

Syndrome effects of NaCl and Epibrassinolide on certain molecular and biochemical activities of salt-sensitive *Phaseolus vulgaris* cv. Brunco L. grown under *in vitro* condition

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Abstract: The present investigation, aimed to evaluate the application efficacy of 24-Epibrassinolide to evoke NaCl stress tolerance of *Phaseolus vulgaris* L. cv. Brunco. *In vitro* plantlets cultured on MS amended with 24-Epibrassinolide and /or different concentrations of NaCl for 3/10 d were analyzed. Sodium chloride (100,150 mmol) reduced photosynthetic pigments, carbohydrates and antioxidant enzymes, however, the 50 mmol alone and along with 24-Epi improved most previous attributes. Salinity stress seriously causes membrane damage evidenced as hydrogen peroxide and malondialdehyde increment, while applying 24-Epi alone and concomitant with salts reduced membrane damages. Proline showed syndrome relation with stress levels with further increment after hormonal application suggesting photosynthetic machinery protection against oxidative stress. High level of sodium and chlorine ions were recorded under salt stress associated with reduction in potassium, calcium ions accumulation, however, 24-Epi successfully alleviated ions toxicity and restore ions balance. All tested plantlets exhibited changes in the endogenous plantlet hormones. SDS-PAGE explore unique protein 77 KDa expressed under 24-Epi alone. Salt stress along with 24-Epi application imply impact on the expression of specific oxidative stress marker genes like peroxidase and polyphenol oxidase and point to priority roles for PPO over POD isozymes under salt stress. Our findings provide integrated evidence that *in vitro* 24-Epi application is effective in amelioration of NaCl stress. [E.M. Nafie, A.A. Khalfallah and R.M. Mansur. **Syndrome effects of NaCl and Epibrassinolide on certain molecular and biochemical activities of salt-sensitive *Phaseolus vulgaris* cv. Brunco L. grown under *in vitro* condition.** *Life Sci J* 2015;12(7):119-136]. (ISSN:1097-8135). <http://www.lifesciencesite.com>. 13

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1. Introduction

Increases in salinity tolerance for the world crops are an important goal and a major challenge as the world's population is increasing more quickly than the area of agricultural land (FAO, 2010). Salt-tolerance may be defined as the ability of a plant to safely and potentially grow and complete its life cycle under stressful salt conditions like NaCl (Yadav *et al.*, 2011). Global food production has to be increased by 38% by 2025 (Wild, 2004) to keep supplying food for developing world populations. Most of the cultivate land has been utilized and spreading into new areas is rarely possible and reasoned as due to urban spreading, climate warming and the continuous accumulation of salt in cultivated soils as a result of land clearing or irrigation causing salinization of previously productive land globally (Rengasamy,2006) so food production enhancements is restricted by human-induced salinity, along with the natural salinity particularly relevant in most arid and semiarid regions of the world (Rengasamy, 2010). A bout 33% of the cultivated land and most extensions of agricultural land in Egypt are already salinized (Ghassemi *et al.*, 1995). The aim therefore, should be an increase in yield per unit of land rather than in the area cultivated.

Various strategies have been considered to improve plant growth and its yield under stressful condition like salt. A fundamental approach is to

develop salt-tolerant plants. Breeding technique for salt tolerance is a long-term endeavor and its extensive processes have delayed development of successful salt-tolerant cultivars in most crop species. The high efficiency of *in vitro* selection technique among various already established techniques is considered as powerful and successful tool because of its regulated conditions whilst additional genetic variations can be induced to elucidate mechanisms involved in salt tolerance (Saleem *et al.*, 2005).

Exploring suitable ameliorants is one of the tasks of plant biologists. In recent decades, an alternative strategy to induce tolerance through exogenous application of protectants like plant growth-regulating compounds among them brassinosteroids (Vardhini and Rao, 1997) potentially showed the capacity to enhance the plant growth, yield as well as stress tolerance under salinity (Ali *et al.*, 2007).

Salinity is one of the most crucial abiotic stresses that not only limit increase in the productivity of food crops but also limit increasing the appropriate areas for agriculture (Reguera *et al.*, 2012). Among the most common effects of salinity is growth inhibition by NaCl impacting the osmotic potential of plants and soils, subsequently affects water availability due to the limitation in plant water uptake, specific ion toxicity, ion disruption which affect its homeostasis and oxidative stress (Tester and Davenport 2003). The

outcome of these effects may cause physiological, biochemical and molecular changes manifested in membrane damage, nutrient imbalance, enzymes and hormones disruptions, metabolic dysfunction including photosynthesis, protein and nucleic acid synthesis which ultimately leads to plant death (Apel and Hirt, 2004; Hasanuzzaman *et al.*, 2012a).

Salinity tolerance initiates adaptation and involves various changes in the plant physiology and biochemistry at the cellular and whole level. These changes include some time the induction of new metabolic pathways as well as switching off others, the biosynthesis of osmolytes (low molecular weight metabolites), the induction of marker proteins, detoxification mechanisms and changes in phytohormone levels (Fujita *et al.*, 2006). Therefore, an integrated approach combining molecular tools along with physiological, biochemical and metabolic aspects are imperative to develop salt-tolerant crop varieties.

Common bean (*Phaseolus vulgaris* L.) is classified as extremely sensitive to soil salinity and suffers yield losses (Maas and Hoffman, 1977) nevertheless, is considered as one of the main leguminous plants as a source of low-cost proteins, which covers 22 % of the total global needs of protein (Delgado-Sanchez *et al.*, 2006). In addition, it forms mutualistic associations with nitrogen fixing bacteria hence improve soil fertility.

BRs, which play an essential role in plant growth and development, have been implicated in many physiological responses (Bajguz and Hayat, 2009). BRs are known to play a vital role in the regulation of ion uptake (Khripach *et al.*, 2000). The growth promotion in *Pelargonium graveolens* L. was associated with elevated levels of chlorophyll pigments, nucleic acids; soluble proteins after 28-homobrassinolide application (Swamy and Rao, 2008). Cross-talk between BRs and other phytohormones have been investigated and that it includes alternation in the expression of hormone biosynthetic genes and/or signaling intermediates (Bajguz and Hayat, 2009). Also, Agami (2013) and Sadeghi and Shekafandeh (2014) found that under salt stress, exogenous application of 24-Epi ameliorate the deleterious effects of salt manifested as increment in total soluble sugars and activities of enzymes and hormones. In addition, Yuana *et al.* (2010) reported that 24-Epi application markedly decreased the contents of H₂O₂ and malondialdehyde in tomato under water stress. Exogenous application of BRs resulted in significant elevation in antioxidant enzymes activities like superoxide dismutase, catalase, glutathione peroxidase and ascorbate peroxidase in salinized rice seedlings (Ozdemir *et al.*, 2004). 24-Epibrassinolide improved *Brassica juncea* growth following the activation of antioxidant enzymes (Arora *et al.*, 2011), possibly due to an increase in transcription and/or

translation processes of specific genes related to stress tolerance (Kagale *et al.*, 2007).

The present paper aimed to explore the role of 24-Epibrassinolide on *in vitro Ph. vulgaris* cv. Brunco plantlets. Assess applying 24-Epi as an alternative strategy for curbing salt deleterious effects and to overcome the sensitivity of *in vitro* regenerated *Ph. vulgaris* plantlets against hazards of salinity stress. Several physiological, biochemical and hormonal parameters were studied to derive conclusion about 24-Epi mode of action at such early growth stage. Expression products as plantlet proteins and antioxidant isozymes like peroxidase and poly phenol oxidase were investigated.

2. Material and Methods

The experiment was carried out at Faculty of Women for Arts, Science and Education of Ain Shams University, Cairo. Source of plant material, culturing mother plant for stem explants preparation; adventitious shoot proliferation and complete plantlet regeneration have been described in detail by Mansur (2015).

2.1. Experiment design

Regenerated plantlets *via* direct organogenesis aged seven weeks (at the physiological age of 9-10 cm in length with four -five roots) were selected and transferred onto MS and on MS augmented with one of the three levels of NaCl (50, 100 and 150 mmol) and/or with 0.1 mg L⁻¹ 24-Epi (Mansur, 2015) as follow:

T ₁ = control (MS free growth regulators)	T ₅ = MS + 0.1 mg L ⁻¹ 24-Epi
T ₂ = MS + 50 mM NaCl	T ₆ = MS + 50 mM NaCl + 0.1 mg L ⁻¹ 24-Epi
T ₃ = MS + 100 mM NaCl	T ₇ = MS + 100 mM NaCl + 0.1 mg L ⁻¹ 24-Epi
T ₄ = MS + 150 mM NaCl	T ₈ = MS + 150 mM NaCl + 0.1 mg L ⁻¹ 24-Epi

The culture vials were incubated under normal condition (16/8 hour's light/dark) at 1500 lux using cool white fluorescent lamps for three/ ten days, at the end of which, plantlets from all treatments and control were harvested, washed with sterilized distilled water. Shoots were excised, carefully separated and further immersed in liquid nitrogen for five min then stored in refrigerator at -20°C until uses.

2.2. Estimation of photosynthetic pigments

Leaf samples (0.2 g) harvested from control and treated plantlets were homogenized in acetone 80% (v/v) following Arnon (1949) method. Pigments content were calculated according to Lichtenthaler and Wellburn (1983) formulae.

2.3. Extraction and estimation of soluble sugars, sucrose and starch

Soluble sugars in control and treated plantlets were extracted using 80% ethanol following Angelov *et al.* (1993) method. Total soluble sugar and sucrose (after hydrolysis) were determined following Riazi *et al.* (1985) method. Estimation of starch was carried out following McCready *et al.* (1950) method using definite dried sugar free pellets and applying 52% (v/v) perchloric acid. The quantity of starch was expressed mg glucose/g DW.

2.4. Estimation of proline

Free proline content was extracted using 3% (w/v) sulfosalicylic acid and estimated by means of a rapid colorimetric method using ninhydrin reagent according to Bates *et al.* (1973). The proline content was calculated on fresh weight basis according to the following formulae:

$$\mu\text{m ol proline g}^{-1}\text{FW} = (\mu\text{g proline ml}^{-1} * \text{ml of toluene} / 115.5) / (\text{g of sample})$$

2.5. Enzymes extraction and assay

For estimating antioxidant enzymes activities, frozen tissues taken from all shoot samples (0.2 g) were crushed into fine powder in a mortar and pestle to which 50 mmol sodium phosphate buffer pH 7.0 containing 1.0 mmol ethylene diamine tetra acetic acid (EDTA) and 1.0% polyvinylpyrrolidone (PVP) was mixed, with the addition of 0.4 mmol ascorbic acid, and 2.0 ml of 0.1 M Tris- HCl buffer pH 7.5, 0.5 ml of 3.0 mM dithiothreitol (DTT) and 0.5 ml of 2.0% casein in case of the ascorbate peroxidase extraction. The homogenate was centrifuged at 4000 rpm for 20 min at 4°C and the supernatant fraction was used to assay the activities of superoxide dismutase, catalase, ascorbate peroxidase, ascorbic acid oxidase, glutathione reductase peroxidase, polyphenol oxidase (Esfandiari *et al.*, 2007b).

2.5.1. Superoxide dismutase assay (SOD; EC 1.15.1.1)

Activity was assayed by measuring its ability to inhibit the photochemical reduction of nitro blue tetrazolium chloride (NBT), as described by Sen Gupta *et al.* (1993). The reaction was started by exposing the mixture to light source for 15 min. and the reaction was stopped by switching off the light. Absorbance of the reaction mixture was read at 560 nm.

2.5.2. Catalase assay (CAT; EC 1.11.1.6)

Activity was measured according to Aebi (1984). The decrease in H₂O₂ absorbance was monitored at 240 nm and quantified by its molar extinction coefficient (36 mol⁻¹ cm⁻¹).

2.5.3. Ascorbate peroxidase assay (APX; EC 1.11.1.11)

Activity was determined according to Nakano and Asada (1981) method. Absorbance was read at 290 nm and enzyme activity was quantified using the molar extinction coefficient for ascorbate (2.8 mmol⁻¹ cm⁻¹).

2.5.4. Glutathione reductase assay (GR; EC 1.6.4.2)

Activity was determined according to Hodges *et al.* (1997) method. Absorbance was read at 340 nm. Enzyme activity was determined using the molar extinction coefficient for NADPH (6.2 mmol⁻¹ cm⁻¹).

2.5.5. Ascorbic acid oxidase assay (AAO; EC 1.10.3.3)

Activity was assayed according to the method of Vines and Oberbacher (1965). Enzyme activity was measured as decrease in absorbance at 265 nm due to ascorbate oxidation. Enzyme activity was quantified using the molar extinction coefficient for ascorbate (2.8 mmol⁻¹ cm⁻¹).

2.5.6. Peroxidase assay (POD; EC 1.11.1.7)

Activity was measured in a reaction mixture consisting from 50 µl enzyme extract, 20 mmol guaiacol and 100 mmol sodium phosphate buffer pH 7.0 in a total volume 3.0 ml. Reaction was initiated by 20 µl H₂O₂ addition following Ranieri *et al.* (1995) method. Activity was determined by measuring the change in absorbance at 470 nm.

2.5.7. Polyphenol oxidase assay (PPO; EC 1.14.18.1)

Activity was assayed according to the method of Luh and Phithakpol (1972). Activity was determined by measuring the change in absorbance at 410 nm.

2.6. Determination of hydrogen peroxide content

Hydrogen peroxide concentration was determined according to Sergiev *et al.* (1997). Absorbance was read at 390 nm. Hydrogen peroxide 50 mmol ml⁻¹ was used as standard.

2.7. Determination of lipid peroxidation

Lipid peroxidation was measured in the terms of malondialdehyde (MDA) content following the method of Heath and Packer (1968). The MDA content calculated following the equation:

$$\frac{\text{Amount of extraction buffer (ml)} \times \text{amount of supernatant (ml)} \times [(A_{532} - A_{600})/155] \times 103}{\text{Amount of sample (g)}}$$

2.8. Estimation of minerals

For determination of calcium, potassium, sodium, and chloride ions, leaves samples (0.5 g) were dried at 70°C, ground and digested by mixture of H₂SO₄-H₂O₂ as the procedure described by Lachica *et al.* (1973).

The extract was used for elements determination. Sodium, potassium, and calcium ions were measured by flame-emission photometry according to Brown and Lilleland (1946). The chloride was estimated according to the method of Higinbotham *et al.* (1967).

2.9. Endogenous hormones analyses

2.9.1. Extraction and estimation of auxins (IAA), gibberellic acid (GA₃), cytokinins and abscisic acid

Extraction, fractionation, identification and determination of auxins (IAA), gibberellic acid (GA₃), cytokinins and abscisic acid as described by Wasfy *et al.* (1975) were practiced with some modifications. Frozen leaves (0.7g) per sample were macerated in cold 80% (v/v) aqueous methanol and equal (20 mlg⁻¹) volumes were added to the macerate before allowing tissue extraction overnight at 4°C. The extract was vacuum filtered. The filter paper and the residue were returned to the container with fresh methanol, shaken for 1 h and filtered again. The previous step was repeated once more and the combined extracts were concentrated under reduced pressure in a rotary evaporator. The aqueous phase was adjusted to 2.5 and then partitioned with equal volumes of ethyl acetate three times, and then passed through anhydrous sodium sulfate. The combined acidic ethyl acetate phase was evaporated under vacuum and the dry residue containing hormones (fraction I) was dissolved in 2.0 ml of methanol and stored in vials at -20°C. This fraction was used for auxins, gibberellins, and abscisic acid determination. On the other hand, the aqueous phase fraction was adjusted to pH 8.0 with some drops of potassium hydroxide (1 mol) and partitioned four times with n-butanol (1/4 of its volume each time). The n-butanolic phase, thereafter, was concentrated to 5 ml (fraction II) and stored at -20°C for cytokinins determination. Kelenet *al.* (2004) detected auxins, gibberellins, and abscisic acid by HPLC isocratic UV analyzer, reverse phase C₁₈ column (RP-C18 μ Bondapak, Waters). The column used included octadecylsilane (ODS) ultra-sphere particle (5 μmol), the mobile phases used were acetonitrile-water (26:74 v/v), pH 4.00; flow rate: 0.8 mlmin⁻¹, detection: UV 208 nm, the standard solution of the individual acid was prepared in the mobile phase and chromatographed. Cytokinins fraction were detected by HPLC isocratic UV analyzer ODS reverse phase C₁₈ column, 20 min gradient from 0.1N acetic acid pH 2.8 to 0.1 N acetic acid in 95% aqueous ethanol, pH 4. The flow rate: 1 ml min⁻¹, detection: UV 254 nm, standards of BA and Kin were used (Muller and Hilgenberg 1986).

2.9.2. Extraction and estimation of jasmonic acid

Jasmonic acid was extracted using methanol (Hong *et al.*, 2011) following Dionex instructions for Accelerated Solvent Extraction (ASE) method. Known frozen weights after ground were placed in a Dionex ASE 150 and extracted using 100% methanol at 100°C and 1500 psi with five min static cycle, flush volume was 60% of the cell volume. Finally, the system was purged with nitrogen for 60 s. Detection and quantitation of JA extract was carried out using a GC

(Agilent Technologies 7890A) interfaced with a mass-selective detector (MSD, Agilent 7000) equipped with a polar Agilent HP-5ms (5%-phenyl methyl poly siloxane) capillary column (30 m × 0.25 mm i. d. and 0.25μm film thickness). The carrier gas was helium with the linear velocity of 1 ml min⁻¹. The oven temperature was set at 85°C for two min. and then programmed until 250°C at a rate of 15°C min⁻¹. The injector and detector temperatures were 150°C and 250°C respectively. Injection mode, split, split ratio 1: 20, volume injected 1μl of the jasmonic acid extract. The MS operating parameters were as follows: Ionization potential 70 eV, interface temperature 250°C, and acquisition mass range 40-600. The identification of jasmonic acid in its extract was based on a comparison of its mass spectra and retention time with its authentic compound and by computer matching with NIST and WILEY library as well as by comparison of the fragmentation pattern of the mass spectral data with those reported in the literature.

2.10. SDS-protein electrophoresis

Frozen ground powder(1.0 g) represent all treatments were transferred to 2 ml Eppendorf tube containing 400 μl of protein extraction buffer [70% of 50 mmol sodium phosphate buffer pH 6.8 and 30% Tris- glycine buffer pH 6.8 (0.3 g of Tris base and 1.45 g glycine100 ml⁻¹ dH₂O)], gently mixed and kept in the fridge overnight. The extracts were vortex, transferred into Eppendorf tubes and centrifuged at 14000 rpm under cooling (4°C) for 15 min. Ninety μl of the protein extract from each sample was heated in water bath with 30 μl of denaturing buffer (5.0 ml of 0.5 mol Tris base pH 8.8, 4.0 ml of 20% sodium dodecyl sulphate 4.0 ml of 50% glycerol, 0.01 g bromophenol blue, 1.0 ml β-mercaptoethanol and 6.0 ml of dH₂O) at 99°C for five min to denature and reduce the proteins and then left in the fridge in -20°C for the next step. The soluble proteins were subjected to SDS-PAGE in gel slabs of 1mm thickness (4% stacking and, 12.5% resolving gels) as described by Laemmli (1970). Gels were run at 80 volt for two h until the bromophenol blue dye reached the resolving gel and then continued at 150 V till the running end and the apparatus switched off. After that, the gel was stained and de stained gel was photographed and scanned. Software data analysis for Bio-Rad Model 620 densitometer and IBM compatible personal computer 165-2072 were used, bands were coded as 0 (absent) and 1 (present).

2.11. Native protein electrophoresis

Native-polyacrylamide gel electrophoresis (Native-PAGE) was conducted following Stegemann *et al.* (1985) method to identify isozyme variations under the study condition using peroxidase and polyphenol oxidase isozymes systems as molecular markers. For isozymes extraction 0.5 g frozen leaves were macerated in 1 ml extraction buffer (10% glycerol). The extract

was then transferred into clean Eppendorf tubes, centrifuged at 12 000 x g for ten min. The supernatant was transferred to new clean Eppendorf tubes and stored at -20°C until isozymes analysis. Extracted proteins were separated using stacking gel 4.5% and separating gel 12%. A volume of 40 μl extract of each sample was mixed with 20 μl of sucrose and 10 μl of bromophenol blue, and then a volume of 50 μl from this mixture was applied to each well. Electrophoresis conditions during the run was performed at 150 V until the bromophenol blue dye has reached the separating gel and then the voltage was increased to 200 V. The apparatus was placed inside a refrigerator during running. After separation, the gels were stained according to their enzyme systems with the appropriate substrate and chemical solutions and incubated at room temperature in dark for complete staining. In most cases incubation for about one to two hours is enough.

Peroxidase was detected by incubating the gel in darkness for one hour at 37°C in a mixture of diaminobenzidine solution (dissolve 0.125 mg of diaminobenzidine in 2.0 ml of glacial acetic and made up to a final volume of 50 ml of d.H₂O). After incubation period the gel was rinsed in d.H₂O and incubated carefully with five drops of 30% H₂O₂. The gel was incubated at room temperature until bands appeared according to Brown (1978) method. Polyphenol oxidase after separation, the gel was soaked in 0.1 mol sodium phosphate buffer pH 6.8 solved in 100 mg of sulfanilic acid then mixed with 30 mg of catechol solved in 1.0 ml of 99% acetone. The gel was incubated at 37°C until bands appeared according to (Flurkey and Jen 1978) method. Gels resulted from isozyme electrophoresis were photographed, scanned using Gel Doc Vilber Lourmat system.

2.12. Statistical analysis

Standard deviations for five replicates of photosynthetic pigments, carbohydrates, proline, and minerals and three replicates of enzymes and hormones were calculated. The studied data were statistically analyzed using one way analysis of variance as described by Snedecor and Cockran (1969). The means were compared by Duncan at 5% using SPSS program version 20.

3. Results and Discussion

Salinity stress is one of the major environmental constraints that retard plant growth and induces divergence in cell metabolism, and in its major biochemical activities to cope with this type of stress. The mechanisms by which plants defend themselves against saline stress are indeed many fold; many are still unclear. Tissue culture among all other techniques is considered as powerful and successful tool to elucidate mechanisms involved in salt tolerance. The present work represents an evaluation of the interaction

between 24-Epibrassinolide (24-Epi) and NaCl in overcoming the sensitivity of *in vitro* regenerated *Ph. vulgaris* cv. Brunco plantlets against hazards of salinity stress.

Sodium chloride treatment at three different levels (50, 100, and 150 mmol) lead to high significant decreases in the content of chlorophyll *a*, chlorophyll *b* and carotenoids of *Ph. vulgaris* leaves to be considered as salt sensitive symptom (Table 1). Lower chlorophyll content was recorded as a typical effect of a biotic stress and symptoms in salt treatment and was attributed to increase activity of ion accumulation (Parida and Das, 2004) or due to the displacement by toxic Na⁺ ions, which caused the degradation of green pigments. Besides, decrease in chlorophyll and carotenoids contents may be attributed to the inhibitory effects of salt on enzymes, associated with chlorophyll biosynthesis (Campos *et al.*, 2012). Previous explanation could be supported by the significant decrease recorded in the activity level of antioxidative enzymes in the present study under salt stress treatments.

Total soluble sugar, sucrose and starch contents showed significant decrease (Fig.1-c) in shoots of stressed *Ph. vulgaris* plantlets (at 100 and 150 mmol levels). The decrease may be due to chlorophylls synthesis impaired events which culminate into a severe loss in the rate of photosynthesis. Consequently energy generated efficiency may be lowered and diverted towards accelerated levels in divers sites of metabolism under stressed condition (Munns and Tester, 2008). Chlorophyll degradation may be a possible cause of the decline in CO₂ fixation under stress conditions leads to a decrease in carbon reduction by the Calvin cycle and decrease in oxidizes NADP⁺ to serve as an electron acceptor in photosynthesis (Khan and Panda 2008). In contrast, 50 mmol NaCl treatments resulted in significant increments in soluble sugar with non-significant increase in sucrose and starch (Fig.1 a-c). This may explain sugar role as signaling and early defense mechanism to counter balance internal osmosis (Saravanavel *et al.*, 2011).

Proline accumulation in shoots of *Ph. vulgaris* plantlets represented in Fig. 2 revealed positive correlation with salt stress levels this response may imply protection mechanism from cell damage induced by free radicals (Ashraf and Foolad, 2007) and or stabilization for cell enzymes and membranes structure (Timasheff and Arakawa, 1989). A similar result for proline under salinity stress has been reported for pepper (Houimli *et al.*, 2010). Besides, proline accumulation implicates it as adaption mechanism, through restoration of cell volume and turgor (Ashraf and Foolad, 2007). Moreover, Hayat *et al.* (2007b) stated that accumulation of proline under stressful

condition is a highly regulated process, which is controlled by both synthesis and degradation at genes

level or resulted from decrease in utilization of proline in proteins synthesis and enhancing proteins turnover.

Table 1. Effect of exogenous amendment of 24-Epi to MS culture media on Photosynthetic pigments chlorophyll *a*, chlorophyll *b* and carotenoids of leaves of *Ph. vulgaris* regenerated plantlets under normal and conditions of different salinity levels at three and ten day harvesting time after application.

Treatments	Photosynthetic pigments (mg g ⁻¹ FW)					
	Chlorophyll <i>a</i>		Chlorophyll <i>b</i>		Carotenoids	
	Harvesting time (day)					
	3	10	3	10	3	10
T ₁	0.66b	0.87b	0.48b	0.52c	0.80b	0.90c
T ₂	0.59c	0.62d	0.44b	0.48c	0.54d	0.81d
T ₃	0.38f	0.45f	0.35d	0.36d	0.44e	0.65e
T ₄	0.34e	0.40f	0.30d	0.31d	0.39e	0.50f
T ₅	0.69b	0.90a	0.54a	0.69b	0.97a	1.11b
T ₆	0.75a	0.92a	0.58a	0.76a	1.02a	1.27a
T ₇	0.46d	0.79c	0.39c	0.50c	0.70c	0.85cd
T ₈	0.43de	0.54e	0.34d	0.47c	0.62cd	0.80d
LSD at 5%	0.056	0.62	0.041	0.064	0.078	0.085

Means having the same letters in a column were not significantly different at p<0.05

T ₁ = control (MS free growth regulators)	T ₅ = MS + 0.1 mg L ⁻¹ 24-Epi
T ₂ = MS + 50 mMNaCl	T ₆ = MS + 50 mMNaCl + 0.1 mg L ⁻¹ 24-Epi
T ₃ = MS + 100 mMNaCl	T ₇ = MS + 100 mMNaCl + 0.1 mg L ⁻¹ 24-Epi
T ₄ = MS + 150 mMNaCl	T ₈ = MS + 150 mMNaCl + 0.1 mg L ⁻¹ 24-Epi

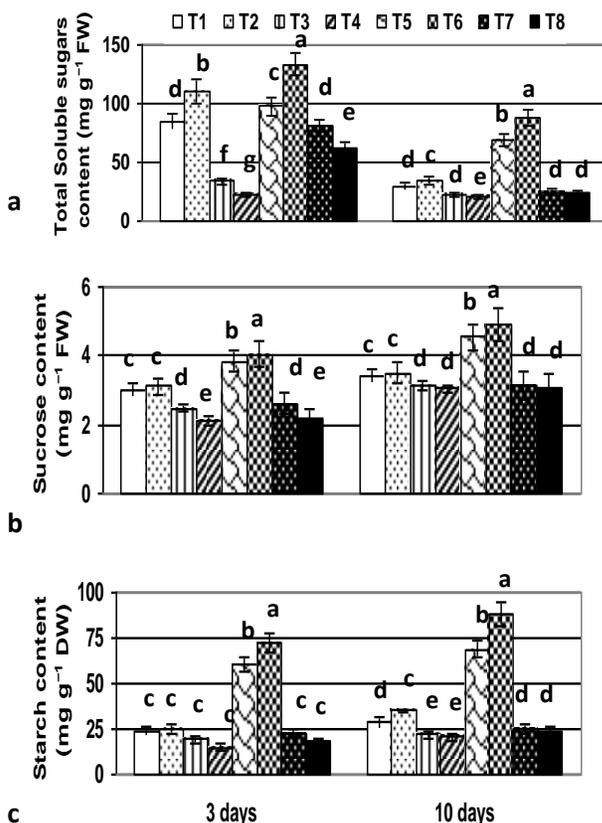


Fig 1. Effects of exogenous amendment of 24-Epi to MS culture media on soluble sugars (a), sucrose (b) and starch (c) contents of *P. vulgaris* shoots regenerated plantlets under normal and conditions of different salinity levels at three and ten days harvesting time after application. T₁-T₈ as illustrated in Table 1.

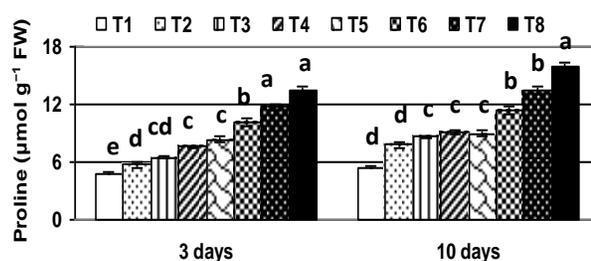
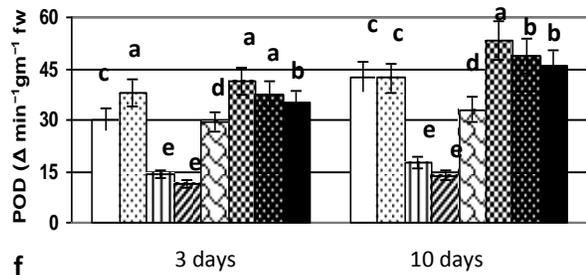
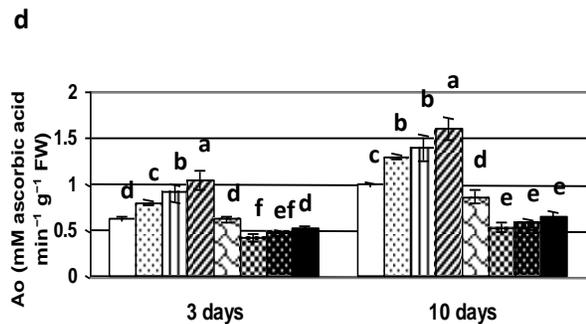
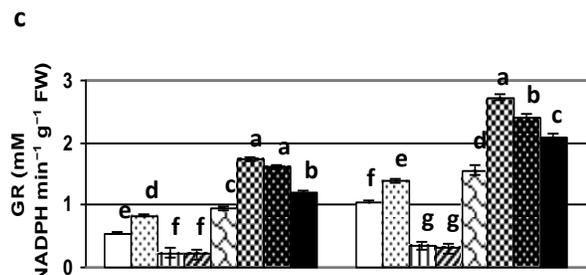
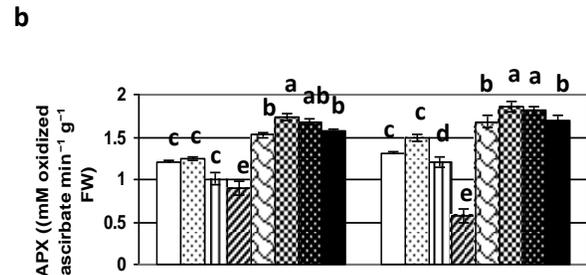
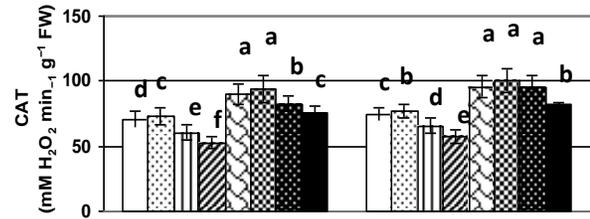
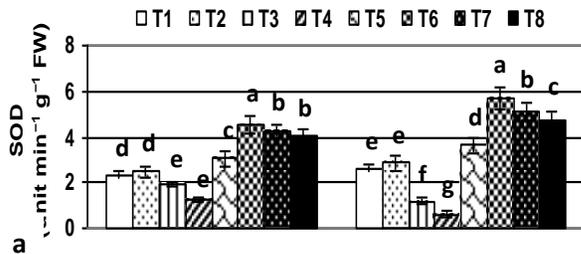


Fig. 2. Effects of exogenous amendment of 24-Epi to MS culture media on proline contents of *P. vulgaris* shoots regenerated plantlets under normal and conditions of different salinity levels at three and ten days harvesting time after application. T₁-T₈ as illustrated in Table 1.

Exposure of plants to various abiotic stresses always accompanied with ROSs production as byproducts, so plants have defend themselves by a series of enzymatic and non-enzymatic detoxification systems to detoxify ROS as possible, and protect cells

from oxidative burst damage. The antioxidant enzymes such as SOD, CAT, APX, GR, AAO, POD, and PPO remove and help in detoxification of super oxide and H₂O₂ (Mittler, 2002). The general increase (Fig.3a-g) in antioxidant activity in shoots of *Ph. vulgaris* plantlets under salt (50 mmol) might result from constitutive activities of preexisting forms or due to synthesis of new isozymes under stress as an adaptive mechanism to reduce free radicals and offer protection against oxidative damage (Agarwal and Pandey, 2004). These results are in accordance with Sairam *et al.* (2005) who reported SOD increment in salt-stressed wheat plants, and it was higher at 100 mmol NaCl than at 200 mmol NaCl. Contrary under higher salt stress levels (100,150 mmol) the capacity and efficiency of the antioxidative defense systems in salt oxidative damage removal was decreased (Fig.3a-g) may be due to enzyme denaturation or assemblies failed in some enzymes under stress imply the sensitivity of the plantlets. Positive correlation between activities of antioxidant enzymes under stress with salt tolerance was reported in certain plant species (Zhu *et al.*, 2005). Various studies reported that over expressed plants containing higher levels of antioxidant enzymes, like SOD, APX, and POX, have improved tolerance to salinity (Alscher *et al.*, 2002).

Hydrogen peroxide and thiobarbituric acid reactive substances (TBARS) contents were significantly increased at all levels of salinity (Fig.4 a, b) may be due to the incapability of antioxidants buffering power to adequately scavenge all the liberated active oxygen species resulted from the oxidative salt stress. Bayram *et al.* (2014) investigate the response of salted common bean hydrogen peroxide and lipid peroxidation products and results solidify the present study. Also, the current results are in agreement with Slathia *et al.* (2012) who observed that lipid peroxidation increased in response to NaCl treatment in tomato.



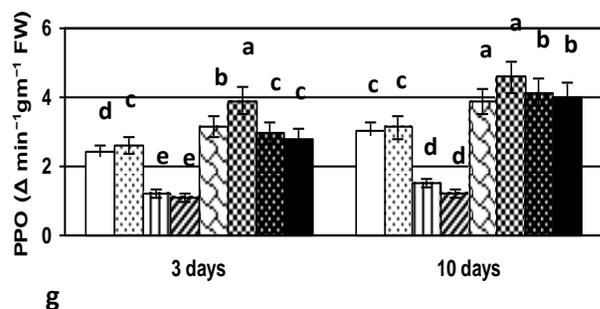


Fig. 3. Effects of exogenous amendment of 24-Epi to MS culture media on (a) superoxide dismutase (SOD), (b) catalase (CAT), (c) ascorbic peroxidase (APX), (d) glutathione reductase (GR) and (e) ascorbate acid oxidase (AAO) (f) peroxidase (POD) and (g) polyphenol oxidase (PPO) enzyme activity of *Ph. vulgaris* shoots regenerated plantlets under normal and conditions of different salinity levels at three and ten days harvesting time after application. T₁-T₈ as illustrated in Table 1.

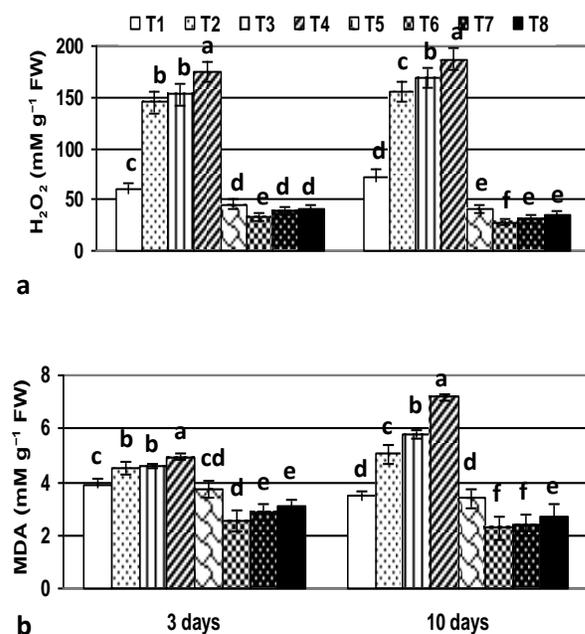


Fig. 4. Effects of exogenous amendment of 24-Epi to MS culture media on (a) hydrogen peroxide (H_2O_2) and (b) malondialdehyde (MDA) of *Ph. vulgaris* shoots regenerated plantlets under normal and conditions of different salinity levels at three and ten days harvesting time after application. T₁-T₈ as illustrated in Table 1.

Since high salt stress induces ion toxicity and nutritional imbalance at both the cellular and the whole-plant levels, differential effects of salt stress on different plant parts may have been due to differences in ion distribution. Sodium (Na^+) and chloride (Cl^-)

ions of shoots of *Ph. vulgaris* indicates that with each increase in $NaCl$ concentration in the growth media, there was a progressive increase in their content of Na^+ and Cl^- ions concentration, meanwhile the content of Ca^{+2} , K^+ and K^+/Na^+ ratio were subjected to appreciable decrease (Table 2). The accumulation of Na^+ associated with marked decrease in K^+ content of shoots of *Ph. vulgaris* exposed to salt stress may be due to a competition between Na^+ and K^+ uptake from the medium, as Na^+ interferes with K^+ selective ion channels in plasma membranes of roots and thus reduce the availability of many ions. Besides, the disruption in membranes function appears to be an important cause of damage by Na^+ (Tester and Davenport, 2003). An increase in Na^+ and a decrease in K^+ content in response to salinity stress were also recorded in green bean (Yasar *et al.*, 2006). In alliance with the marked decrease encountered in K^+/Na^+ ratio of *Ph. vulgaris* shoots in response to salinity stress, Babu *et al.* (2012) found that tomato plants under salt stress, exhibited a decrease of K^+/Na^+ ratio. On the basis of these studies, it can be postulated that K^+/Na^+ ratio shows positive relationship with salt tolerance and it might be a valid selection criterion for evaluating the salt tolerance or sensitivities of different crop species (Kusvuran *et al.*, 2007). Calcium levels in *Ph. vulgaris* grown under salinity stress exhibit significant decrease, participate in its sensitivity to its hazards. Calcium among other effects is important in maintaining a proper balance of nutrients in plants and Ca^{+2} functions to maintain the integrity and permeability of cellular membranes and they could reduce or prevent undesirable leakage induced by salt stress (Taiz and Zeiger, 2006). Physiological evidence indicates that Ca^{+2} enhance K^+ over Na^+ uptake through a high-affinity system (Epstein, 1998) and so calcium deficiency will negatively affect potassium uptake. Potassium activates more than 50 enzymes and the decrease in K^+ content obtained in the present work may affect cells protein as prerequisite for binding tRNA to ribosome (Blaaha *et al.*, 2000).

Environmental signals often influence endogenous concentration and sensitivity to plant hormones. Hormones also are reported to mediate genetically programmed developmental changes or responses to environmental stimuli (Davies, 2010). The results obtained in the present study (Table 3) indicated that *Ph. vulgaris* exposition to salinity stress induced a significant decrease in its content of GA_3 and cytokinins with a concomitant increase in auxins and ABA content and no significant change in JA content. Auxins are one of the unique signaling molecules, which convert environmental information into a cellular context; the recorded increase in auxins obtained in salt sensitive *Ph. vulgaris* is a prerequisite for activating and regulating the intracellular signal

transmission. In other words, the correlative increment under salinity stress may represent trial for adaptation. The present results are consolidated by the work of Babu *et al.* (2012) where an appreciable increase in IAA content of tomato was obtained with the increase in salt treatment. A pivotal role for the gibberellins (GAs) class of growth hormones in response to abiotic stress is becoming accumulated. Under stress environment, growth promotion may occur to escape as a strategy mechanisms act to enhance GAs biosynthesis through up regulation of specific dioxygenase genes or may be involve growth inhibition associated with retard GAs synthesis implicated in some cases *via* DELLA genes. Attenuation of GAs concentration allows stabilization of the DELLA growth retardation,

which would otherwise be degraded by the 26S proteasome. *Arabidopsis thaliana* studies investigate a central role for specific family of transcription factors in regulating GAs metabolism in response to abiotic stress (Dubois *et al.*, 2013). Reduction in GAs contents and its signaling pathway has been implicated with plant growth restriction under salt stress (Colebrook *et al.*, 2014). Sharp decrease encountered in GA₃ content of *Ph. vulgaris* in response to NaCl, was in alliance with stressed *Arabidopsis thaliana* exhibited a reduction in endogenous bioactive gibberellins (Achard *et al.*, 2006) which coincided with DELLA protein accumulation implicated with growth restraint under stress (Claeys *et al.*, 2012).

Table 2. Effects of exogenous amendment of 24-Epi to MS culture media on certain minerals contents as Ca²⁺, K⁺, Na⁺ and Cl⁻ (estimated as percentage) of leaves of *ph. vulgaris* regenerated plantlets under normal and conditions of different salinity levels at three and ten day harvesting time after application. T₁-T₈ as illustrated in Table 1.

Treatments	Mineral contents (%)									
	Ca ²⁺		K ⁺		Na ⁺		Cl ⁻		K ⁺ /Na ⁺	
	Harvesting time									
	3	10	3	10	3	10	3	10	3	10
T ₁	0.14ab	0.19b	0.30b	0.48b	0.13ab	0.14b	0.035b	0.037c	2.31c	3.43cd
T ₂	0.10bc	0.14cd	0.22c	0.32c	0.14a	0.18a	0.045ab	0.047c	1.57bc	1.78de
T ₃	0.08c	0.12cd	0.20c	0.24d	0.16a	0.17ab	0.049b	0.067b	1.25c	1.38e
T ₄	0.07c	0.10d	0.19c	0.21d	0.17a	0.19a	0.052a	0.086a	1.12c	1.14d
T ₅	0.17a	0.30a	0.33ab	0.53b	0.10bc	0.10c	0.020c	0.023c	3.30b	5.30b
T ₆	0.14ab	0.18bc	0.36a	0.59a	0.05d	0.06d	0.020c	0.025c	7.20a	9.83a
T ₇	0.13ab	0.16bc	0.24c	0.46b	0.07cd	0.08cd	0.026bc	0.028c	3.43b	5.75b
T ₈	0.12bc	0.14c	0.23c	0.44b	0.09c	0.10c	0.028bc	0.028c	2.56b	4.63bc
LSD_{5%}	0.044	0.045	0.51	0.54	0.032	0.035	0.013	0.015	1.22	1.85

Means having the same letters in a column were not significantly different at $p < 0.05$

Where: Ca²⁺= calcium ions; K⁺= potassium ions; Na⁺= sodium ions and Cl⁻= chloride ions.

Many of the studies point to crosstalk between the GA-signaling molecule DELLA with components in the signaling pathway of jasmonic acid suggesting additional mechanisms by which GA signaling may integrate information of other hormone signaling pathways under stress (Colebrook *et al.*, 2014). Abrupt change in endogenous content of cytokinins, zeatin and abscisic acid was recorded in plants subjected to salt stress (Javid *et al.*, 2011). In the present work, with each increase of NaCl concentration in MS media, there was a progressive decrease in cytokinins content of *Ph. vulgaris* plantlets. As salt stress induces oxidative stress, this decrease in cytokinins content may be attributed to accelerated degradation *via* oxidation which represents an important pathway for its inactivation and destruction (Kamínek *et al.*, 1997). The classical stress hormones ABA appears to be closely integrated with GA signaling in a number of systems. In *A. thaliana* inhibition of root growth in seedlings treated with ABA was associated with the

accumulation of DELLA proteins, and was reduced in the quadruple- della mutant (Achard *et al.*, 2006). Divi *et al.* (2010) speculated that BR augments ABA levels and ABA-related effects during heat stress. Increased level of ABA in *Ph. vulgaris* in response to salinity stress could play a role, *via* retarding the rate of water uptake from the medium, and regulating the Na⁺ uptake from the medium (Babu *et al.*, 2012). Marked increase in ABA content of tomato was also observed upon its exposure to salt stress (Babu *et al.*, 2012). The rapidly expanding information on the mechanism of DELLA signaling involves it with GA, ABA and JA signaling pathways in response to stress (Yang *et al.*, 2012). Studies of the BR signaling pathway and BR gene-regulating properties point to cross-talk between BRs and jasmonic acid (Divi *et al.*, 2010). Jasmonic acid resistance and growth inhibition responses, was suggested to be *via* interaction with DELLA signaling (Yang *et al.*, 2012). In the present study changes in endogenous JA may affects with other deterrents plant

resistance to abiotic stresses such as salinity. Similar to our results, Kramell *et al.* (1995) stated that

endogenous JA levels did not increase when treated with a high NaCl concentration.

Table 3. Effects of exogenous amendment of 24-Epi to MS culture media on hormones IAA, GA₃, CKs, ABA (mg/ 100 g FW) and JA (ppm) of shoots of *Ph. vulgaris* regenerated plantlets under normal and conditions of different salinity levels at 3 and 10 day harvesting time after application. T₁-T₈ as illustrated in Table 1.

Treatments	IAA		GA ₃		CKs		ABA		JA	
	Harvesting time (days)									
	3	10	3	10	3	10	3	10	3	10
T ₁	0.44e	0.5e	1.69b	2.58b	0.3b	0.44b	0.10bcd	0.11ef	1.13c	1.25b
T ₂	0.52d	0.6e	0.69d	1.11de	0.18de	0.16de	0.12bc	0.21abc	1.21c	1.42b
T ₃	0.65c	0.70e	0.56de	0.97ef	0.16e	0.14de	0.14ab	0.22ab	1.15c	1.34b
T ₄	0.71b	0.85cd	0.44e	0.75f	0.13e	0.09e	0.17a	0.24a	1.13c	1.26b
T ₅	0.60c	0.72de	2.40a	2.69b	0.48a	0.59a	0.07d	0.09f	1.44b	1.67a
T ₆	0.74b	1.18b	2.62a	6.43a	0.47a	0.52a	0.09cd	0.14de	1.62a	1.80a
T ₇	0.82a	1.34a	1.36c	1.66c	0.26cd	0.27c	0.12bc	0.17cd	1.15c	1.35b
T ₈	0.75b	0.93c	1.31c	1.31d	0.21cd	0.19d	0.12bc	0.19bc	1.14c	1.27b
LSD 5%	0.03	0.14	0.26	0.33	0.09	0.072	0.041	0.044	0.15	0.21

Means having the same letters in a column were not significantly different at $p < 0.05$. Where: IAA= Indole-3-acetic acid; GA₃=Gibberellic Acid; CKs= cytokinins; ABA=abscisic acid and JA= asmonic acid.

Comparing the protein profiles in control and salt treated plantlet leaves using SDS-PAGE (Fig. 5 and Table 4) revealed that NaCl treatment did induce changes in cells gene expressions. In the present study, modifications were assessed as *de novo* bands, disappearance of some, and selective expression in others. Present study analysis clearly point to that all treatments and regardless of age comprised fourteen bands as common bands (monomorphic), represented 46.66% polymorphism. On the other hand, scanning the gel explore twelfth non –unique protein bands represent 40% polymorphism. Meanwhile, four bands were assessed as unique bands having molecular weights 77.6, 71.4, 55.2 and 18.1 KDa represent 13.3% polymorphism. Interestingly three high molecular weight proteins (80.5, 67.2, 65.1KDa) and low molecular weight one (30.8 KDa) were specifically expressed under saline treatments compared to control non-treated (Table 4) as stress inducible proteins collaborate in refolding the damaged or denatured proteins *via* cell chaperones systems. Salt stress proteins have been reported in many plant species, such as in *Brassica* Jain *et al.* (1993): 56.1- 70.8 kDa, 93.8 kDa. Also, Kong-ngern *et al.* (2005) investigate 31 KDa protein expressed in rice specifically under salt stress. During the first age, elevated number of expressed peroxidase isozyme under salt stress (Fig.6a and Table 5) was found be correlated with salt concentrations and that the encoding gene (S) was accelerated in response to salt stress. In alliance with the present results El-Baz *et al.* (2003) who stated that peroxidase isozymes can be used as marker for salt stress tolerance in cucumber plants and they found that the peroxidase isozyme profile was altered during salt stress conditions. Also, Sreenivasulu *et al.* (1999)

found increment in POD isozymes activity in salt tolerant cultivar versus susceptible of fox-tail millet explained as salt adaptation mechanism. During the second age, elevated numbers of expressed PPO isozymes (Fig.6b and Table 5) under salt (50,100 mmol NaCl) were recorded compared with the control. However, number of banding patterns of peroxidase isozymes revealed slightly higher numbers (eight) with levels of polymorphism 75% while polyphenol oxidase exhibit lesser number of isozymes patterns (seven) with levels of polymorphism 85.7%. Such observations may be point to PPO sensitivity to salt treatments more than POD.

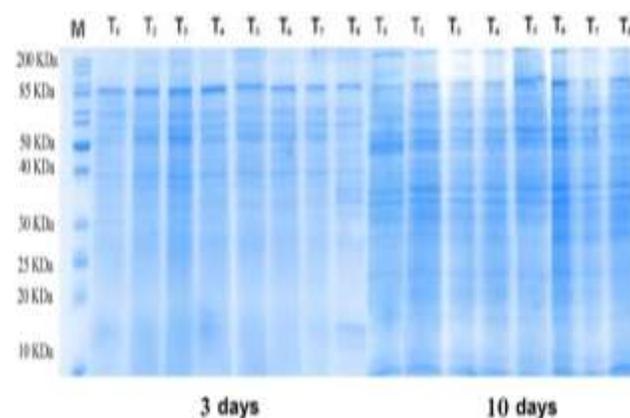


Fig. 5: Electrophoretic banding patterns separated by SDS of leaves of *in vitro* of *P. vulgaris* regenerated plantlets cultured in free MS and /or MS invested with different concentrations of NaCl alone or along with 24-Epi, harvested three and ten days later. T₁-T₈ as illustrated in Table 1.

Table 4. The presence (1) and absence (0) of bands of leaves proteins electrophoretic banding patterns and molecular weights (MW) of SDS proteins in *in vitro* *Ph. vulgaris* regenerated plantlets cultured on free MS and or/ MS invested with different salt levels and 24-Epi and harvested three and ten days after application. T₁-T₈ as illustrated in Table 1.

Harvesting time (day)	3 days								10 days								
Band number	MW	T ₁	T ₂	T ₃	T ₄	T ₅	T ₆	T ₇	T ₈	T ₁	T ₂	T ₃	T ₄	T ₅	T ₆	T ₇	T ₈
1	90.9	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
2	84.3	1	1	1	1	1	1	0	0	1	1	1	1	1	1	1	1
3	80.5	0	1	1	0	0	0	0	0	1	0	0	0	0	0	0	0
4	77.6	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0
5	75.3	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
6	71.4	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
7	68.5	0	0	0	1	1	1	1	1	1	1	1	1	1	1	1	1
8	67.2	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0
9	65.1	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0
10	62.7	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
11	58.2	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
12	55.2	1	1	1	1	1	1	1	0	1	1	1	1	1	1	1	1
13	53.5	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
14	50.8	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
15	48.6	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
16	45.3	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
17	42.0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
18	40.2	0	0	0	0	1	0	1	1	1	0	0	0	0	0	0	0
19	37.6	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
20	35.0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
21	33.0	0	0	0	0	0	0	0	0	1	1	0	0	0	0	0	0
22	32.5	1	1	1	1	1	1	1	1	0	0	0	0	0	0	0	0
23	30.8	0	0	1	1	1	1	1	1	0	0	0	0	0	0	0	0
24	28.1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
25	25.3	0	0	0	0	0	0	0	0	1	1	1	1	1	1	1	1
26	23.8	0	0	0	0	0	0	0	0	1	1	1	1	1	1	1	1
27	21.8	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
28	18.1	1	1	1	1	1	1	1	0	1	1	1	1	1	1	1	1
29	17.1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
30	14.5	0	0	0	0	1	0	0	1	1	1	1	1	1	1	1	1
Total bands		18	22	23	21	23	20	21	21	25	23	22	22	23	22	22	22

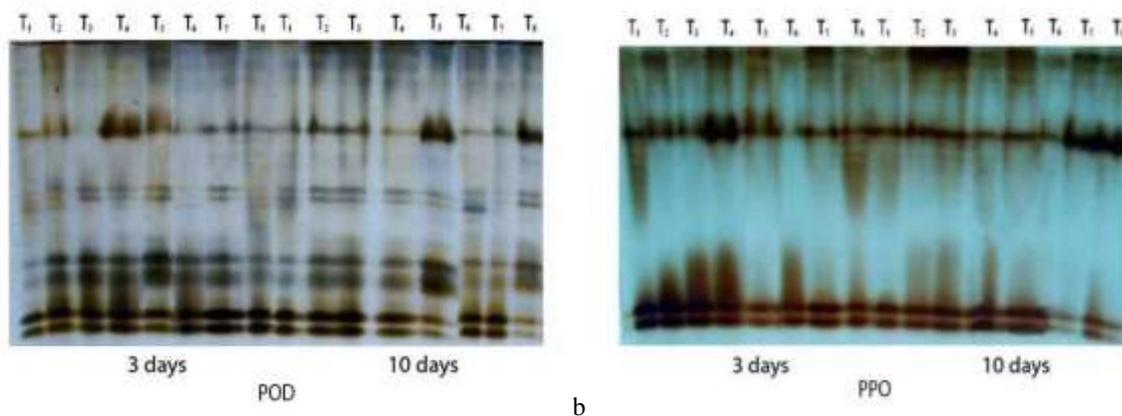


Fig. 6: Electrophoretic patterns of (a) peroxidase (POD), and (b) polyphenol oxidase (PPO), isoforms of leaves of *in vitro* of *Ph. vulgaris* regenerated plantlets cultured in free MS and /or MS invested with different concentrations of NaCl alone or along with 24-Epi, harvested three and ten days later. T₁-T₈ as illustrated in Table 1.

Table 5. The presence (+) and absence (-) of bands and relative mobilities (*Rf*) in two isozymes, peroxidase (POD) and Polyphenol oxidase (PPO) of leaves of *in vitro* of *Ph. vulgaris* regenerated plantlets cultured in free MS and /or MS invested with different concentrations of NaCl alone or along with 24-Epi, harvested three and ten days later. T₁-T₈ as illustrated in Table 1.

Harvesting time (day)	Relative mobility	3 days								10 days							
		T ₁	T ₂	T ₃	T ₄	T ₅	T ₆	T ₇	T ₈	T ₁	T ₂	T ₃	T ₄	T ₅	T ₆	T ₇	T ₈
POD 1	0.104	+	+	-	+	+	-	+	+	+	+	+	+	+	+	+	+
POD 2	0.332	-	-	+	+	+	-	+	-	+	+	+	+	+	+	-	+
POD 3	0.433	-	-	+	+	+	-	+	-	+	+	+	+	+	+	-	+
POD 4	0.521	-	-	-	-	+	-	-	-	-	-	-	-	-	+	-	-
POD 5	0.642	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
POD 6	0.731	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
POD 7	0.834	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
POD 8	0.900	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Total bands		3	5	6	7	8	4	7	5	7	7	7	7	7	8	5	7
Polyphenol oxidase (PPO) group	Relative mobility	T ₁	T ₂	T ₃	T ₄	T ₅	T ₆	T ₇	T ₈	T ₁	T ₂	T ₃	T ₄	T ₅	T ₆	T ₇	T ₈
		PPO1	0.102	-	+	-	-	+	-	+	-	-	+	+	-	-	-
PPO2	0.211	-	+	-	+	+	-	+	-	-	+	+	-	-	-	-	-
PPO3	0.331	-	-	-	-	+	-	-	-	+	+	-	-	-	-	-	-
PPO4	0.520	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+
PPO5	0.631	+	-	-	-	+	-	+	-	+	+	+	+	+	+	+	+
PPO6	0.800	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
PPO7	0.902	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+
Total bands		4	5	3	4	7	2	6	3	4	7	7	3	4	4	2	3

Implication of plant growth regulators in biotic and abiotic stresses management is among the widely known traditional strategies. Brassinosteroids are a group of steroidal hormones that play crucial roles in different developmental stages of plants and in their interaction with the abiotic stresses (Fariduddin *et al.*, 2014). Although much has been learned about their roles in plant development, their mechanisms to control plantlets responses under stress tolerance and consequently to regulate their responsive gene expression is scarce. Brassinosteroids are widely used to overcome salinity stress in plants (Slathia *et al.*, 2012). The results obtained in the present work refer to the immense significance of 24-Epi in alleviating most physiological and biological attributes which measure the sensitivity of a plantlets to salinity stress (same trend as normal plants), following in this regard different mechanisms and protocols. The change in leaf content of chlorophyll *a*, chlorophyll *b*, carotenoids and carbohydrate fractions (Table 1 and Fig.1 a-c) in response to 24-Epi applied alone or in combination with NaCl indicated its potential application even alone and in hampering salt effects on plant normal metabolism as photosynthesis (generally with the lowest applied concentration). However, in case of the other applied concentrations of NaCl (100, 150 mmol), the pigment content was significantly lower or higher

compared with the control or salt treatments solely, respectively. These results are in agreement with Alam *et al.* (2007) who found that 28-homobrassinolide increased the total chlorophyll contents and its fractions in *Brassica juncea*. In addition, 24-Epi was found to protect pigment-protein complexes resulting in decreased degradation of chlorophyll (Fariduddin *et al.*, 2009). Supporting our results Jiang *et al.* (2012) results in which he recorded significant increment after 24-Epi application in cucumber Rubisco, sucrose, soluble sugars, and starch contents explained by Epibrassinolide ability to regulate Calvin cycle and sugar metabolism *via* redox signaling and thus increase the photosynthesis potential. Epibrassinolide *via* activating the antioxidative enzymes has diminished the elevated level of generated ROS induced under salinity stress and thus protected the photosynthetic pigments and /or chloroplasts membranes from destructive effects of ROS and by discarding toxic ions. In addition, Epibrassinolide by increasing the activity level of antioxidative enzymes protect cytokinins *via* oxidative pathway in which the free radicals play a central role, so, their contents exhibited significant increase in response to 24-Epi treatment alone or in combination with the lowest NaCl dose.

The accumulation of proline in plants exposed to stressful conditions repeatedly recorded. In the present

work, the presence of NaCl in the growth culture media of *Ph. vulgaris* plantlets, in presence or absence of 24-Epi lead to marked increase in proline content of shoots (Fig.2), with a magnitude of response obtained in the presence of 24-Epi. In accordance Houimli *et al.* (2010) found that applying 24-Epi to salt stressed *Capsicum annuum* showed increment in proline accumulation. Also, Sadeghi and Shekafandeh (2014) in a study to investigate 24-Epi mechanism in inducing salt tolerance to loquat recorded considerable proline increment. Proline acts as a signaling molecule initiating adaptation protocols copes with syndrome of salinity stress (Maggio *et al.*, 2002). In the present study, chlorophylls content increments may result from proline-thylakoid membranes preservation attack by ROS as reported by Kavi Kishore *et al.* (2005).

Analyzing oxidative enzyme activity suggests an immense mechanism for 24-Epibrassinolide in ameliorating oxidative stress generated by salt stress. Adding 24-Epi alone and in combination with NaCl lead to general marked increase in activity level of SOD, CAT, APX, GR, POD and PPO except AAO exhibited retard response compared to non-treated (Fig.3a-g). These results are in agreement with El-Mashad and Mohamed (2012) who found that, cowpea treatment as foliar spray with BR successfully attenuated salt stress deleterious effects *via* increasing activities of antioxidant enzymes like superoxide dismutase, peroxidase, and polyphenol oxidase. In the same context, Arora *et al.* (2008) showed that the levels of SOD, CAT, APX, GR and POD activity was elevated after 28-homo BL application which boosted salt resistance of *Zea mays*. A preponderance of literatures reported that APX coordinated with CAT and does a central protective role during salt stress (Bajguz and Hayat, 2009). Also, Ozdemir *et al.* (2004) concluded that differential activities of antioxidant enzymes in salt-sensitive rice cultivar suggest possible role for APX in salt stress tolerance induced by EB. Increase in GR activity has been reported as essential reason in salt tolerance in pea (Bor *et al.*, 2003), eggplant (Yasar, 2003) and *Chlorella vulgaris* (Bajguz and Hayat 2009).

As a result of the elevated activity of antioxidative enzymes induced by 24-Epi amendments to the salt-sensitive *Ph. vulgaris*, a significant decrease in H₂O₂ and MDA content was recorded which indicate retard hydrogen peroxide accumulation and a lower lipid peroxidation in salt-treated regenerated plantlets (Fig.4 a, b). Similarly lower lipid peroxidation resulting from elevated activities of antioxidative enzymes and antioxidants under salt stress was also reported in cowpea (El-Mashad and Mohamed, 2012). After 24-Epi application Jiang *et al.* (2012) recorded potential accumulation in H₂O₂.

Mineral analysis of *Ph. vulgaris* provided another potential protocol of 24-Epi in lowering its sensitivity to salt stress. The significant increase recorded in K⁺ ions of *Ph. vulgaris* shoots in response to 24-Epi provided alone to MS culture medium or in presence of low applied concentration of NaCl, may be attributed to 24-Epi ability to change membrane selectivity and through increasing K⁺ content accompanied with a significant decrease in Na⁺ alleviated the servers' competition between Na⁺ and K⁺ ions resulted upon exposure to high concentrations of NaCl and which in turn affect ion receptor and disturbs the ion transport system of plant roots (Yokoi *et al.*, 2002).

Although BR roles in plant responses to abiotic stress have become documented over the last decade, there is still obvious shortage in reports indicating how BR interacts with other stress-related hormones and also their signaling pathways in acquisition stress tolerance. In response to 24-Epi addition to MS medium in absence of NaCl or its presence accompanied with low concentration there was a significant increase in *Ph. vulgaris* content of IAA, GA₃, cytokinins and jasmonic acid associated with concomitant decrease in ABA content. The present results seemed likely to be in alliance with those obtained by others, where an appreciable increase in IAA content of squash seedling was obtained in response to BR (Eun *et al.*, 1989). Yang *et al.* (1999) suggested that the increase in auxins content induced by BRs could be achieved *via* increasing its rate of synthesis, de conjugation of auxins or *via* increasing the activity of auxin receptors. Research points to interaction between auxin and BR *via* share a number of target genes, many of which are involved in growth-related processes (Hardtke *et al.*, 2007). The marked increase obtained in GA₃ content of 24-Epi -treated *Ph. vulgaris* associated with concomitant decrease in ABA level could refer to interference of 24-Epi with the control mechanisms prevailed at the branching points of mevalonate pathway which result its deviation toward GAs. The appreciable increase encountered in cytokinins content in response to 24-Epi -treated *Ph. vulgaris* was also recorded in *Cucurbita pepo* seedlings (Metwally 2008). In this connection also, Hu *et al.* (2000) showed that Epibrassinolide can substitute for the effects of cytokinin in *A. thaliana* cultured cells. Molecular analysis provided a strong evidence for a cross-talk between BRs and ABA included an alteration in the expression of hormone biosynthetic genes and/or signaling 'intermediates' (Lin *et al.*, 2003). Zhu (2002) reported ABA as key molecule implicated in salt stress. Reduction in ABA content in response to BRs was observed in squash and rice (Ono *et al.*, 2000). The present study assigned positive increment in *Ph. vulgaris* plantlets JA treated with 24-Epi and notably also when coupled with low salt dose confirm

assistant role for acquisition salt stress tolerance in regenerated plantlets. Müssig *et al.* (2000a) reported a possible relationship between BRs action and JA synthesis. Experimental evidence points to interactions of BR with JA (Ren *et al.*, 2009). With the exception of BR-auxin interaction, little is known in terms of genes how BR interacts with other hormones (Divi *et al.*, 2010).

Gene functions are manifested in the form of proteins. They are central for the various biological activities. Newly synthesized proteins in response to interaction between environmental stress and growth substances applied may correctly fold and named stress proteins or sometimes misfold. Many of these stress proteins were suggested to play protect role inside the cell. Molecular chaperones which are proteins naturally found in cells are being used to decrease or prevent misfolding of other proteins. Changes in protein synthesis under the 24-Epi treatment alone or along with salt stress treatments may be due to modifications in gene expression at the mRNA translation or *via* regulation of RNA transcription. The protein band which has molecular weight 77.6 kDa was *de novo* synthesized in hormone alone treatment in leaves of *Ph. vulgaris* plantlets only during the second harvesting time. In accordance with the present result, brassinolide 77 KDa protein was investigated by Lapeyre *et al.* (1987). Electrophoreses patterns of peroxidase isozyme expressed under 24-Epi application along with salts exhibited more isozyme bands compared to the control peaked with treatment received the hormone alone. Changing in POD isozyme forms explore 24-Epi role in mediating oxidative stress not only under stress but also under non-stress growth culture (Gapper and Dolan 2006). Such behavior point to that 24-Epi exogenous application can evoke tolerance against salt stress in *in vitro Ph. vulgaris* plantlets which may cause some shift in the cell gene expression. Also, Sreenivasulu *et al.* (1999) found increment in POD isozymes activity in salt tolerant cultivar versus susceptible of fox-tail millet explained as salt adaptation mechanism. Also, Sharma *et al.* (2013) reported that 24-Epibrassinolide application on rice; under salt stress conditions evoke the expression of various oxidative stress marker genes, although to different levels. Electrophoreses patterns of polyphenol oxidase (PPO) isozyme showed under supplementation of 24-Epi along with salt less number of isozymes bands never the less higher percent of polymorphism (85.7%). Such observations may be point to PPO sensitivity to salt treatments more than POD.

4. Conclusions

Salt stress has immense effects on many physiological, biochemical, hormonal and molecular events at cellular and the whole plant level, though

tolerance is considered as a complex trait regulated with numerous genes. Keeping in mind the multifaceted role of BRs, the conclusion of the present study is that 24-Epibrassinolide (putative BRs member) application as an alternative strategy for curbing ill effect of salt can be used to alleviate the harmful effect of salt stress and trigger salt tolerance responses applying *in vitro* technique with salt sensitive regenerated *Ph. vulgaris* plantlets prior acclimation period. Relevant information on physiological, biochemical and molecular indicators at the cellular level may represent selection criteria for tolerance of salts in economical vital crops. Therefore, the use 24-Epiviva the suggested mechanism (s) can help to solve the production problems caused by high salinity.

References

1. Achard P, Cheng H, De Grauwe L, Decat J, Schoutteten H, Moritz T, Van Der Straeten D, Peng, J, Harberd NP (2006) Integration of plant responses to environmentally activated phytohormonal signals. *Science* 311: 91-94.
2. Aebi H (1984) Catalase *in vitro*. *Methods in Enzymology* 105: 121-126.
3. Agami RA (2013) Alleviating the adverse effects of NaCl stress in maize seedlings by pretreating seeds with salicylic acid and 24-epibrassinolide. *African Journal of Botany* 88: 171-177.
4. Agarwal S, Pandey V (2004) Antioxidant enzyme responses to NaCl stress in *Cassia angustifolia*. *Biology Plant* 48: 555-560.
5. Alam MM, Ali B, Ahmad A (2007) Effect of 28-homobrassinolide treatment on nickel toxicity in *Brassica juncea*. *Photosynthetica* 45 (1): 139-142.
6. Ali B, Hayat S, Ahmad A (2007) 28-Homobrassinolide ameliorates the saline stress in chickpea (*Cicerarietinum* L.). *Environmental and Experimental Botany* 59: 217-223.
7. Alscher RG, Ertürk N, Heath LS (2002). Role of superoxide dismutases in controlling oxidative stress in plants. *Journal of Experimental Botany* 53: 1331-1341.
8. Angelov MN, Sun J, Byrd GT, Brown RH, Black CC (1993). Novel characteristics of cassava (*Manihotesculenta* Crantz), a reputed C₃-C₄ intermediate photosynthetic species. *Photosynthesis Research* 38: 61-72.
9. ApelK, HirtH (2004) Reactive oxygen species: metabolism, oxidative stress and signal transduction. *Annual Review of Plant Biology* 55: 373-399.
10. Arnon DI (1949) Copper enzyme in isolated chloroplasts: Polyphenol oxidase in *Beta vulgaris*. *Journal of Plant Physiology* 24: 1-15.
11. Arora N, Bhardwaj R, Sharma P, Arora HK (2008) 28-Homobrassinolide alleviates oxidative stress in salt treated maize (*Zea mays* L.) plants. *Journal of Plant Physiology* 20: 153-157.

12. Arora P, Bhardwaj R, Kanwar MK (2011) Presoaking seed treatment of 24-epiBL modulates Cr stress in *Brassica juncea* L. *Terraqua Environmental Toxicology* 5(1): 14-18.
13. Ashraf M, Foolad MA (2007) Improving plant abiotic-stress resistance by exogenous application of osmoprotectants glycine betaine and proline. *Environmental and Experimental Botany* 59: 206–216.
14. Babu MA, Singh D, Gothandam KM (2012) The effect of salinity on growth, hormone and mineral elements in leaf and fruit of tomato cultivar PKM1. *The Journal of Animal and Plant Science* 22: 159–164.
15. Bajguz A, Hayat S (2009) Effects of brassinosteroids on the plant responses to environmental stresses. *Plant Physiology and Biochemistry* 47: 1-8.
16. Bates LS, Waldren RP, Teare LD (1973) Rapid determination of free proline for water-stress studies. *Plant and Soil* 39: 205–207.
17. Bayram D, Dinler BS, Tasci E (2014) Differential response of bean (*Phaseolus vulgaris* L.) roots and leaves to salinity in soil and hydroponic culture. *Notulae Botanicae Horti Agrobotanici Cluj-Napoca* 42 (1): 219-226.
18. Blaha G, Stelzl U, Spahn CM, Agrawal RK, Frank J, Nierhaus KH (2000) Preparation of functional ribosomal complexes and effect of buffer conditions on tRNA positions observed by cryoelectron microscopy. *Methods in Enzymology Journal* 317: 292–309.
19. Bor M, Ozdemir F, Turkan I (2003) The effect of salt stress on lipid peroxidation and antioxidants in leaves of sugar beet *Beta vulgaris* L. and wild beet *Beta maritima* L. *Plant Science* 164(1): 77-84.
20. Brown AH (1978) Isozymes, plant population, genetic structure and genetic conservation. *Theoretical and Applied Genetic* 52:145-157.
21. Brown JD, Lilleland O (1946) Rapid determination of potassium and sodium in plant material and soil extract by flame photometry. *Proceedings American Society of Horticultural Science* 73:813.
22. Campos MLDO, De Hsie BS, Granja JADA, Correia RM, Cortez JSDA, Pompelli MF (2012) Photosynthesis and antioxidant activity in *Jatropha curcas* L. under salt stress. *Journal of Plant Physiology* 24 (1): 55-67.
23. Claeys H, Skirycz A, Maleux K, Inzé D (2012) DELLA signaling mediates stress-induced cell differentiation in *Arabidopsis* leaves through modulation of anaphase-promoting complex cyclosome activity. *Journal of Plant Physiology* 159: 739-747.
24. Colebrook EH, Thomas, SG, Phillips AL, Hedden P (2014) The role of gibberellin signaling in plant responses to abiotic stress. *The Journal of Experimental Biology* 217: 67-75.
25. Cramer GR, Lauchli A, Polito VS (1985) Displacement of Ca²⁺ by Na²⁺ from the plasma lemma of root cells. A primary response of salt stress. *Journal of Plant Physiology* 79: 207-277.
26. Davies PJ (2010) The plant hormones: their nature, occurrence, and function. In: Davies PJ (ed) *Plant Hormones: Biosynthesis, Signal Transduction, Action*. Springer, Dordrecht, pp 1-15.
27. Delgado-Sanchez P, Saucedo-Ruiz M, Guzman-Maldonado SH, Villordo-Pineda E, Gonzalez-Chavira M, Fraire-Vela'zquez S, Acosta-Gallegos JA, Mora-Avile's A (2006) Anorganogenic plant regeneration system for common bean (*Phaseolus vulgaris* L.). *Plant Science* 170: 822–827.
28. Divi UK, Rahman T, Krishna P (2010) Brassinosteroid-mediated stress tolerance in *Arabidopsis* shows interactions with abscisic acid, ethylene and salicylic acid pathways. *Biomed Central Plant Biology* 10:151.
29. Dubois M, Skirycz A, Claeys H, Maleux K, Dhondt S, De Bodt S, Vanden Bossche R, De Milde L, Yoshizumi T, Matsui M (2013) The ethylene response factor 6 acts as central regulator of leaf growth under water limiting conditions in *Arabidopsis thaliana*. *Journal of Plant Physiology* 162: 319-332.
30. El-Baz FK, Mohamed AA, Aly AA (2003) Development of biochemical markers for salt stress tolerance in cucumber plants. *Pakistan Journal Biological Science* 6 (1): 16–22.
31. El-Mashad AAA, Mohamed HI (2012) Brassinolide alleviates salt stress and increases antioxidant activity of cowpea plants (*Vignasinensis*). *Protoplasma* 249: 625–635.
32. Epstein E (1998) How calcium enhances plant salt tolerance. *Science* 280: 1906–1907.
33. Esfandiari E, Shekari F, Shekari F, Esfandiari M (2007b) The effect of water stress on the antioxidant content, protective enzyme activities, proline content and lipid peroxidation in wheat seedling. *Notulae Botanicae Horti Agrobotanici Cluj-Napoca* 35: 48-56.
34. Eun J-S, Kuraishi S, Sakurai N (1989) Changes in levels of auxin and abscisic acid and the evolution of ethylene in squash hypocotyls after treatment with brassinolide. *Plant and Cell Physiology* 30:807–810.
35. FAO (2010) FAO (Food and Agricultural Organization of the United Nations, Rome) Land and Plant Nutrition Management Service. <http://www.fao.org>.
36. Fariduddin Q, Yusuf M, Ahmad I, Ahmad A (2014) Brassinosteroids and their role in response of plants to abiotic stresses. *Plant Biology* 58 (1): 9-17.
37. Fariduddin Q, Khanam S, Hasan SA, Ali B, Hayat S, Ahmad A (2009) Effect of 28-homobrassinolide on the drought stress-induced changes in photosynthesis and antioxidant system of *Brassica juncea* L. *Actaphysiologiae Plantarum* 31: 889–897.
38. Flurkey WH, Jen JJ (1978) Peroxidase and polyphenol oxidase activities in developing peaches. *Journal of Food Science* 43 (6): 1826–1828.

39. Fujita M, Fujita Y, Noutoshi Y, Takahashi F, Narusaka Y, Yamaguchi-Shinozaki K, Shinozaki K (2006) Crosstalk between abiotic and biotic stress responses: a current view from the points of convergence in the stress signaling networks. *Current Opinion in Plant Biology* 9: 436–442.
40. Gapper C, Dolan L (2006) Control of plant development by reactive oxygen species. *Journal of Plant physiology* 141(2):341–345.
41. Ghassemi F, Jakeman A, Nix H (1995) Salinization of land and water resources: Human causes, extent, management and case studies. University of New South Wales Press: CABI Sydney, Australia, and UK.
42. Gopal R, Khurana N (2011) Effect of heavy metal pollutants on sunflower. *African Journal of Plant Science* 5(9): 531-536.
43. Hardtke CS, Dorcey E, Osmond KS, Sibout R (2007) Phytohormone collaboration: zooming in on auxin-brassinosteroid interactions. *Trends in Cell Biology Journal* 17 (10): 485-92.
44. Hasanuzzaman M, Hossain MA, da Silva JAT, Fujita M (2012a) Plant responses and tolerance to abiotic oxidative stress: antioxidant defenses are key factors. In: Bandi V, Shanker AK, Shanker C, Mandapaka M (eds) *Crop stress and its management: perspectives and strategies*. Springer, Berlin, pp 261–316.
45. Hayat S, Ali B, Hasan SA, Ahmad A (2007b) Effect of 28-homobrassinolide on salinity induced changes in growth, ethylene and seed yield in mustard. *Journal of Plant Physiology* 12: 207–211.
46. Heath RL, Packer L (1968) Photo peroxidation in isolated chloroplasts I. Kinetics and stoichiometry of fatty acid peroxidation. *Archives of Biochemistry and Biophysics* 125:189–198.
47. Higinbotham N, Etherton B, Foster RJ (1967) Mineral ion contents and cell trans membrane electro potential of Pea and Oat seedling tissue. *Journal of Plant Physiology* 42: 37-46.
48. Hodges D, Andrews C, Johnson DA, Hamilton RI (1997) Antioxidant enzyme responses to chilling stress in differentially sensitive inbred maize lines. *Journal of Experimental Botany* 48: 1105-13.
49. Hong FU Ji, Hong SX, De W Ji, Fang C Jin, Yu YC (2011) Progress in quantitative analysis of plant hormones. *Chinese Science Bulletin* 56(4-5): 355-366.
50. Houimli SIM, Denden M, Mouhandes BD (2010) Effects of 24-epibrassinolide on growth, chlorophyll, electrolyte leakage and proline by pepper plants under NaCl-stress. *Eur Asian Journal of Bioscience* 4: 96-104.
51. Hu YX, Bao F, Li JY (2000). Promotive effects of brassinosteroids on cell division involves a distinct Cyc D₃ induction pathway in *Arabidopsis*. *The plant Journal* 24, 693 – 701.
52. Jain S, Nainawatee HS, Jain RK, Chowdhury JB (1993) Salt-tolerance in *Brassica juncea* L. II. Salt stress induced changes in polypeptide pattern of *in vitro* selected NaCl-tolerant plant. *Euphytica* 65: 107-112.
53. Javid MG, Sorooshzadeh A, Moradi F, Sanavy SAMM, Allahdadi I (2011) The role of phytohormones in alleviating salt stress in crop plants. *Australian Journal of Crop Science* 5: 726-734.
54. Jiang Yu-ping, Cheng Fei, Zhou Yan-hong, Xia Xiao-jian, Mao Wei-hua, Shi Kai, Chen Zhi-xiang, Yu Jing-quan (2012) Hydrogen peroxide functions as a secondary messenger for brassinosteroid-induced CO₂ assimilation and carbohydrate metabolism in *Cucumis sativus*. *Journal of Zhejiang University-SCIENCE B (Biomedicine & Biotechnology)* 13(10): 811-823.
55. Kagale S, Divi UK, Krochko JE, Keller WA, Krishna P (2007) Brassinosteroids confers tolerance in *Arabidopsis thaliana* and *Brassica napusta* a range of abiotic stresses. *Planta* 225: 353-364.
56. Kamínek M, Motyka V, Vaňková R (1997) Regulation of cytokinin content in plant cells. *Physiologia Plantarum* 101(4): 689-700.
57. Kelen M, Demiralay EC, Sen S, Ozkan G (2004) Separation of abscisic acid, indole-3-acetic acid, gibberellic acid in 99 R (*Vitisber landieri* x *Vitis rupestris*) and rose oil (*Rosa damascena* Mill.) by Reversed Phase Liquid Chromatography. *Turkish Journal of Chemistry* 28: 603 -610.
58. Khan MH, Panda SK (2008) Alterations in root lipid peroxidation and antioxidative responses in two rice cultivars under NaCl-salinity stress. *Acta Physiologiae Plantarum* 30: 81-89.
59. Khrupach V, Zhabinskii VN, de Groot AE (2000) Twenty years of brassinosteroids: steroidal plant hormones warrant better crops for the XXI century. *Annals of Botany* 86: 441-447.
60. Kavi Kishore PB, Sangam S, Amrutha RN, Sri Laxmi P, Naidu KR, Rao KRSS, Rao S, Reddy, KJ, Theriappan P, Sreenivasulu N (2005) Regulation of proline biosynthesis, degradation, uptake, and transport in higher plants: its implications in plant growth and abiotic stress tolerance. *Current Science* 88: 424–438.
61. Kong-ngern K, Daduang S, Wongkham CH, Bunnag S, Kositrakun M, Theerakulpisut P (2005) Protein profiles in response to salt stress in leaf sheaths of rice seedlings. *Science Asia* 31: 403–408.
62. Kramell R, Atzorn R, Schneider G, Miersch O, Bruckner C, Schmidt J, Sembdner G, Parthie, B (1995) Occurrence and identification of jasmonic acid and its amino acid conjugates induced by osmotic stress in barley leaf tissue. *Journal of Plant Growth Regulation* 14: 29-36.
63. Kusvuran S, Yasar F, Ellialtioglu S, Abak K (2007) Utilizing some of screening methods in order to determine of tolerance of salt stress in the melon (*Cucumis melo* L.). *Research Journal of Agriculture and Biological Science* 3: 40-45.

64. Lachica M, Aguilar A, Y´anez J (1973) Análisis foliar. Métodos utilizados en la Estación Experimental del Zaidín. *Anales de Edafología y Agrobiología* 32:1033-1047.
65. Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227: 680-685.
66. Lapeyre B, Bourbon H., Amalric F (1987) Nucleolin, the major nucleolar protein of growing eukaryotic cells: an unusual protein structure revealed by the nucleotide sequence. *Proceedings in National Academy of Science* 84: 1472- 1476.
67. Lichtenthaler H, Wellburn A (1983) Determinations of total carotenoids and chlorophylls a and b of leaf extracts in different solvents. *Biochemical Society Transactions* 11: 591-592.
68. Lin J, Tarr PT, Yang R, Rhee J, Puigserver P, Newgrad CB, Spiegelman BM (2003) PGC – I beta in the regulation of hepatic glucose and energy metabolism. *Journal of Biological Chemistry* 278: 30843 – 30848.
69. Luh BS, Phithakpol B (1972) Characteristics of polyphenol oxidase related to browning in cling peaches. *Journal of Food Science* 37: 264–268.
70. Maas EV, Hoffman GJ (1977) Crop salt tolerance-current assessment. *Journal of Irrigation Drainage Engineering- ASCE* 103: 115-134.
71. Maggio A, Miyazaki S, Veronese P, Fujita T, Ibease JI, Damsz B, Narasimhan ML, Hasegawa PM, Joly RJ, Bressan RA (2002) Does proline accumulation play an active role in stress-induced growth reduction? *Plant Journal* 31:699–712.
72. Mansur RMA (2015) Physiological and Molecular Studies on the 24-Epibrassinolide Mechanism (s) in Ameliorating Effects of Salted *Phaseolus vulgaris* L. cv. Brunco Tissues. Dissertation, University of Ain Shams, Cairo.
73. McCready RM, Guggloz J, Silviora V, Owens HS (1950) Determination of starch and amylase in vegetables. Application to peas. *Analytical Chemistry* 29: 1156-1158.
74. Metwally MH (2008) Physiological and molecular studies of brassinosteroids, 24-epibrassinolide on *Cucurbita pepo* L. Dissertation, University of Ain Shams, Cairo.
75. Mittler R (2002) Oxidative stress, antioxidants and stress tolerance. *Trends in Plant Science* 7: 405-410.
76. Muller P, Hilgenberg W (1986) Isomers of zeatin and zeatin riboside in club root tissue: evidence for trans-zeatin biosynthesis by *Plasmodiophora brassicae*. *Physiologia Plantarum* 66:245–250.
77. MunnsR, Tester M (2008) Mechanisms of salinity tolerance. *Annual Review of Plant Biology* 59: 651-681.
78. Müssig C, Biesgen C, Lisso J, Uwer U, Weiler EW, Altmann T (2000a) A novel stress inducible 12-oxophytodienoate reductase from *Arabidopsis thaliana* provides a potential link between brassinosteroid-action and jasmonic-acid synthesis. *Journal of Plant Physiology* 157: 143–152.
79. Nakano Y, Asada K (1981) Hydrogen peroxide is scavenged by ascorbate specific peroxidase in spinach chloroplasts. *Plant Cell Physiology* 22: 867-880.
80. Ono EO, Nakamura T, Machado SR, Rodrigues JD (2000) Application of brassinosteroid to *Tabebuia alba* (Bignoniaceae) plants. *Revista Brasileira de Fisiologia Vegetal* 12: 187-194.
81. Ozdemir F, Bor M, Demiral T, Turkan I (2004) Effect of 24- epibrassinolide on seed germination, seedling growth, lipid peroxidation, proline content and antioxidant system of rice (*Oryza sativa* L.) under salinity stress. *Journal of Plant Growth Regulation* 42 (3): 203–211.
82. Parida AK, Das AB (2004) Effects of NaCl stress on nitrogen and phosphorus metabolism in a true mangrove *Bruguiera parviflora* grown under hydroponic culture. *Journal of Plant Physiology* 161: 921–928.
83. Ranieri A, Nali G, D’Urso G (1995) Peroxidase activity in *Cucurbita pepo* L. leaves exposed to ozone. *Agricoltura Mediterranea (Special volume)*, 47-54.
84. Reguera, M., Peleg, Z., Blumwald, E., 2012. Targeting metabolic pathways for genetic engineering abiotic stress-tolerance in crops. *Biochimica et Biophysica Acta* 1819 (2): 186–194.
85. Ren C, Han C, Peng W, Huang Y, Peng Z, Xiong X, Zhu Q, Gao B, Xie D (2009) A leaky mutation in DWARF4 reveals an antagonistic role of brassinosteroid in the inhibition of root growth by jasmonate in *Arabidopsis*. *Journal of Plant physiology* 151: 1412-1420.
86. Rengasamy P (2006) World salinization with emphasis on Australia. *Journal of Experimental Botany* 57: 1017-1023.
87. Rengasamy P (2010) Soil processes affecting crop production in salt-affected soils. *Functional Plant Biology* 37: 255-263.
88. Riazi A, Matsuda K, Arslan A (1985) Water-stress induced changes in concentrations of proline and other solutes in growing regions of young barley leaves. *Journal of Experimental Botany* 172: 1716–25.
89. Sadeghi F, Shekafandeh A (2014) Effect of 24-epibrassinolide on salinity-induced changes in loquat (*Eriobotrya japonica Lindl*). *Journal of Applied Botany and Food Quality* 87: 182 - 189.
90. Sairam RK, Srivastava GC, Agarwal S, Meena RC (2005) Differences in antioxidant activity in response to salinity stress in tolerant and susceptible wheat genotypes. *Biologia Plantarum* 49: 85–91.
91. Saleem MY, Mukhtar Z, Cheema AA, Atta BM (2005) Induced mutation and *in vitro* techniques as a method to induce salt tolerance in Basmati rice (*Oryza sativa* L.). *International Journal of*

- Environmental Science & Technology 2(2): 141-145.
92. Saravanavel R, Ranganathan R, Anantharaman P (2011) Effect of sodium chloride on photosynthetic characteristic of *Avicennia officinalis* seedlings. Recent Research in Science and Technology 3(4): 177-180.
 93. Sen Gupta A, Webb RP, Holaday AS, Allen RD (1993) Over expression of superoxide dismutase protects plants from oxidative stress. Journal of Plant Physiology 103: 1067-1073.
 94. Sergiev I, Alexieva V, Karanov E (1997) Effect of spermine, atrazine and combination between them on some endogenous protective systems and stress markers in plants. Proceedings of Bulgarian Academy of Science 51:121-124.
 95. Sharma I, Ching E, Saini S, Bhardwaj R, Pati PK (2013) Exogenous application of brassinosteroid offers tolerance to salinity by altering stress responses in rice variety Pusa Basmati. Plant Physiology and Biochemistry 69: 17-26.
 96. Slathia S, Sharma A, Choudhary SP (2012) Influence of exogenously applied epibrassinolide and putrescine on protein content, antioxidant enzymes and lipid peroxidation in *Lycopersicon esculentum* L. under salinity stress. American Journal of Plant Science 3: 714-720.
 97. Snedecor GM, Cochran WC (1969) Statistical Methods, 6th ed. Iowa Univ. press: Ames. Iowa USA.
 98. Sreenivasulu N, Ramanjulu S, Ramachandra-Kini K, Prakash HS, Shekar-Shetty H, Savithri HS, Sudhakar C (1999) Total peroxidase activity and peroxidase isoforms as modified by salt stress in two cultivars of foxtail millet with differential salt tolerance. Plant Science 141: 1-9.
 99. Stegemann H, Afify AMR, Hussein KRF (1985) Cultivar identification of dates (*Phoenix dactylifera*) by protein patterns. Second International Symposium of Biochemical Approaches to Identification of Cultivars. Braunschweig, West Germany, 44.
 100. Swamy KN, Rao SSR (2008) Influence of 28-Homobrassinolide on growth, photosynthesis metabolite and essential oil content of Geranium [*Pelargonium graveolens* (L.) Herit]. American Journal of Plant Physiology 3: 173-179.
 101. Taiz L, Zeiger E (2006) Plant Physiology, 4th edn. Sinauer Associates, Inc. Publishers, Sunderland, Massachusetts, 468-557, 594-611.
 102. Tester M, Davenport R (2003) Na⁺ tolerance and Na⁺ transport in higher plants. Annals of Botany 91: 503-527.
 103. Timasheff SN, Arakawa T (1989) Stabilization of protein structure by solvents. In: Creighton TE(ed) Protein Structure: A practical Approach. IRL Press, Oxford, pp 331-334.
 104. Vardhini BV, Rao SSR (1997) Effects of BRs on salinity induced growth inhibition of groundnut seedlings. Indian Journal of Plant Physiology 2: 156-157.
 105. Vines HM, Oberbacher MF (1965) Response of oxidation and phosphorylation in citrus mitochondria to arsenate. Nature 206: 319-320.
 106. Wasfy W, Shind Y, Orrin E Smith (1975) Identification of plant hormones from cotton ovules. Journal of Plant Physiology 55: 550-554.
 107. Wild A (2004) Soils, land and food: managing the land during the twenty-first century. Annals of Botany 93 (6): 785-786. doi: 10.1093/aob/mch104.
 108. Yadav PV, Kumari M, Ahmed Z (2011) Seed priming mediated germination improvement and tolerance to subsequent exposure to cold and salt stress in Capsicum. Research Journal of Seed Science 4: 125-136.
 109. Yang DL, Yao J, Mei CS, Tong XH, Zeng LJ, Li Q, Xiao LT, Sun TP, Li J, Deng XW (2012) Plant hormone jasmonate prioritizes defense over growth by interfering with gibberellin signaling cascade. Proceedings of National Academy of Science USA, 109:E1192-E1200.
 110. Yang YH, Zhang H, Cao RQ (1999) Effect of brassinotide on growth and shikonin formation in cultured on *Osoma paniculatum* cell. Journal of Plant Growth Regulation 18: 89 -92.
 111. Yaşar F (2003) Investigation of some antioxidant enzyme activities in eggplant genotypes grown under salt stress *in vitro* and *in vivo*. Dissertation, University of Yuzuncu Yil, Turkey.
 112. Yasar F, Uzal O, Tufenkci S, Yildiz K (2006) Ion accumulation in different organs of green bean genotypes grown under salt stress. Plant Soil and Environment 52: 476-480.
 113. Yokoi S, Quintero FJ, Cubero B, Ruiz MT, Bressan RA, Hasegawa PM, Pardo JM (2002) Differential expression and function of *Arabidopsis thaliana* NHX Na⁺/H⁺ antiporters in the salt stress response. Plant Journal 30: 529-539.
 114. Yuana G, Cheng-Guo J, Zhen C, Li A, Suna B, Zhanga L, Qiao-Mei NL, Wanga B (2010) Effect of brassinosteroids on drought resistance and abscisic acid concentration in tomato under water stress. Scientia Horticulturae 126:103-108.
 115. Zhu JK (2002) Salt and drought stress signal transduction in plants. Annual Review of Plant Biology 53: 247-273.
 116. Zhu JK, Chinnusamy V, Jagendorf A (2005) Understanding and improving salt tolerance in plants. Crop Science 45:437 -448.