ORIGINAL ARTICLE

RECORDS OF PHARMACEUTICAL AND BIOMEDICAL SCIENCES

Enhancement of Growth and Alkaloids Accumulation in *Hyoscyamus muticus* L. Callus Cultures by High Salt Concentration

Eman Abdelrazik[a*], Mohamed A. El-hamahmy[b], Enas E. Eltamany [c], Islam H. Abo-Elsoud [d], Elham M. Ali[e], Akram. A. Aboseidah[a]

[a] *Botany and Microbiology Department, Faculty of Science, Suez University, Suez, Egypt,*
[b] *Agric. Botany Department, Faculty of Agriculture, Suez Canal University, Ismailia, 41522, Egypt,*
[c] *Pharmacognosy Department, Faculty of Pharmacy, Suez Canal University, Ismailia, Egypt,*
[d] *Horticulture Department, Faculty of Agriculture, Suez Canal University, Ismailia, 41522, Egypt,*
[e] *Environmental Sciences Department, Suez University, Suez, Egypt.*

Received on: 10. 02. 2019

Revised on: 19. 02. 2019

Accepted on: 23. 02. 2019

Correspondence Author:

Tel:+2 01140485181.

E-mail address:

abdelrazikeman1@gmail.com

Abstract

The effect of different concentrations of NaCl (6, 8 and 10 dS/m) on growth and alkaloids production in 21 days-old callus of Egyptian henbane (*Hyoscyamus muticus* L.) was studied. Results showed that 10 dS/m of NaCl increased fresh and dry weight by 4.1 and 2.8 fold more than control, respectively. Total alkaloids increased by 2 fold at 10 dS/m compared to control or wild leaves. In contrary, wild leaves had the highest amount of hyoscyamine (2.8 mg/g DW) followed by 6 dS/m stressed-callus (1.14 mg/g DW) as detected by HPTLC. Peroxidase and superoxide dismutase activity were increased by 2.6 and 2.3 fold, respectively in highly salt stressed callus than control while catalase activity (CAT) was decreased. Anthocyanin concentration was increased 3 fold in 10 dS/m of NaCl stressed callus compared to control. Glutathione content was increased by 33.8% under low level of NaCl more than control. Reduction of photosynthetic pigments under all NaCl levels led to decrease the total carbohydrate and protein content. Protein band with molecular weight 109 KDa was a unique band in 10dS/m of NaCl treated callus with high expression of protein bands with MW 44, 38 and 32 KDa. Histologically, callus cells under high level of NaCl had amoeboid shape and start in senescence with formation of lysiogenous intercellular spaces. It can be concluded that, using of NaCl as elicitor was effective method for enhancing the growth and alkaloids

Keywords: Hyoscyamine, *in vitro*, protein electrophoresis, HPTLC, salt stress, biochemical compounds

1. Introduction

Salt stress is an important abiotic elicitor affecting plant growth, development, morphogenesis and the accumulation of secondary metabolites such as phenols, terpenes and alkaloids (Naik and Al-khayri, 2016; Selmar, 2008; Haghighi *et al.*, 2012). NaCl stressor had stimulating or inhibiting effect on growth or synthesis of natural products according to its concentration, exposure period and plant species. In this respect, low concentration of NaCl (2000 ppm) enhanced the production of secondary metabolites in *Mentha longifolia in vitro* cultures but high concentration (6000 ppm) inhibited growth and yellowing cells (El-shennawy *et al.*, 2017). Also, low NaCl level (1000 ppm) increased the total phenolics, total flavonoids and total tannins in callus culture of Fenugreek (*Trigonella*

foenum-graecum), while the higher concentration (3000 ppm) significantly decreased the secondary metabolite accumulation (Hussein and Aqlan, 2012). However, 25mM of NaCl stressed callus produced about three times of pilocarpine more than the unstressed callus (Abreu *et al.*, 2005). Although root growth was decreased, secondary metabolite accumulation was increased with elevating level of NaCl compared control in madder plants (Asci *et al.*, 2018). Sitosterol content was increased in *Nitraria tangutorum* cell suspension at 250 mM NaCl treatment (Ni *et al*., 2015). Low increment of canavanine content was recorded in *Sutherlandia frutescens in vitro* shoot culture after exposure to 100 mM NaCl (Colling *et al.*, 2010). Using of NaCl as an elicitor improved the synthesis of both vinblastine and vincristine in *Catharanthus roseus* embryogenic tissue culture (Fatima *et al.*, 2015).

Tropane alkaloids are plant derived organic compounds that contain a tropane ring in their chemical structure and they are among the oldest medicines known to man. Tropane alkaloids have been found in different plant families, Brassicaceae, Convolvulaceae, Erythroxylaceae, Euphorbiaceae, Olacaceae, Proteaceae, and Rhizophoraceae, but they are best known for their occurrence in the family Solanaceae (Griffin and Lin, 2000). *Hyoscyamus muticus*, commonly known as the Egyptian henbane, is a shrub belongs to family Solanaceae, is a rich source of tropane alkaloids which have mydriatic, anticholinergic and antispasmodic properties. Hyoscyamine represents 90% of the total alkaloids in addition to small amounts of hyoscine in *Hyoscyamus muticus* (Batanouny *et al.*, 1999). The present study was carried out to investigate the effect of salt stress by different concentration of NaCl on callus growth, histological structure, biochemical parameters, and alkaloid production from *Hyoscyamu muticus* L.

2. Materials and methods

2.1. Seeds sterilization and germination:

Wild *Hyoscyamus muticus* L. seeds were collected from Saint Catherine peninsula and identified according to Tackholm, (1974). To break a seed dormancy, it were immersed in 250 mg l^{-1} of gibberellic acid (GA_3) for 24 h at lab. temperature (25±0.5˚C) (Alaghemand *et al.*, 2013). Then washed with tap water and surface-sterilized with 70 % ethanol for 2 min., then in 25 % commercial bleach (containing 5.25 % sodium hypochlorite) with a drops of tween-80 for 20 min and finally rinsed 3 times with double distilled sterilized water. The sterilized seeds were cultured in hormone-free MS medium (Murashige and Skoog), supplemented with 7 g l^{-1} agar and 15 g l^{-1}sucrose and maintained at 25 ± 1 ℃ under light condition (1,500 lux, 16 h/day) for germination. After germination, plantlets have been obtained for explants preparation.

2.2. Explant culture and callus induction:

Shoot tip (18 days-old) were excised and cultured in MS media supplemented with different concentrations of BAP (0.5 mg l^{-1}) and NAA (0.5, 1 and 2mg l^{-1}), pH (5.7-5.8) for callus induction with 10 replicate. The cultures were incubated at (25±1C°) with (16/8) light/dark cycle. After 4 weeks more suitable callus were selected for further work.

2.3.Elicitation treatments:

After four weeks, callus were removed from vessels under aseptic condition and cut a suitable weight (500 mg) and subculture on the same medium for further proliferation. NaCl added to the medium at different concentrations 6, 8 and 10 dS/m to initiate salt stress. Callus cultures were incubated at 25 ± 1 ℃ under light condition (1,500 lux, 16 h/day). After 21 days treatment callus were harvested and washed with distilled water to remove remains agar and dried by filter paper.

2.4.Vegetative measurements:

Forty pieces of callus fresh weight (FW) and dry weight (DW) after drying at 70^{o}C until constant weight was recorded. Water content (WC) of callus was calculated using FW and DW values according to Henson *et al.* (1981).

2.5.Biochemical determinations:

Photosynthetic pigments (mg 100 g^{-1} FW) in callus (chl. a, b and carotenoids) were extracted with 85% of acetone and estimated spectrophotometrically at 662, 644 and 440.5 nm (Arnon, 1949). Anthocyanins concentration (mg g^{-1} FW) was determined after digesting callus with 1% of HCl (v/v) in methanol and kept overnight at 4ºC (Lange *et al.*, 1971). Then centrifuged at 3000 rpm for 10 min at 5°C. The values are calculated as E_{535} - 0.25 (E_{650}) /g FW. Total carbohydrates (mg g^{-1}DW) were determined according to Hedge and Hofreiter, (1962), dried samples digested by 5 ml of 2.5 N HCl for 3 hours at 100^{o}C .Then naturalized with sodium carbonate. Sample was made volume up to 10 ml and then filtrated. 50 µl was diluted to 1ml with distilled water. To each tube, 1ml of 5%

phenol and 5ml of 96% H2SO4 were added. After 10 min, tubes were shaken at 25-30 ^{0}C for 20 min. Optical density was measured at 490 nm as reported in. Total protein (mg/g FW) was determined by Bradford method (Bradford, 1976) at 595 nm. Proline as mg g^{-1}FW was estimated with ninhydrin reagent as described by Bates *et al.* (1973), the red color intensity was measured at 520 nm against the toluene blank. Total glutathione (µmol /mg protein) was determined at 412 nm by 5-5'-Dithiobis (2-nitrobenzoic acid) (DTNB) reagent (Griffith, 1980). For determination of total free amino acids and free phenols alcohol extraction of callus was prepared as Abdel-Rahman *et al.* (1975).Free phenolics (mg g^{-1} FW) were determined by a modified Folin-Ciocalteu method and measured at 650 nm according to Horwitz *et al.*, (1970). Total free amino acids (mg g^{-1} FW) was estimated using the method of Rosen colour (1957) with ninhydrin reagent .The blue colored was measured against blank sample at 570 nm. Hydrogen peroxide (mmol/g FW) was determined at 390 nm by the modified method according to Shi *et al.*, (2007) from Sun *et al.*, (2007). Malondialdehyde **(**µmol.g^{-1} FW) determined by the thiobarbituric acid (TBA) reaction as described by Gallego *et al.*, (1996) from Heath and Packer, (1968). All spectrophotometric analyses were done using UV/VIS spectrophotometer, PG instrument Ltd, USA.

2.6.Enzymatic antioxidants activity:

Enzymes solutions were prepared according to Urbanke *et al*., (1991). Catalase (CAT, E.C.:1.11.1.6) activity was determined by measured the oxidation of H_2O_2 at 240 nm (Urbanek *et al*., 1991). The unit of CAT activity was defined as the amount of enzyme, which decomposes 1 mM H_2O_2 per mg^{-1} protein.minute. Peroxidase (POD, E.C.: 1.11.1.7) activity estimated with 0.1% O-dianisidine and 0.2 M hydrogen peroxide at 430 nm (Urbanek *et al*., 1991). One unit of peroxidase activity was taken as the change of 1.0 unit of optical density per mg^{-1} protein.minute**.** Superoxide dismutase (SOD, E.C.: 1.15.1.1) activity was assayed by measuring its ability to inhibit reduction of nitro blue tetrazolium at 560 nm as described with (Beauchamp and Fridovich, 1971). One unit of enzyme activity represents the amount of enzyme required for 50 % inhibition of NBT reduction.

2.7. SDS-PAGE of soluble proteins

One dimensional SDS-PAGE gel electrophoresis based on the method of Laemmli, (1970) was used to fractionate the soluble proteins in callus. Twenty milligrams of callus were dispersed in 1 ml SDS 10% with 100 µl β-mercaptoethanol for 15 min, then centrifuged at 11000 rpm for 10 min. Twenty µl of extraction were mixed with 20 µl of SDS-loading sample buffer (SDS 4%, β-mercaptoethanol 3%, glycerol 20%, Tris HCl 50 mM pH 6.8 and bromophenol blue traces), heated at 96 °C for 3 min and 10 µl aliquot was electrophoresed (10 µl of protein/ lane). The resolving and stacking gels were prepared according to the standard procedure of Davis, (1964). The electrode buffer contained 50 mM TRIS, glycine 0.384 M and SDS 0.1%. The protein bands were developed with Commassie Brilliant Blue R-250 dye (0.2% solution, freshly prepared in 45% methanol, 10% glacial acetic acid and 45% distilled water) at room temperature overnight. The gel was photographed and made by scan apparatus as densitometric (optical density) analysis at 600 nm using standard maker protein (Pharmacia).

2.8. Histological investigations

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 (Johansen, 1940). Sections were cut with steel blade on rotary microtome. The fixed sections were stained with Safranin O-Fast-green double stain. After staining, sections mounted in Canada balsam (Sass, 1961). Observation and photomicrographs were achieved using research microscope (LEICA DM500) fitted with digital camera (LEICA ICC50). For staining alkaloid, callus was macerated and stained with Dragendorff's reagent**.**

2.9.Alkaloids determination

For alkaloids extraction, powdered dry callus was percolated overnight in methanol till complete exhaustion, and then methanol was removed by distillation under For alkaloids extraction. The residue obtained was stirred with 0.1 N HCl and extracted with CH_3Cl. The chloroform layer was washed with 0.1 N HCl and discarded. The combined acidic layer was then rendered alkaline with NH_4OH and extracted with CH_3Cl. The organic layer was collected then dried under vacuum to afford the crude alkaloid extract

(Karawya *et al.*, 1975). Total alkaloids concentration was determined using the method

reported in Ajanal et al., 2012; which in brief depends on the reaction between Bromocresol green and alkaloids in pH 4.7 to form a yellow colored complex which is extractable in chloroform and the color intensity obey Beer Lambert's law.

Atropine standard solution was made by dissolving 1 mg of Atropine (Sigma) in 10 ml distilled water. The absorbance of the complex in chloroform was measured at spectrum of 470 nm in UV/VIS spectrophotometer, PG instrument Ltd, USA against the blank prepared as above but without atropine.

Table 1: Description of *Hyoscyamus muticus* L. callus after 21 days as affected by different concentrations of NaCl.

NaCl (dS/m)	Cell			Symmetric of cells	Intercellular space
	Shape	length µm	width µm		
0.0 (control)	regular ovate	72 b	54 c	Symmetric	schizogenous
6	irregular amoeboid	108 a	81 a	Symmetric	schizogenous
8	spherical	109 a	73 b	symmetric	schizogenous
10	rregular amoeboid	73 b	51 c	symmetric	Lysiogenous

2.10. Determination of Hyoscyamine concentration:

High-Performance Thin-Layer Chromatography (HPTLC) was performed on 20 cm ×10 cm HPTLC silica gel 60 F_{254} plates (Merck) with a mobile phase consisting of chloroform : methanol : acetone : aqueous ammonia (25%) 75 : 15 : 10 : 1.6 (v/v/v/v) (Jaremicz *et al.*, 2014). Hyoscyamine standard (Sigma) was diluted by chloroform to final concentration 20, 40, 60, 80, 120, 160 µg/ml. All samples and standards were applied to the plates by means of CAMAG Linomat 5 with dosing syringe 100 µL as 7 mm bands with 10.5 mm distance between tracks, application X 15 mm and 13 mm application Y edges of plate and the application volume was 1–20 µL for samples and 2-8 µL for standard. Loaded HPTLC plates were developed to a distance of 50 mm in Camag Automatic Developing Chamber CADC 2 at room temperature. The development occurring in a two- steps (Preconditioning with 10 ml mobile phase for 5 minutes and development with 25ml mobile phase for 20 minutes). The plate was developed to a distance of 50 mm and dried for 5 min by a stream of warm air, then the plates derivatized with 200 ml Dragendorff's reagent using Chromatogram Immersion Device. Then the plate was scanned and examined densitometry at l = 550 nm by means of CAMAG TLC Scanner 4 with slit dimension of 6 x 0.30 mm.

2.11. Statistical analysis

All data were statistically analyzed as randomized complete blocks design (Steel *et al*., 1997). Analysis of variance (one-way analysis; ANOVA) and means comparisons (Duncan's multiple range tests, 5%) were performed using the MSTAT-C statistical pack-age (M-STAT, 1990).

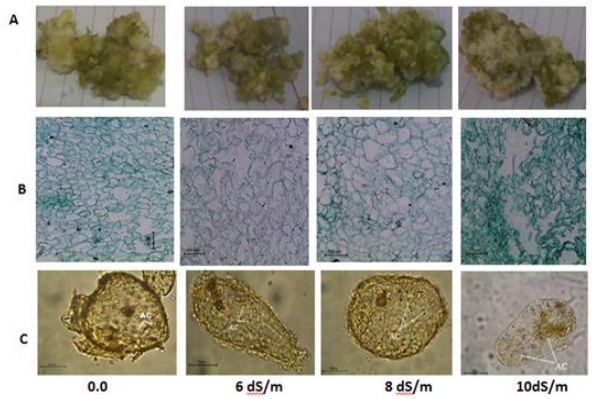

Figure 1: Effect of different concentrations of NaCl on growth (A) , histology (B) and orange-stained alkaloid crystals (C) in callus of *Hyoscyamus muticus* L., after 21 days

3. Results and discussion:

3.1. Results

3.1.1. Growth, histological description and alkaloid crystals of callus cells:

The stimulating effect of different levels of NaCl on callus growth after 21days showed in **Figure (1)**. Shape of callus cells differed according to NaCl levels as shown in **Figure (1B)**. Cells had ovate or spherical shape in 8dS/m of NaCl and unstressed callus while it was amoeboid in low or high levels of salt stressed one. The highest values of cell length and width were found in 6 and 8 dS/m of NaCl stressed callus compared to control or High level of NaCl stressed one. Lysiogenous

intercellular space was abundant in high level of NaCl exposed callus compared to schizogenous one in other treatments. Higher abundance of orange-stained crystals of alkaloids in callus cells was found in all salt stressed callus cells especially under 10 dS/m of NaCl as shown in **Figure (1C)**.

3.1.2. The vegetative growth and total carbohydrates of callus:

The fresh (FW) and dry (DW) weight of henbane callus were gradually increased with increment of NaCl level in MS medium. High level of NaCl (10 dS/m) increased the FW and DW by 4.1 and 2.8 fold more than control, respectively **(Figure 2A,B)**. Slight increment (1%) of water content in salt stressed callus compared to unstressed callus was observed **(Figure 2C)**. Total carbohydrate content significantly reduced under all NaCl levels. The highest reduction of total carbohydrates (24.3%) was determined in 10dS/m of NaCl stressed callus compared to control as showed in **(Figure 2D)**.

3.1.3. pigments:

Photosynthetic pigments (Chl. a, b, and carotenoid) concentrations were gradually decreased in callus under all NaCl levels compared to untreated one. The highest reduction of Chl.a and carotenoid concentration by 31.5 and 26 % was observed at high NaCl level (10 dS/m) lower than control, respectively **(Figure 3A,C)**. Also, the highest reduction of Chl. b (28.1%) was observed in callus treated with moderate level of NaCl (8 dS/m) compared to control **(Figure 3B)**. In contrary, anthocyanin content was increased 3 fold in 10 dS/m of NaCl stressed callus compared to untreated one as shown in **Figure (3D)**.

3.1.4.Nonenzymatic antioxidants and H_2O_2

Both free phenolics and glutathione concentrations had opposite trend in response to NaCl treatments. So free phenolics were decreased by 21 and 30% **(Figure 4B)** the glutathione content was increased by 34 and 20 % **(Figure 4A)** in 6 and 8 dS/m of NaCl stressed callus respectively compared to control. Also, the content of both phenolics and glutathione increased by 8 and 4.6% in 10 dS/m of NaCl streesed callus more than control, respectively but without significant differences. Data in **Figure (4C)** indicated the slight reduction in proline content at 6 and 8 dS/m of NaCl stressed callus, but marked reduction occurred at 10 dS/m by 50.6 % in relation to control. The lowest value (1.53 mmol/g FW) of H_2O_2 was determined in 10 dS/m of NaCl stressed callus with reduction by 16.8 % compared to untreated one **(Figure 4D)**.

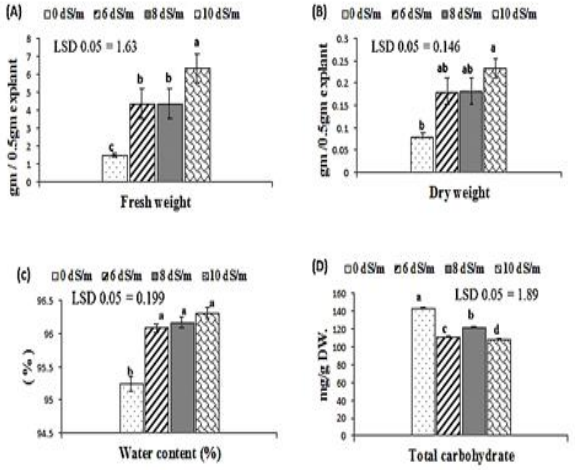

Figure 2: Effect of different concentrations of NaCl on fresh, dry weight ,water content and total carbohydrate of callus of *Hyoscyamus muticus* L., after 21 days.

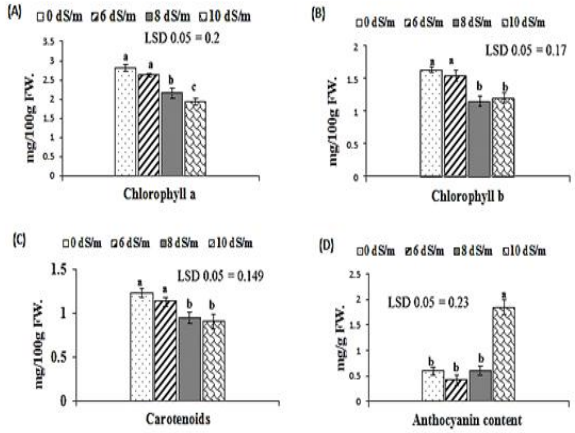

Figure 3: Effect of different concentrations of NaCl on pigments (Chl a, Chl b, carotenoid and anthocyanin) of callus of *Hyoscyamus muticus* L., after 21 days.

3.1.5.Enzymatic antioxidants and MDA

The activity of peroxidase (POD) and superoxide dismutase (SOD) were increased with rising of NaCl level in MS medium, while the catalase (CAT) activity was decreased as shown in **Figure (5)** . Activity of POD and SOD increased by 2.6 and 2.3 fold in 10dS/m of NaCl stressed callus more than untreated callus, respectively. However, malondialdehyde (MDA) content was decreased

under salt stress condition compared to normal one. The minimum concentration (1.27 µmol/g FW) of MDA was found in 8 dS/m of NaCl stressed with 56.7% reduction lower than control **(Figure 5D)**.

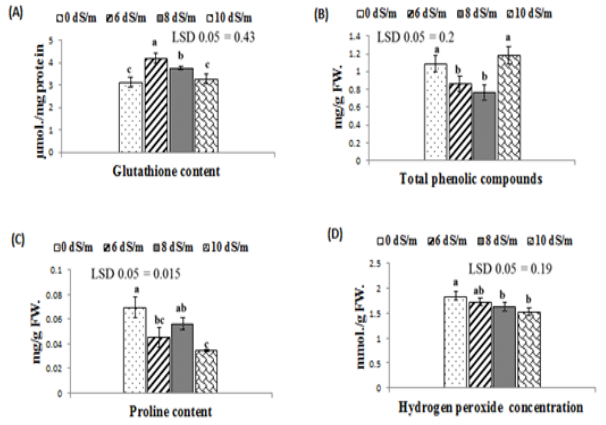

Figure 4: Effect of different concentrations of NaCl on glutathione (A), total phenolic compound (B), proline (C), and H_2O_2 (D) of callus of *Hyoscyamus muticus* L., after 21 days.

3.1.6. Alkaloids and nitrogenous compounds

Protein content was gradually decreased by 41.2, 17.1 and 11.7 % in, 6, 8 and 10 dS/m of NaCl stressed callus respectively, compared to control **(Figure 6B)**. The concentration of free amino acids was decreased in callus under both low and moderate level by 48 and 18.4 % compared to control, respectively. Increment of free amino acids content was observed in 10 dS/m of NaCl stressed callus without significant differences with control **(Figure 6A)**. Total alkaloids content was increased 2-fold in callus treated with 10dS/m of NaCl compared to untreated callus or wild leaves. Moreover, low and moderate level of NaCl reduced the total alkaloid by 22.7 and 11.5%, respectively compared to control or wild plants **(Figure 6C)**. In contrary, hyoscyamine content was decreased under all NaCl levels. In this respect, wild leaves synthesized the highest amount (2.8 mg/g DW) of hyoscyamine which was double amount as low NaCl level **(Figure 7)**.

3.1.7.protein profile

Soluble protein of NaCl stressed or unstressed callus were electrophoretic analyzed by one dimensional SDS-PAGE gel as showed in **Figure (8)**. Densitometric analysis revealed that soluble protein was fractioned into 7 varied bands with molecular weights (MW) 240, 109 , 44, 38, 32, 16 and 10 KDa. The unique band (109 KDa) was only expressed in 10 dS/m stressed callus. Bands with MW 240, 16 and 10 had high expression (as high optical density) in 8 dS/m of NaCl stressed callus. While, bands with MW 44, 38 and 32 had high expression in 10 dS/m of NaCl stressed callus. Protein bands were similar by 100% between unstressed callus and 6 or 8 dS/m of NaCl stressed one, then similarity % was low with 10 dS/m of NaCl stressed one.

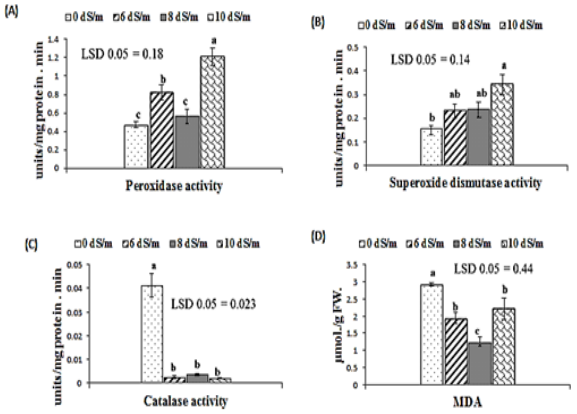

Figure 5: Effect of different concentrations of NaCl on POD (A), SOD (B), CAT (C), and MDA (D) of callus of *Hyoscyamus muticus* L., after 21 days.

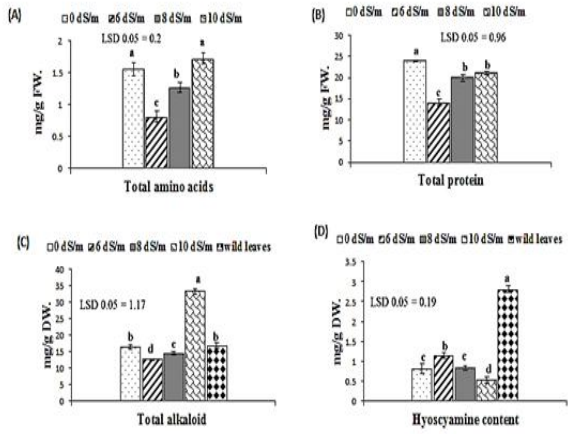

Figure 6: Effect of different concentrations of NaCl on free amino acids (A), protein (B), total alkaloid (C), and hyoscyamine (D) of callus of *Hyoscyamus muticus* L., after 21 days.

3.2. Disscusion:

Creating a case of salt stress in the medium of plant callus cultures is a potential method for

secondary metabolites accumulation which may be much more effective than studies performed on intact plant due to the absence of root barrier (Vanishree *et al.*, 2004; Namdeo, 2007). Results reported herein showed that the total alkaloids concentration had doubled under high level of NaCl (8 dS/m) compared to unstressed callus or wild leaves, while the hyoscyamine accumulation was higher in wild leaves compared to all NaCl stressed callus **(Figure 7)**. However, the concentration of hyoscyamine was increased by 40 % in callus treated with low level of NaCl (6dS/m) compared to untreated one. Results were agreed with Mahajan *et al.*, (2005) who reported that high salt stress may be increase or decrease a specific secondary metabolite in plants.

The data obtained from our investigation revealed that callus cells of Egyptian henbane were tolerant to NaCl salt stress due to the enhancement of both fresh and dry biomass as shown in **(Figure 2)**. These findings were found in different plant species such as *in vitro* Cassava plantlets treated with 10mM NaCl (Cheng *et al.*, 2018). In contrary, Aljibouri *et al.*, (2012) found a reduction of both FW and DW of *Hyoscyamus niger* callus after application of abiotic elicitors. Results cleared that Photosynthetic pigments such a Chl a, b and carotenoids were susceptible to salt stress due

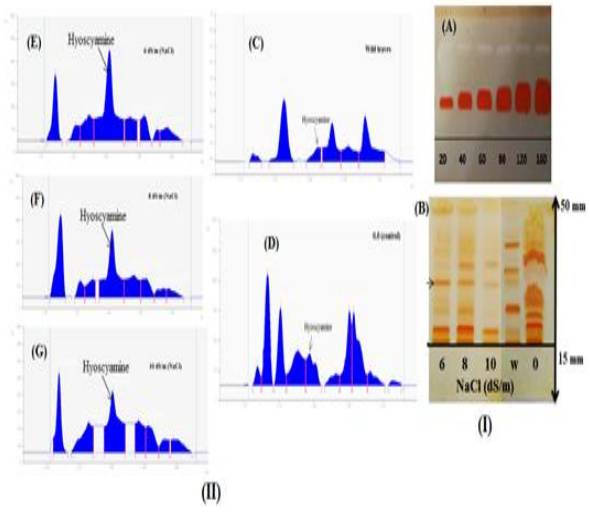

Figure 7: (I) Chromatogram of alkaloid fraction from (A) hyoscyamine standard solutions (µg/ml) and (B) *Hyoscyamus muticus* callus. (II) Densitogram of alkaloid fraction from *Hyoscyamus muticus* callus recorded at λ=550nm after derivatisation with Dragendorff's reagent, (C) wild leaves, (D) untreated callus, (E) 6 dS/m NaCl, (F) 8 dS/m NaCl and (G) 10 dS/m NaCl.

to its reduction under all NaCl levels, while anthocyanin was a stable pigments, so it increased by 3.1 fold compared to unstressed callus **(Figure 3)**. Decreasing the Chls. content in the salt-affected plants could be due to the destruction of the alkaloid protein complex or degradation of Chls. by chlorophyllase (Rao and Rao, 1981). Under salt stress the photosynthetic pigments were decreased in callus of different species such as of *Capsicum annuum* (Sen and Alikamanoglu, 2011) and wheat (El-Kaaby *et al.*, 2017).

Our results reinforced the hypothesis that anthocyanin participated in salt resistance mechanism in tolerant plant species as tomato and red cabbage (Steyn *et al.*, 2002 and Winkel-Shirley, 2002, Eryılmaz, 2006).

High salt concentrations resulted in oxidative stress due to the formation of reactive oxygen species. Effective enzymatic and nonenzymatic antioxidant system is one of the most common protection mechanism that plants evolve to stabilize important macromolecules which deleteriously affected by the ROS. (Shi *et al.* 2006). Results cleared that henbane callus depend on peroxidase (POD) and superoxide dismutase (SOD) more than catalase (CAT) as enzymatic antioxidant mechanism to quench the ROS under salt stress, as POD and SOD were significantly increased by 2.6 and 2.3 fold in 10 dS/m of NaCl stressed callus compared to control, respectively, while CAT activity was decreased **(Figure 5)**. This findings were agreed with Foyer and Noctor (2000) who reported that, SOD is a key enzyme that plays an important role in cellular defense and catalyze the dismutation of superoxide radicals to H_2O_2 and superoxide. Also, Yamasaki *et al.*, (1997) reported that POD is oxidoreductase, which efficiently catalyze the oxidation of H_2O_2 with phenolic compounds or ascorbic acid. Enhancement of POD and SOD activity was detected in different plant species under salt stress as *Jatropha curcas* callus(Kumar *et al.*, 2008) and *Punica granatum* callus (Bonyanpour and Khosh-Khui, 2013) *Capsicum annuum* callus (El-Kaaby *et al.*, (2017) callus cultures of sugarcane (Munir and Aftab, 2013). Reduction of CAT activity also reported in callus of different species such as *Solanum tuberosum* (Queirós *et al.*, 2011) and *Salsola baryosma* (Sharma and Ramawat, 2014). Results also showed that, glutathione content was significantly increased by 34 and 20 % at 6 and 8 dS/m,

respectively compared to control. This finding was in line with Queirós *et al.*, (2011) who found the reduction of glutathione reductase activity in salt-exposed callus of *Solanum tuberosum*. Free amino acids were slightly increased by 10.7 % in 10 dS/m of NaCl stressed callus compared to control **(Figure 6A)**. Results were coordinated with Hartzendorf and Rolletschek, 2001 who found a significantly increment of total free amino acids after application of NaCl in *Phragmites australis*. However, both free phenolics and proline content were decreased in callus under all NaCl levels **(Figure 4)**. This result was in opposite with Ahmed *et al*., (2012). who found increment of total phenolic in Garden cress cultured under NaCl treatment, in *Trianthema triquetra* callus cultures (Sharma and Ramawat, 2014). Also, proline increased in callus culture of *Salsola baryosma*, *Trianthema triquetra* and *Zygophyllum simplex* under the salinity stress (Sharma and Ramawat, (2014).

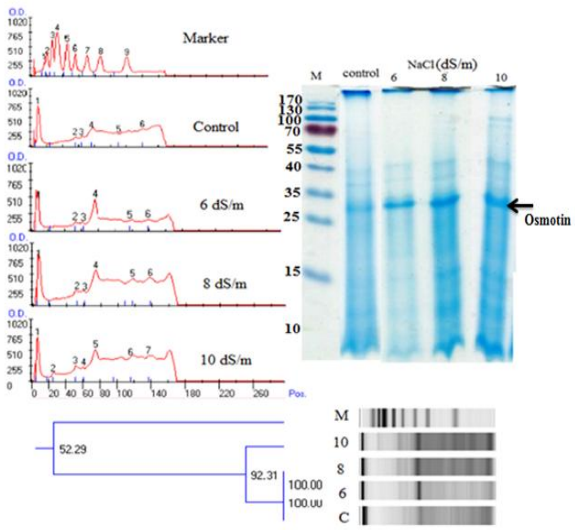

Figure 8: SDS-PAGE of protein profile , optical densities of each protein band and similarity of each concentration of NaCl of callus of *Hyoscyamus muticus* L., after 21 days.

Decreasing of Malondialdehyde (MDA) and H_2O_2 concentration to minimum values (1.265 µmol. /gFW and 1.53 mmol /gFW, respectively) in henbane callus under all NaCl levels may be attributed with effective function of anthocyanin, glutathione, POD and SOD activity in scavenging the ROS under salt stress. This result was in line with Queirós *et al.*, (2011) who found that lipids peroxidation were found in salt sensitive callus in *Solanum tuberosum*. The H_2O_2 concentration was increased in four cassava cultivars *in vitro* shoots and decreased in another one under salt stress (Cheng *et al.*, 2018).

Protein **(Figure 6B)** and carbohydrate content **(Figure 2D)** were reduced in all NaCl levels. This reduction may be due to its degradation by active ROS (El-Mashad and Mohamed, 2012). Protein degradation may be essential to provide amino acids for synthesis of new proteins as osmotin suited for growth or survival under stress conditions and also substrates for energy production (Kocisko *et al.*, 1994). Reduction of protein content was occurred in different plants under salt stress as wheat (Sen and Alikamanoglu, 2011), *Salsola baryosma*, *Trianthema triquetra* and *Zygophyllum simplex* (Sharma and Ramawat, 2014). In contrary, soluble sugars content increased in *Atriplex halimus* NaCl stresses (Martinez *et al*., 2004). Results reported herein showed that osmotin protein with molecular weight 32 KDa was highly expressed in 10 dS/m of NaCl stressed callus. Results were in line with Hassanein, (1999) who reported that proteins with MW 127 and 52kDa highly induced in peanut under NaCl stress. Also, Kumar *et al.*, (2008) found that protein bands with MW 98, 69, 66 and 64 kDa were highly expressed in NaCl treated callus of *Jatropha curcas* while other bands with MW as 83, 58 and 43 kDa proteins were low expressed.

4. Conclusion:

NaCl at 10 dS/m gave the highest content of total alkaloids in Egyptian henbane callus culture. Hyoscyamine concentration was high in wild leaves than salt stressed callus. High accumulation of alkaloids and antioxidant agents under saline condition improved callus growth. Protein bands can be used as stable indicator for callus tolerance to salt stress.

5. References

Abdel-Rahman, M., Thomas, T. H., Doss, G. J., Howell, L. 1975: Changes in endogenous plant hormones in cherry tomato fruits during development and maturation. *Physiologia plantarum*, 34(1), 39-43.

Abreu, I. N. Sawaya, A.C.H. F., Eberlin, M. N., Mazzafera, P. 2005. Production of pilocarpine in callus of jaborandi (*pilocarpus microphyllus* stapf)', *In Vitro Cellular and Developmental Biology - Plant*, 41(6), pp. 806–811.

Ahmed, A. R., Gabr, A. M. M., Sayed, M. A. 2012.

Effect of Drought and Salinity Stress on Total Phenolic, Flavonoids and Flavonols Contents and Antioxidant Activity in *in vitro* Sprout cultures of Garden cress (*Lepidium sativum*). *Journal of Applied Sciences Research*, 8(8), pp. 3934–3942.

Ajanal, M., Gundkalle, M. B. , Nayak, S. U. 2012. Estimation of total alkaloid in Chitrakadivati by UV-Spectrophotometer. *Ancient science of life*, 31(4), pp. 198–201.

Alaghemand, A., Ghorbanpour, M. , Moghaddasian, B. 2013. The Role of Gibberellic Acid, Sulfuric Acid and Cold Stratification. *Bulletin of Environment, Pharmacology and Life Sciences*, 2, 99–103.

Aljibouri, A. M. J., Al-samarraei, K. W., Abd, A. S., Mageed, D. M., Ali, A. J. A. 2012. Alkaloids Production from Callus of Hyoscyamus niger L . *in Vitro. Journal of Life Sciences*, 6, 874–882.

Arnon, D. I. 1949. Copper enzymes in isolated chloroplasts: polyphenol oxidase in *Beta vulgaris*. *Plant physiology*, 24(1), 1.

Aşci, Ö. A., Demirci, T., Baydar, N. G. 2018. Effects of NaCl applications on root growth and secondary metabolite production in madder (*Rubia tinctorum* L.) root cultures. *International journal of secondary metabolite*, 5(3), 210-216.

Batanouny, K. H., Aboutabl, E., Shabana, M., Soliman, F. 1999. Wild medicinal plants in Egypt. *With support of the Swiss Development Co-operation (SDC). Academy of Scientific Research and Technology, Egypt. The World Conservation Union (IUCN), Switzerland*, 60-64.

Bates, L. S., Waldren, R. P., Teare, I. D. 1973. Rapid determination of free proline for water-stress studies. *Plant and soil*, 39(1), 205-207.

Beauchamp C., Fridovich I. 1971. Superoxide dismutase: Improved assays and an assay applicable to acrylamide gels. *Analytical biochemistry*, 44:276-287.

Breusegem, F. V., Dat, J. F. 2006. Reactive Oxygen Species in Plant Cell Death. *Plant Physiology*. 141 (2): 384–390.

Bonyanpour, A. , Khosh-Khui, M. 2013. Effects of salt and drought stress conditions on callus growth, proline content and antioxidant enzyme avtivity of *Punica granatum* (Nana). *BioTechnology: An Indian Journal*, 7(7), pp. 257–262.

Bradford, M. M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical biochemistry*, 72(1-2), 248-254.

Chalker-Scott, L. 1999. Environmental significance of anthocyanins in plant stress responses. *Photochemistry and photobiology*, 70(1), 1-9.

Cheng, Y. E., Dong, M. Y., Fan, X. W., Nong, L. L., Li, Y. Z. 2018. A study on cassava tolerance to and growth responses under salt stress. *Environmental and Experimental Botany*, 155, 429–440.

Colling, J., Stander, M. A. Makunga, N. P. 2010. Nitrogen supply and abiotic stress influence canavanine synthesis and the productivity of *in vitro* regenerated *Sutherlandia frutescens* micro shoots. *Journal of plant physiology*, 167(17), pp. 1521–1524.

Davis, B. J. 1964. Disc electrophoresis–II method and application to human serum proteins. Annals of the New York academy of sciences, 121(2), 404-427.

El -Kaaby, E. ., AI-Anny, J. , Almaliky, E. S. 2017. *In Vitro* effect of exogenous NaCl on enzyme activity, chlorophyll and carotene accumulation in callus of chilli pepper (*Capsicum annuum* L .) explants. *international journal of multidisciplinary and current research*, 5, pp. 253–255.

El-Mashad, A. A. A. and Mohamed, H. I. 2012. Brassinolide alleviates salt stress and increases antioxidant activity of cowpea plants (*Vigna sinensis*). *Protoplasma*, 249(3), 625-635.

El-shennawy, O. A., El-torky, M. G., El-mokadem, H. E., Abass, B. I. 2017. Effect of NaCl and Phenylalanine on the Production of some Secondary Metabolites in In Vitro Cultures of *Mentha longifolia*. *Alexandria science exchange journal*, 38(3), 577-587.

Eryılmaz, F. 2006. The relationships between salt stress and anthocyanin content in higher plants. *Biotechnology and biotechnological equipment*, 20(1), 47–52.

Fatima, S., Mujib, A., Tonk, D. 2015. NaCl amendment improves vinblastine and vincristine

synthesis in *Catharanthus roseus*: a case of stress signalling as evidenced by antioxidant enzymes activities. *Plant Cell, Tissue and Organ Culture*, 121(2), pp. 445–458.

Foyer, C. H., Noctor, G. 2000. Tansley Review No. 112 Oxygen processing in photosynthesis: regulation and signalling. *The New Phytologist*, 146(3), 359-388.

Gallego, S. M., Benavides, M. P., Tomaro, M. L. 1996. Effect of heavy metal ion excess on sunflower leaves: evidence for involvement of oxidative stress. *Plant Science*, 121(2), 151-159.

Griffin, W. J., Lin, G. D. 2000. Chemotaxonomy and geographical distribution of tropane alkaloids. *Phytochemistry*, 53(6), 623-637.

Griffith, O. W. 1980. Determination of glutathione and glutathione disulfide using glutathione reductase and 2-vinylpyridine. *Analytical Biochemistry*, 106(1), 207-212.

Haghighi, Z., Modarresi, M. and Mollayi, S. 2012. Enhancement of compatible solute and secondary metabolites production in Plantago ovata Forsk. by salinity stress. *Journal of Medicinal Plants Research*, 6(18), 3495-3500.

Harborne, J. B., Williams, C. A. 2000. Advances in flavonoid research since 1992. *Phytochemistry*, 55(6), 481-504.

Hartzendorf, T. and Rolletschek, H. 2001. Effects of NaCl-salinity on amino acid and carbohydrate contents of *Phragmites australis*. *Aquatic Botany*. 69(2–4), pp. 195–208.

Hassanein, A. M. 1999. Alterations in protein and esterase patterns of peanut in response to salinity stress. *Biologia plantarum*, 42(2), 241-248.

Heath, R. L., Packer, L. 1968. Photoperoxidation in isolated chloroplasts: I. Kinetics and stoichiometry of fatty acid peroxidation. *Archives of biochemistry and biophysics*, 125(1), 189-198.

Hedge, J. E. , Hofreiter, B. T. 1962. In carbohydrate chemistry 17 (Eds whistler RL and Be Millee, JN) Academic press. New York.

Henson, I. E., Mahalakshmi, V., Bidinger, F. R. , Alagarswamy, G. 1981. Genotypic variation in pearl millet (*Pennisetum americanum* L. Leeke), in the ability to accumulate abscisic acid in response to water stress. *Journal of experimental botany*, 899-910.

Horwitz, W., Chichilo, P. ,Reynolds, H. 1970. Official methods of analysis of the Association of Official Analytical Chemists. *Official methods of analysis of the Association of Official Analytical Chemists*.

Jaremicz, Z., Luczkiewicz, M., Kisiel, M., Zárate, R., Jaber-Vazdekis, N. E., Migas, P. 2014. Multi–development–HPTLC Method for Quantitation of Hyoscyamine, Scopolamine and their Biosynthetic Precursors in Selected Solanaceae Plants Grown in Natural Conditions and as *In vitro* Cultures. *Phytochemical Analysis*, 25(1), 29-35.

Johansen, D. A. 1940. Plant microtechique. McGraw-Hill Book Company, Inc.; London, 523p.

Karawya, M. S., Abdel-Wahab, S. M., Hifnawy, M. S., & Ghourab, M. G. (1975). Colorimetric and gas-liquid chromatographic determination of atropine-hyoscyamine and hyoscine (scopolamine) in solanaceous plants. *Journal-Association of Official Analytical Chemists*, 58(5), 884-887.

Kennedy, B. ,De Filippis, L. F. 1999. Physiological and oxidative response to NaCl of the salt tolerant Grevillea ilicifolia and the salt sensitive Grevillea arenaria. *Journal of plant physiology*, 155(6), 746-754.

Khavari-Nejad, R. A. , Mostofi, Y. 1998. Effects of NaCl on photosynthetic pigments, saccharides, and chloroplast ultrastructure in leaves of tomato cultivars. *Photosynthetica*, 35(1), 151-154.

Kocisko, D. A., Come, J. H., Priola, S. A., Chesebro, B., Raymond, G. J., Lansbury, P. T. , Caughey, B. 1994. Cell-free formation of protease-resistant prion protein. *Nature*, 370(6489), 471.

Kumar, N., Pamidimarri, S. D., Kaur, M., Boricha, G. , Reddy, M. P. 2008. Effects of NaCl on growth, ion accumulation, protein, proline contents and antioxidant enzymes activity in callus cultures of Jatropha curcas. *Biologia*, 63(3), 378.

Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*, *227*(5259), 680.

Lange, H., Shropshire, W., Mohr, H. 1971. An analysis of phytochrome-mediated anthocyanin synthesis. *Plant Physiology*, 47(5), 649-655.

LaRosa, P. C., Singh, N. K., Hasegawa, P. M. ,

Bressan, R. A. 1989. Stable NaCl tolerance of tobacco cells is associated with enhanced accumulation of osmotin. *Plant Physiology*, 91(3),855-861.

Mahajan, S. ,Tuteja, N. 2005. Cold, salinity and drought stresses: an overview. *Archives of biochemistry and biophysics*, 444(2), 139-158.

Mansour, M. M. F. 2000. Nitrogen containing compounds and adaptation of plants to salinity stress. *Biologia Plantarum*, 43(4), 491-500.

Martìnez, J. P., Lutts, S., Schanck, A., Bajji, M. , Kinet, J. M. 2004. Is osmotic adjustment required for water stress resistance in the Mediterranean shrub *Atriplex halimus* L. *Journal of Plant Physiology*, 161(9), 1041-1051.

M-STAT. 1990. A Microcomputer program for the design management and analysis of agronomic research experiments. Michigan state university.

Munir, N. , Aftab, F. 2013. Changes in activities of antioxidant enzymes in response to NaCl stress in callus cultures and regenerated plants of sugarcane. *Journal of Animal and Plant Sciences*, 23(1), pp. 203–209.

Naik, P. M. ,Al-Khayri, J. M. 2016. Impact of abiotic elicitors on in vitro production of plant secondary metabolites: a review. *Journal of advanced Research in Biotechnology*, 1(2), 7.

Namdeo, A. G. 2007. Plant cell elicitation for production of secondary metabolites: a review. *Pharmacognosy Review*, 1(1), 69-79.

Ni, J., Yang, X., Zhu, J., Liu, Z., Ni, Y., Wu, H. , Liu, T. 2015. Salinity-induced metabolic profile changes in *Nitraria tangutorum* Bobr. suspension cells. *Plant Cell, Tissue and Organ Culture* (PCTOC), 122(1), 239-248.

Queirós, F., Rodrigues, J. A., Almeida, J. M., Almeida, D. P., Fidalgo, F. 2011. Differential responses of the antioxidant defence system and ultrastructure in a salt-adapted potato cell line. *Plant Physiology and Biochemistry*, 49(12), 1410-1419.

Rao, G.G., Rao, G.R. 1981. Pigment composition & chlorophyllase activity in pigeon pea (*Cajanus indicus* Spreng) & Gingelley (*Sesamum indicum* L.) under NaCl salinity. *Indian Journal of Experimental Biology*, (19), 768-770.

Rosen, H. 1957. A modified ninhydrin colorimetric analysis for amino acids. *Archives of biochemistry and biophysics*, *67*(1), 10-15.

Sajid Aqeel Ahmad, M., Javed, F. , Ashraf, M. 2007. Iso-osmotic effect of NaCl and PEG on growth, cations and free proline accumulation in callus tissue of two indica rice (*Oryza sativa* L.) genotypes. *Plant growth regulation*, 53(1), pp. 53–63.

Sass, J.E. 1961. Botanical Microtechnique.Third edition.The IOWA State University Press.Amess. Iowa.

Selmar, D. 2008. Potential of salt and drought stress to increase pharmaceutical significant secondary compounds in plants. *Landbauforschung Volkenrode*, 58(1–2), pp. 139–144.

Sen, A. , Alikamanoglu, S. 2011. Effect of salt stress on growth parameters and antioxidant enzymes of different wheat (*Triticum Aestivum* L .) varieties on *in vitro* tissue culture. *Fresenius Environmental Bulletin*, 20(2), 489–495.

Sharma, V. , Ramawat, K. G. 2014. Salt stress enhanced antioxidant response in callus of three halophytes (*Salsola baryosma, Trianthema triquetra, Zygophyllum simplex*) of Thar Desert. *Biologia (Poland)*, 69(2), 178–185.

Shi, Q., Ding, F., Wang, X. , Wei, M. 2007. Exogenous nitric oxide protect cucumber roots against oxidative stress induced by salt stress. *Plant physiology and biochemistry*, 45(8), 542-550.

Singh, N. K., Bracker, C. A., Hasegawa, P. M., Handa, A. K., Buckel, S., Hermodson, M. A. , Bressan, R. A. 1987. Characterization of osmotin: a thaumatin-like protein associated with osmotic adaptation in plant cells. *Plant physiology*, 85(2), 529-536.

Steel, R. G. D., Torrie, J. H. ,Dickey, D. A. 1997. Principles and procedures of statistics: a biometrical approach., 3rd ed. (McGraw-Hill: New York).

Steyn, W. J., Wand, S. J. E., Holcroft, D. M. ,Jacobs, G. 2002. Anthocyanins in vegetative tissues: a proposed unified function in photoprotection. *New Phytologist*, 155(3), 349-361.

Sun, B., Jing, Y., Chen, K., Song, L., Chen, F., Zhang, L. 2007. Protective effect of nitric oxide on iron deficiency-induced oxidative stress in maize (*Zea mays*). *Journal of plant physiology*, 164(5), 536-543.

Tackholm, V. 1974. Students flora of Egypt. 2nd Ed. Cairo University, Cairo, 888p.

Takahama, U., Oniki, T. 1997. A peroxidase/phenolics/ascorbate system can scavenge hydrogen peroxide in plant cells. *Physiologia Plantarum*, 101(4), 845-852.

Urbanek, H., Kuzniak-Gebarowska, E., Herka, K. 1991. Elicitation of defence responses in bean leaves by Botrytis cinerea polygalacturonase. *Acta Physiologie Plantarum (Poland)*,13:43-50.

Vanishree M., Lee C.Y., Lo S.F., Nalawade SM, Lin C.Y. , Tsay S.H. 2004. Studies on the production of some important metabolites from medicinal plants by plant tissue cultures. *Botanical Academic Science* 45: 1-22.

Wang, L., Burhenne, K., Kristensen, B. K. , Rasmussen, S. K. 2004. Purification and cloning of a Chinese red radish peroxidase that metabolise pelargonidin and forms a gene family in Brassicaceae. Gene, 343(2), 323-335.

Winkel-Shirley, B. 2002. Biosynthesis of flavonoids and effects of stress. *Current opinion in plant biology*, 5(3), 218-223.

Yamasaki, H., Sakihama, Y., Ikehara, N. 1997. Flavonoid-peroxidase reaction as a detoxification mechanism of plant cells against H_2O_2. *Plant Physiology*, 115(4), 1405-1412.