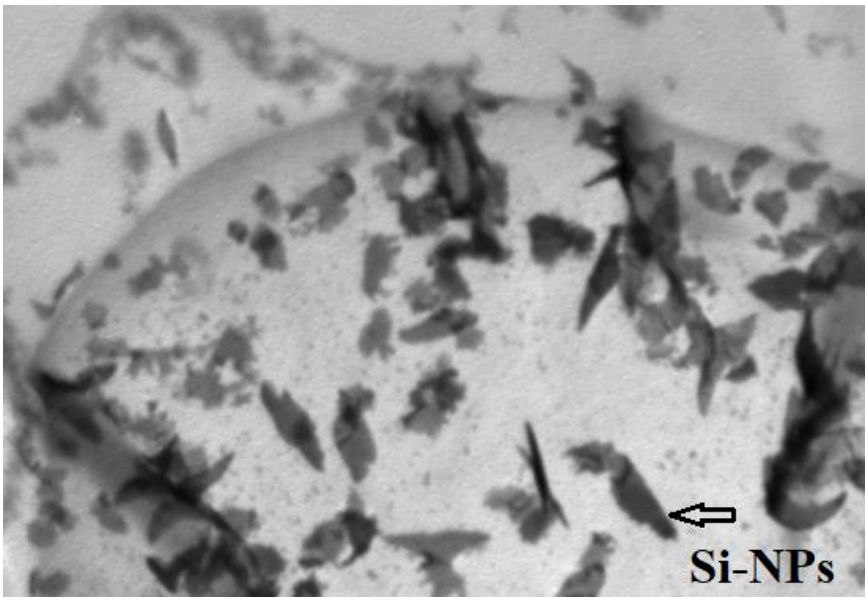


Fig. 1. Overall view of Si-NPs under TEM

by measuring its ability to inhibit reduction of nitro blue tetrazolium (NBT) at 560 nm as described by **Beauchamp and Fridovich (1971).** Enzymes activity estimated as units / 100 g protein. min.

Soluble protein determination

Soluble protein concentration determined according to **Bradford (1976)** using Coomassie brilliant blue G-250 (Sigma).

Histological Investigation

For longitudinal sections (15 µm thick), callus was fixed in formalin acetic acid (FAA), then dehydrated with ethanol series and cleared with ethanol-xylene. Then samples were embedded in paraffin wax at 45-55ºC **(Willey, 1971)**. The fixed sections were stained with Safranin O-Fast-green double stain. Observation and photomicrographs were achieved using research microscope (LEICA DM500) fitted with digital camera (LEICA ICC50).

SCoT Analysis

Genomic DNA of callus was extracted by CTAB buffer according to **Doyle and Doyle (1973).** The amplification of DNA was performed in an automated thermal cycle (model Techno 512) programmed for one cycle at 95ºC for 5 min, followed by 40 cycles of 30 sec. at 95ºC, 30 sec. at 57ºC (annealing temperature), and 30 sec. at 72ºC. Nucleotide sequence of different primers was of SCoT 26, 5'ACCATGGC TACCACCGTC'3; SCoT 66, 5' ACCAT GGCTACCAGCGAG'3; SCoT77, 5'CCA TGGCTACCACTACCC'3. DNA ladder was used as standard DNA with molecular weights of 100 to1500 bp. The run was performed for about 30 min at 80 V in mini submarine gel BioRad. The polymorphism percentage was calculated, according to **Patra *et al.* (2008)**.

Experimental Design and Statistical Analysis

There were five replications each replicate contained 3 explants for each treatment at a completely randomized design (CRD) and data were tested by using Co-STAT software V.6.13 (Cohort software, Berkeley, CA 94701). Mean values of treatments were differentiated by using least significant range (Duncan's multiple range testes) at 0.05% level probability **(Duncan, 1975).**

RESULTS AND DISCUSSION

Callus Weight (g), Callus Formation (%) and Callus Color at Normal Conditions

Results in Table 1 and Fig. 2 illustrate the interaction effect of cultivars, Kin and 2,4-D concentrations on callus FW, callus formation and callus color. The highest value of callus FW (6.76 g) and callus formation (100 %) occurred in Balady cv. treated with 4.0 mgl^{-1} 2,4-D + 0.4 mgl^{-1} Kin, followed by MM 106 cv. as shown in Fig. 2. However, the minimum values of callus FW (0.5 g) and callus formation (17.00 %) achieved in MM 111 cv. after application of 1.0 mgl^{-1} 2,4-D + 0.1 mgl^{-1} Kin. Callus color was white in all treatments. Results were in line with **Saito and Suzuki (1999)** who found that calli were induced using the apical meristem of apples (*Malus domestica*) treated with 2 mgl^{-1} 2,4-D and mgl^{-1} BA. Also, **Onofrio and Morini (2005)** found that, callus of quince (*Cydonia oblonga* Mill.) induced using leaves as an explant during 2 days by 11.3 mM of 2,4 D.

Callus Weight (g) and Callus Color as Affected by Si-NPs Application under Salt Stress Conditions

As shown in Table 2, salinity, Si-NPs and cultivars had a clear significant effect on callus FW in all cultivars under study. The highest significant value of callus FW (7.44 g) was obtained by Balady cv. treated by 2000ppm NaCl + 100 mgl^{-1} Si-NPs, followed by MM 106 cv. which recorded (7.23 g), then MM 111 cv. with 6.80 g. The minimum significant value (1.82 g) was recorded under 4000ppm of NaCl + 0 mgl^{-1} Si-NPs with MM 111 cv., followed by MM 106 cv. which recorded (2.40 g), then Balady cv. with 3.50 g. Results showed that both Balady and MM111 cvs. were more tolerant to salinity at moderate level of salinity (2000 ppm of NaCl) and MM111 was more tolerant at high level of salt (4000 ppm) with application of Si-NPs. FW was increased by more than 80% after application of 10 and 100 mg/l of Si-NPs in Balady and MM111 cvs., respectively under 2000 ppm of NaCl.

Keeping of white color for callus in all treatments, obvious that application of Si-NPs preserved the viability of callus cells under salinity conditions. This result was agreed with **Liu *et al.* (2006)** who cleared that callus FW of apple (*Malus domestica* Borkh.) decreased 6-folds compared to control after exposure to 200 mM NaCl for 7 d. Also, high concentration of NaCl was declined the callus FW and its growth was completely ceased at 125 mM of NaCl in date palm (*Phoenix dactylifera* L.) as showed by **Al-Khayri and Ibraheem (2014)**.

Biochemical Parameters

Total antioxidants (%), peroxidase (POD) and superoxide dismutase (SOD)

Salinity, Si-NPs, and cultivars had a significant effect on total antioxidants (%), peroxidase (POD) and superoxide dismutase (SOD) activity in all cvs. as shown in Table 3. The maximum significant value of total antioxidants (47.21%), POD (6.81) and SOD (1.886) was shown in Balady cv. treated with 4000 ppm NaCl + 100 mgl^{-1} Si-NPs, followed by MM 106 cv. which gave (47.19%) of total antioxidants, POD (0.576) and SOD (0.580) activity, then MM 111 cv. which gave (47.16%) of total antioxidants, (5.33) of POD and (0.563) of SOD activity. Meanwhile, MM 111 cv. showed the minimum value of total antioxidants (21.21%), POD (0.35) and SOD (0.102) activity. On the other hand, the lowest significant value of total antioxidants (15.56), POD (0.04) and SOD (0.043) was recorded in untreated MM 111 cv., followed by MM 106 cv. which gave (15.59%) of total antioxidants, POD (0.05) and SOD (0.056) activity, then Balady cv. which gave (15.61%) of total antioxidants,

Table 1. Effect of 2,4-D and Kin on callus fresh weight (g), callus formation (%) and callus color under non-salt stressed conditions in three apple cultivars

	Treatment		Callus fresh weight (g)	Callus formation (%)	Callus color
Balady	2,4-D 0.0 mg/l	Kin 0.0 mg/l	0.00 n	0.00 f	No callus
		Kin 0.1 mg/l	0.00 n	0.00 f	No callus
		Kin 0.2 mg/l	0.00 n	0.00 f	No callus
		Kin 0.4 mg/l	0.00 n	0.00 f	No callus
	2,4-D 1 mg/l	Kin 0.0 mg/l	0.00 n	0.00 f	No callus
		Kin 0.1 mg/l	1.14 klm	22.00 def	White
		Kin 0.2 mg/l	1.00 lm	29.00 de	White
		Kin 0.4 mg/l	1.96 g-k	36.00 de	White
	2,4-D 2 mg/l	Kin 0.0 mg/l	1.88 g-k	83.00 abc	White
		Kin 0.1 mg/l	3.48 cd	88.00 abc	White
		Kin 0.2 mg/l	3.38 cde	84.00 abc	White
		Kin 0.4 mg/l	3.00 def	73.60 bc	White
	2,4-D 4 mg/l	Kin 0.0 mg/l	2.64 d-h	90.00 abc	White
		Kin 0.1 mg/l	2.36 f-i	90.00 abc	White
		Kin 0.2 mg/l	5.12 b	94.00 abc	White
		Kin 0.4 mg/l	6.76 a	100.00 a	White
MM 106	2,4-D 0.0 mg/l	Kin 0.0 mg/l	0.00 n	0.00 f	No callus
		Kin 0.1 mg/l	0.00 n	0.00 f	No callus
		Kin 0.2 mg/l	0.00 n	0.00 f	No callus
		Kin 0.4 mg/l	0.00 n	0.00 f	No callus
	2,4-D 1 mg/l	Kin 0.0 mg/l	1.64 i-l	75.00 abc	White
		Kin 0.1 mg/l	0.60 mn	13.00 ef	White
		Kin 0.2 mg/l	1.62 i-l	23.00 def	White
		Kin 0.4 mg/l	1.98 g-k	38.00 d	White
	2,4-D 2 mg/l	Kin 0.0 mg/l	1.00 lm	29.00 de	White
		Kin 0.1 mg/l	2.94 def	84.00 abc	White
		Kin 0.2 mg/l	2.70 d-g	74.00 bc	White
		Kin 0.4 mg/l	3.26 de	86.00 abc	White
	2,4-D 4 mg/l	Kin 0.0 mg/l	3.30 de	72.00 bc	White
		Kin 0.1 mg/l	2.00 g-j	90.00 abc	White
		Kin 0.2 mg/l	3.20 def	90.00 abc	White
		Kin 0.4 mg/l	5.44 b	94.00 abc	White
MM 111	2,4-D 0.0 mg/l	Kin 0.0 mg/l	0.00 n	0.00 f	No callus
		Kin 0.1 mg/l	0.00 n	0.00 f	No callus
		Kin 0.2 mg/l	0.00 n	0.00 f	No callus
		Kin 0.4 mg/l	0.00 n	0.00 f	No callus
	2,4-D 1 mg/l	Kin 0.0 mg/l	0.00 n	0.00 f	No callus
		Kin 0.1 mg/l	0.50 mn	17.00 def	White
		Kin 0.2 mg/l	1.26 j-m	24.00 def	White
		Kin 0.4 mg/l	1.30 j-m	27.00 de	White
	2,4-D 2 mg/l	Kin 0.0 mg/l	1.80 h-l	96.00 ab	White
		Kin 0.1 mg/l	2.54 e-h	84.00 abc	White
		Kin 0.2 mg/l	3.18 def	86.00 abc	White
		Kin 0.4 mg/l	3.10 def	70.00 c	White
	2,4-D 4 mg/l	Kin 0.0 mg/l	2.64 d-h	70.00 bc	White
		Kin 0.1 mg/l	3.16 def	84.00 abc	White
		Kin 0.2 mg/l	2.90 def	84.00 abc	White
		Kin 0.4 mg/l	4.10 c	88.00 abc	White

- **Mean values of treatments were differentiated by using Least Significant Range (Duncan's multiple range test) at 0.01 level probability**

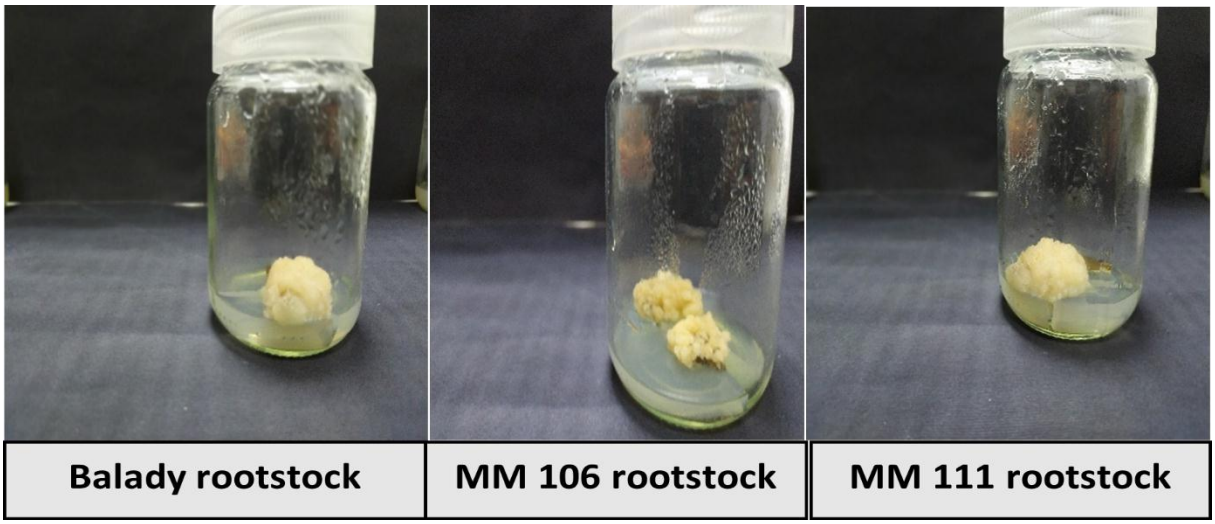


Fig. 2. Effect of the highest concentration of 2,4-D and Kin on apple callus induction

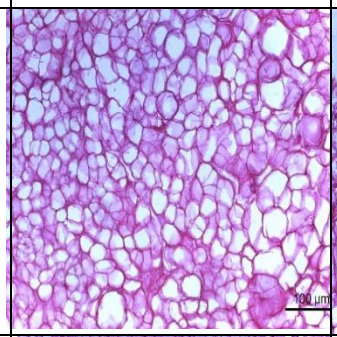
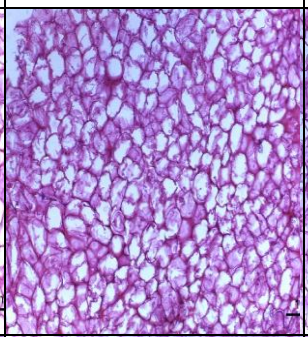
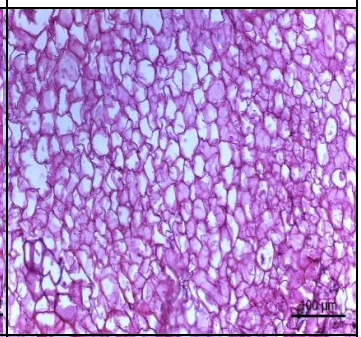
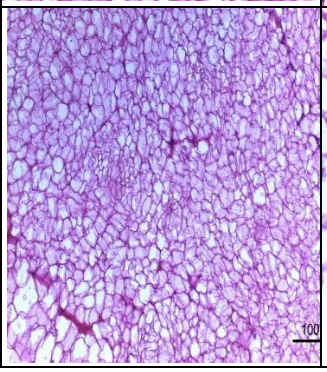
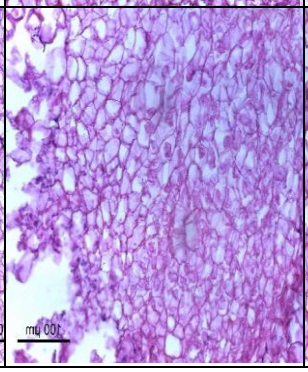
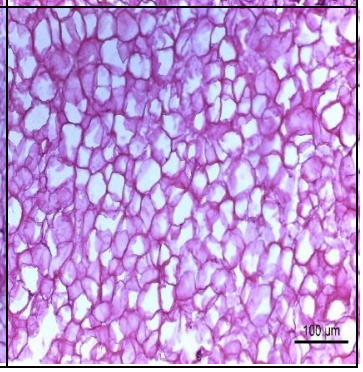
Cultivar Treatment	Balady	MM 106	MM 111
Control			
4000 ppm NaCl+ 100 mgl^{-1} Si-NPs			

Fig. 3. Histological modifications of callus formed after exposure to high concentration of Si-NPs and NaCl in three cvs. of apple

Table 2. Impact of Si-NPs application on callus fresh weight (g) and (%) of callus increment or decrement compared to control under salinity conditions in three apple cultivars

	Treatment		Callus weight (g)	Increment or decrement compared to control (%)
Balady	0 ppm (control)	Nano-Si 0 mgl^{-1}	3.72 ghi	0.0
		Nano- Si 10 mgl^{-1}	3.86 gh	3.7
		Nano- Si 100 mgl^{-1}	6.16 cd	65.6
	Salinity stress 2000 ppm	Nano-Si 0 mgl^{-1}	4.44 fg	0.0
		Nano- Si 10 mgl^{-1}	6.50 abc	46.4
		Nano- Si 100 mgl^{-1}	7.44 a	67.6
	Salinity stress 4000 ppm	Nano-Si 0 mgl^{-1}	3.50 gh	0.0
		Nano- Si 10 mgl^{-1}	6.40 bc	82.8
		Nano- Si 100 mgl^{-1}	2.82 i-l	-19.4
MM 106	0 ppm (control)	Nano-Si 0 mgl^{-1}	3.62 ghi	0.0
		Nano- Si 10 mgl^{-1}	3.46 g-j	-4.4
		Nano- Si 100 mgl^{-1}	3.76 ghi	3.9
	Salinity stress 2000 ppm	Nano-Si 0 mgl^{-1}	4.16 gh	0.0
		Nano- Si 10 mgl^{-1}	5.44 de	30.8
		Nano- Si 100 mgl^{-1}	7.23 ab	73.8
	Salinity stress 4000 ppm	Nano-Si 0 mgl^{-1}	2.40 kl	0.0
		Nano- Si 10 mgl^{-1}	2.82 i-l	17.5
		Nano- Si 100 mgl^{-1}	2.60 jkl	8.3
MM 111	0 ppm (control)	Nano-Si 0 mgl^{-1}	2.56 jkl	0.0
		Nano- Si 10 mgl^{-1}	3.38 h-k	32.0
		Nano- Si 100 mgl^{-1}	2.58 jkl	0.80
	Salinity stress 2000 ppm	Nano-Si 0 mgl^{-1}	3.62 ghi	0.0
		Nano- Si 10 mgl^{-1}	5.22 ef	44.1
		Nano- Si 100 mgl^{-1}	6.80 abc	87.8
	Salinity stress 4000 ppm	Nano-Si 0 mgl^{-1}	1.82 l	0.0
		Nano- Si 10 mgl^{-1}	2.60 jkl	42.8
		Nano- Si 100 mgl^{-1}	2.26 l	24.1

- **Mean values of treatments were differentiated by using Least Significant Range (Duncan's multiple range test) at 0.01 level probability**

Table 3. Total antioxidants (%), peroxidase (POD), superoxide dismutase (SOD) activity (units/100g protein. min.), free amino acids, proline, soluble protein, reducing sugars and free phenolics (mg/g 100FW) as affected by Si-NPs application in three apple cultivars under salt stress conditions

	Treatment		Total antioxidants (%)	POD	SOD	Free amino acids	Proline	Soluble protein (mg/g FW)	Reducing sugars	Free phenolics
Balady	0 ppm (control)	Nano-Si 0 mgl^{-1}	15.61 d	0.08 c	0.086 b	2.60 r	2.59 p	107.90 a	1.85 q	11.47 h
		Nano- Si 10 mgl^{-1}	15.66 d	0.13 c	0.070 b	2.98 p	2.96 o	108.58 a	3.24 p	27.47 fgh
		Nano- Si 100 mgl^{-1}	19.23 cd	0.18 c	0.063 b	3.11 o	3.25 n	116.95 a	4.69 no	33.81 fgh
	Salinity stress 2000 ppm	Nano-Si 0 mgl^{-1}	28.90 bc	0.28 c	0.120 b	3.27 n	3.45 l	101.93 a	5.68 m	35.81 fgh
		Nano- Si 10 mgl^{-1}	33.01 b	0.61 c	0.136 b	3.57 l	3.90 j	102.86 a	11.03 k	60.15 e-h
		Nano- Si 100 mgl^{-1}	38.64 ab	0.91 c	0.176 b	4.07 i	4.62 h	103.29 a	13.66 i	87.15 d-h
	Salinity stress 4000 ppm	Nano-Si 0 mgl^{-1}	45.19 a	0.91 c	0.233 b	4.41 g	7.21 f	78.72 a	15.05 d	108.60 c-f
		Nano- Si 10 mgl^{-1}	46.36 a	2.15bc	0.326 b	10.12 d	8.66 d	89.23 a	18.26 d	189.84 abc
		Nano- Si 100 mgl^{-1}	47.21 a	6.81 a	1.886 a	16.04 a	12.99a	91.99 a	27.22 a	227.50 a
MM 106	0 ppm (control)	Nano-Si 0 mgl^{-1}	15.59 d	0.05 c	0.073b	2.43 s	2.22 r	107.90 a	1.70 q	10.42 h
		Nano- Si 10 mgl^{-1}	15.59 d	0.09 c	0.063 b	2.73 q	2.48 q	108.54 a	2.90 p	23.01 fgh
		Nano- Si 100 mgl^{-1}	19.17 cd	0.15 c	0.056 b	2.60 r	3.25 n	116.91 a	4.45 no	32.38 fgh
	Salinity stress 2000 ppm	Nano-Si 0 mgl^{-1}	28.83 bc	0.26 c	0.113 b	3.15 o	3.36m	101.93 a	5.09 vn	33.81 fgh
		Nano- Si 10 mgl^{-1}	33.01 b	0.61 c	0.133 b	3.44 m	3.90 j	102.86 a	9.79 l	59.52 e-h
		Nano- Si 100 mgl^{-1}	38.55 ab	0.90 c	0.166 b	3.99 j	4.05 i	103.18 a	13.48 i	74.28 e-h
	Salinity stress 4000 ppm	Nano-Si 0 mgl^{-1}	45.17 a	0.90 c	0.206 b	4.32 h	5.06 g	78.57 a	14.81 g	106.98 c-f
		Nano- Si 10 mgl^{-1}	46.32 a	1.49bc	0.323 b	6.76 e	8.72 e	89.09 a	17.62 e	164.92 a-d
		Nano- Si 100 mgl^{-1}	47.19 a	5.76ab	0.580 b	11.13 b	9.52 b	91.95 a	26.03 b	199.68 ab
MM 111	0 ppm (control)	Nano-Si 0 mgl^{-1}	15.56 d	0.04 c	0.066 b	1.43 t	2.05 s	107.80 a	1.70 q	5.23 h
		Nano- Si 10 mgl^{-1}	15.56 d	0.09 c	0.063 b	2.98 q	2.45 q	108.51 a	2.22 q	20.14 fgh
		Nano- Si 100 mgl^{-1}	19.17 cd	0.14 c	0.043 b	2.98 p	3.25 n	116.77 a	4.30 o	27.14 fgh
	Salinity stress 2000 ppm	Nano-Si 0 mgl^{-1}	28.83 bc	0.21 c	0.113 b	3.11 o	3.22 n	101.82 a	4.70 no	33.01 fgh
		Nano- Si 10 mgl^{-1}	32.95 b	0.56 c	0.123 b	3.40 m	3.56 k	102.72 a	9.67 l	59.04 e-h
		Nano- Si 100 mgl^{-1}	38.53 ab	0.82 c	0.150 b	3.82 k	4.02 i	103.18 a	11.84 j	72.85 e-h
	Salinity stress 4000 ppm	Nano-Si 0 mgl^{-1}	45.14 a	0.90 c	0.203 b	4.11 i	5.04 g	78.54 a	14.81 g	105.71 c-g
		Nano- Si 10 mgl^{-1}	46.30 a	1.45bc	0.323 b	4.53 f	7.24 f	89.05 a	16.85 f	143.0 a-e
		Nano- Si 100 mgl^{-1}	47.16 a	5.33ab	0.563 b	10.79 c	8.93 c	91.92 a	19.61 c	190.79 abc

- **Mean values of treatments were differentiated by using Least Significant Range (Duncan's multiple range test) at 0.01 level probability**

(0.08) of POD and (0.063) of SOD activity. These findings reported herein obvious that, Balady cv. was more tolerant to salt stress followed by MM 106 cv. but MM111 cv. was more sensitive to salt stress. The results were in harmony with **Molassiotis *et al.* (2006)** who showed that activity of SOD was increased in explants of apple MM 106 cv. after exposure to NaCl stress compared to other cultivars. Also, POD activity was increased under salt stress compared to unstressed explants.

Free amino acids, proline, and soluble protein concentrations (mg/g FW)

Salinity, Si-NPs, and cultivars also, had significant effect on free amino acids, proline, and soluble protein concentrations (mg/100g FW) in all cvs. as shown in Table 3. The highest significant value for each free amino acids (16.04) and proline (12.99) mg/100g FW was recorded in Balady cv. under 4000 ppm NaCl + 100 mgl^{-1} Si-NPs, followed by MM 106 cv. which gave 11.13 and 9.52 mg/100g FW of free amino acids and proline, respectively then MM 111 cv. which had 16.04 and 8.93 mg/100g FW of free amino acids and proline, respectively. The maximum value of soluble protein (116.95 mg/100g FW) was recorded in Balady cv. which exposed to 0 ppm NaCl + 100 mgl^{-1} Si-NPs, followed by MM 106 cv. which had 116.91 mg/g FW, then MM 111 cv. by116.77 mg/100g FW. Results were in line with (**Kishor *et al.*, 2005; Jalili *et al.*, 2009)** who obvious that, proline normally accumulated in large quantities under different environmental stresses as a protector of cell membranes and proteins.

Reducing sugars and free phenolics

Salinity, Si-NPs, and cultivars had significant effect on reducing sugars and free phenolics in all cvs. as shown in Table 3. The highest significant value for each reducing sugars (27.22 mg/100g FW) and free phenolics (227.50 mg/100g FW) was achieved with 4000 ppm NaCl + 100 mgl^{-1} Si-NPs with Balady cv., followed by MM 106 cv. which gave (26.03 mg/100g FW) of reducing sugars and (199.68 mg/100g FW) of free phenolics, then MM 111 cv. which gave (19.61 mg/100g FW) of reducing sugars and (190.79 mg/100g FW) of free phenolics..

These results agreed with **Sotiropoulos (2007)** who reported that soluble sugar content in leaves of apple (*Malus domestica* Borkh) cv. M 4 significantly increased after exposure to 100 and 200 mM NaCl in comparison to control. Also, **Badran and Savin (2019)** achieved that salinity at 3 and 5 dsm^{-1} increased the accumulation of total carbohydrate in the bitter almond rootstock. **Ye *et al.* (2016)** found that the concentration of soluble sugars significantly increased in peach seedlings under 100 mM of NaCl compared to control. Moreover, free phenolics increased with increment of salinity level to 90mM of NaCl in callus of red-fleshed apple (**Zahed *et al.*, 2018).**

Histological structure of callus as affected by high levels of Si-NPs and NaCl

All three apple cultivars under control or salinity stress with Si-NPs were significantly differed in all histological parameters as shown in Table 4 and Fig. 3. The maximum polar (P) and equatorial (E) diameter of callus cells was recorded in untreated callus of MM111 cv. (72 and 53 µm, respectively) compared to other treatments. Application of Si-NP at 100 ppm to salt-stressed calli of both Balady and MM 106 cvs. gave the high P/E ratio, with oblate shape of cells. Oblate shape may be more rapidly differentiate to xylem tissue compared to spherical shape (**Lyndon, 1990**). Callus had multiseriate symmetric cells and shizogenous intercellular spaces under all treatments. Application of Si-NPs increased division of cells under salinity conditions in all three cultivars compared to control. Addition of Si-NPs to salt-stressed calli of Balady cv. gave the highest number of cells/ mm^2 and number of cells formed/day (2049 and 22.8, respectively) compared to other treatments. Silicon had a vital role in cell division and elongation by increasing the levels of GA3 in the cells in Salvia splendens under high temperature (**Soundararajan *et al.*, 2014).**

Molecular response of cultivars to high Si-NPs and salinity conditions

Table 5 and Fig. 4 show that three cultivars under study were genetically differed according to the overall bands which formed after PCR amplification using three SCoT primers , 66 , 77 and 26. The maximum

Table 4. Histological variation among Si-NPs treated and control callus of apple cultivars under NaCl stress for 90 days:

Cultivar	NaCl (ppm)	Si	Average (µm) of the		P/E ratio	Cells		Intercellular spaces	Average		Increment (%)
			Maximum Polar (P) diameter of cell	Maximum equatorial (E) diameter of cell		Shape	Symmetry		Number of cells / mm^2	Number of cells formed/day	
Balady	0.0	0.0	48 c	37 b	1.3 : 1	Prolate	symmetrical multiseriate	shizogenous	579 d	6.4 d	253.9
	4000	100	32 e	17 d	1.9 : 1	Oblate	multiseriate symmetrical	Shizogenous	2049 a	22.8 a	
MM 106	0.0	0.0	49 c	36 b	1.4 : 1	Semi-prolate	symmetrical multiseriate	Shizogenous	724 c	8.0 c	59.4
	4000	100	44 d	23 c	2 :1	Oblate	symmetrical multiseriate	Shizogenous	1154 b	12.8 b	
MM 111	0.0	0.0	72 a	53 a	1.4 : 1	Semi-prolate	symmetrical multiseriate	Shizogenous	339 e	3.8 e	70.8
	4000	100	59 b	54 a	1.1 : 1	spheroid	symmetrical multiseriate	Shizogenous	579 d	6.4 d	

Table 5. Variations among Si-NPs treated and control callus of apple cultivars in the number of amplicon bands using three SCoT primers under NaCl stress.

Cultivar			SCoT 66							SCoT 77						SCoT 26						
			MW (bp)							MW (bp)						MW (bp)						
			500	300	250	200	150	100	Total bands	350	300	250	200	150	Total bands	400	300	200	150	100	Total bands	Overall bands
	NaCl (ppm)	Si-NPs																				
Balady	**0.0**		-	+	+	+	+	+	**5**	+	+	+	+	+	**5**	+	+	+	+	-	**4**	**14**
	4000	**100**	-	-	-	-	-	+	**1**	-	+	+	+	+	**4**	-	-	+	+	-	**2**	**7**
MM 106	**0.0**		-	-	-	-	+	+	**2**	-	-	-	-	-	**0**	-	+	+	+	-	**3**	**5**
	4000	**100**	-	-	-	+	+	+	**3**	+	+	+	+	+	**5**	-	+	+	+	-	**3**	**11**
MM 111	**0.0**		-	-	-	+	-	-	**1**	-	+	+	+	-	**3**	-	+	+	+	-	**3**	**7**
	4000	**100**	-	-	-	+	+	-	**2**	-	+	+	+	-	**3**	-	-	+	+	-	**2**	**7**
Monomorphic bands (%)					**16.6%**							**0.0%**						**60%**				
Polymorphic bands (%)					**83.3%**							**100%**						**40%**				

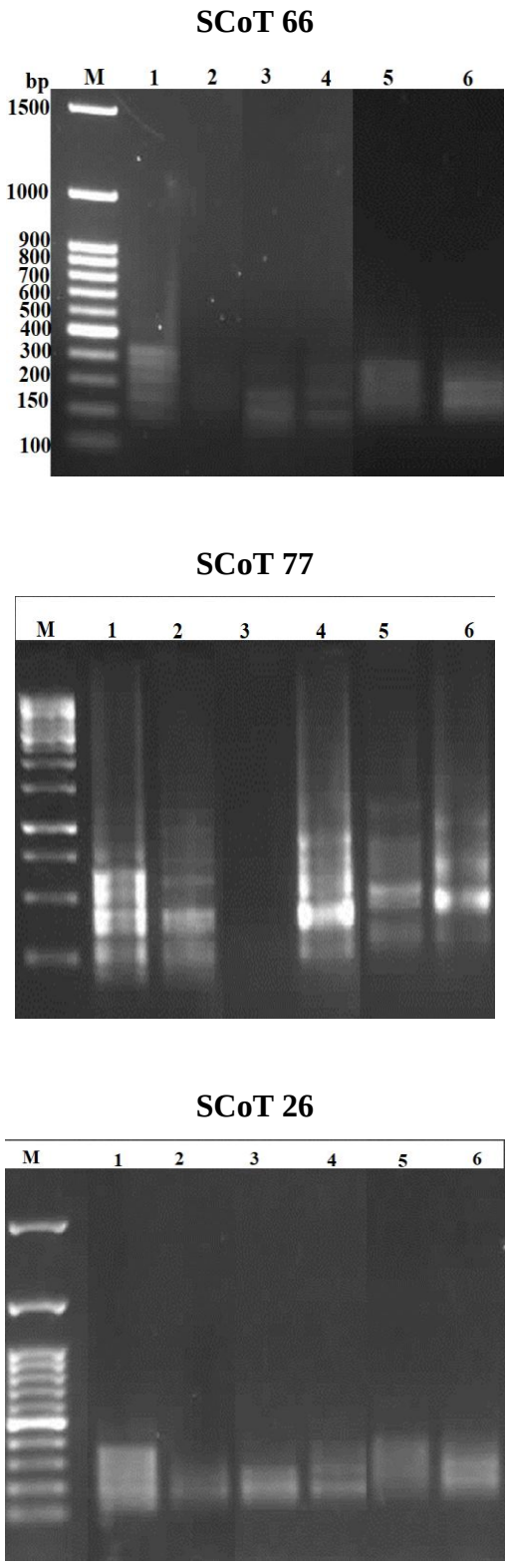


Fig. 4. SCoT amplification profile of Si-NPs treated callus and control of three apple cultivars produced by 3 different primers as affected by salt stress. M, marker; 1, 2 Balady cv.; 3,4 MM106 cv.; 5,6 MM111 cv.; 1,3,5 control; 2, 4, 6 Si-NPs + 4000 ppm of NaCl

amplicon bands (14) were shown in control callus of Baldy cv., and 5 bands in MM106 cv. as well as 7 bands in MM111cv. Response of cultivars was differed at genetic level under high concentration of Si-NPs and salinity treatments. Although overall bands were decreased from 14 to 7 bands in Balady cv., it increased from 5 to 11 bands in MM106 cv. but it stabled in MM111 cv. SCoT 77 primer was the highest efficient primer for differentiation among cultivars and treatment due to the high polymorphic bands as 100% compared to other primers. Addition of Si-NPs to salt-stressed callus led to presence or disappear specific bands. Results were agreed with **Hazman *et al.* (2015)**, who reported that salinity may induced DNA mutation due to formation of different ROS. Exposure callus to both salinity and Si-NPs changed the genetic amplicon which amplified by different SCoT primers. Bands with 100 to 300 bp amplified by ScoT 66, 350 bp by SCoT 77 and both 300,400 bp by SCoT 26 which found in unstressed callus of Balady cv. were absent in salt stressed one after application of Si-NPs. However, bands with 200 bp produced by SCoT 66 and bands with 150 to 350 bp formed by SCoT 77 were present as application of Si-NPs to salt-stressed callus of MM106 cv. Also, band with 150bp formed by SCoT 66 was present as function of Si-NPs in salt stressed callus, while band with 300 bp formed by SCoT 26 was absent in salt stressed callus of MM111 cv.

Conclusion

Using 2,4-D (4 mgl^{-1}) and Kin (0.4 mgl^{-1}) was suitable concentrations for callus induction in apple cultivars rootstocks. Balady rootstock was more tolerant to salinity stress by showing the highest value for each of total antioxidants, POD, SOD, free amino acids, proline, soluble protein content, reducing sugars and free phenolics content, followed by MM 106 rootstock but MM111 rootstock was more sensitive to stress. Using Si-NPs at (100 mgl^{-1}) can induce callus growth and tolerance under salinity stress.

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الملخص العربي

الاستجابة الفسيولوجية والجزيئية لإضافة جزيئات النانو سليكون على أصول التفاح النامية تحت ظروف الملوحة معملياً

ندا سيد محمد[1]، محمد دياب الديب[1]، هاني محمد سامي حسن[2]، محمد على الحماحمي[2]

1. قسم الانتاج النباتي، كلية العلوم الزراعية البيئة، جامعة العريش، مصر.

2. قسم النبات الزراعي، كلية الزراعة، جامعة قناة السويس، الاسماعيلية، مصر.

تم دراسة استجابة ثلاثة أصول للتفاح هي (البلدي – MM 106 – MM 111) بتركيزات مختلفة من كلوريد الصوديوم كمحفز للإجهاد الملحي هي (صفر – 2000 – 4000 جزء في المليون)، وتركيزات مختلفة من النانو سيليكون هي (صفر – 10 – 100 ملجم/لتر) ودراسة تأثيرهم على نمو الكالس والمكونات البيوكيميائية له. أظهرت النتائج أن التركيز الأعلى من 2,4-D (4 ملجم/ لتر) مع التركيز الأعلى للكينيتين (0.4 ملجم/ لتر) أظهر أعلي معدل للوزن الطازج وتشكل الكالس مع جميع الأصول تحت ظروف عدم الاجهاد الملحي. اظهر الصنف البلدي وMM111 تحمل للملوحة على مستوى نمو الكالس تحت ظروف الملوحة المتوسطة (2000 جزء في المليون) بينما اظهر الصنف MM111 تحملا أعلى للملوحة عند التركيز العالي للملوحة (4000 جزء في المليون). حيث زاد وزن الكالس بمقدر أعلى من 80% بعد اضافة السليكون النانو بتركيز 10 و100 ملجرام لكل لتر في الصنف البلدي وMM111 عند 2000 جزء في المليون من كلوريد الصوديوم على التوالي. لوحظ اعلى قطر قطبي وعرضي (72 و 53 ميكروميتر على التوالى) لخلايا الكالس تشريحيا في الصنف MM111 تحت ظروف الملوحة العالية. كما سجل وجود 7 حزم وراثية (امبليكون) باستخدام تكنيك SCoT مع 3 بوادئ مختلفة في التتابع النيوكليتيدى هى SCoT 26, 66, 77 تحت كلا الظروف الطبيعية والملوحة العالية في الصنف MM111. كما ادت اضافة النانو سيليكون بتركيز (100 مليجرام/لتر) إلى الحصول على أعلى قيم لكل من مضادات الأكسدة الكلية، واعلى نشاط للبيروكسيديز، والسوبر أوكسيد ديسميوتيز، وأعلى تركيز للأحماض الأمينية الحرة، البرولين، السكريات المختزلة والفينولات الحرة لجميع الأصول بالمقارنة مع معاملة الكنترول. حيث ارتفع نشاط انزيم البيروكسيديز والسوبراوكسيد ديسميوتيز بمقدار 6.5 و 5.7 مرة كما زاد تركيز السكريات المختزلة والبرولين بنسبة 80% نتيجة اضافة النانوسليكون بتركيز مرتفع لكالس الصنف البلدى تحت ظروف الملوحة المرتفعة. يمكن التوصية بإضافة النانوسليكون بتركيز 10 جزء في المليون لزيادة تحمل الكالس الناتج من اصول التفاح للملوحة حتى 2000 جزء في المليون خاصة مع الصنف البلدي وMM111.

الكلمات الاسترشادية: التفاح، كلوريد الصوديوم، النانوسليكون، SCoT.

REVIEWERS:
Dr. Nabil I.A. ElSheery | nshery@yahoo.com
Dept. Agric. Botany, Fac. Agric., Tanta Univ., Egypt.
Dr. AbdelRahman E. Hassan | abdelrhamanhassan9@gmail.com
Dept. Agric. Botany, Fac. Agric., Suez Canal Univ., Ismailia, Egypt.