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# EFFECT OF 24-EPIBRASSINOLIDE ON THE PHYSIOLOGICAL AND GENETIC CHANGES ON TWO VARIETIES OF PEPPER UNDER SALT STRESS CONDITIONS

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**Abstract**

This study investigates the effects of using 24-epibrassinolide on two varieties of pepper (*Capsicum annuum* L.) to decrease the adverse effects of salinity stress. The growth parameters; carotenoid; proline; total anthocyanins; minerals concentrations; isozymes electrophoresis; RAPD-PCR and some leaf anatomical characters in the tested pepper plants (*Capsicum annuum* L.) varieties were evaluated. The data showed that EBR reduces the reduction in both fresh and dry weights of the 2 varieties of pepper induced by NaCl-stress on 2 varieties of pepper. Proline increased in 2 varieties in the studied in response to NaCl and EBR treatments. Carotenoid decreased with salinity stress, whereas application of 24-epibrassinolide significantly increased carotenoids in the 2 varieties at salinity and EBR 0.1-ppm. Anthocyanins significantly increased with salt and EBR in both varieties. In addition, foliar application of EBR upon NaCl stressed plants, improved the content and uptake of the nutrients (N, P and K) and decreased Na+ percentage in the 2 studied varieties. The thickness of mesophyll width, dimensions of main vascular bundle and number of stomata were decreased at salt stress in comparison with the control .On the other hand these anatomical characters increased as increasing in concentrations 24-epibrassinolide (EBR). Moreover, the diameter of vascular bundle in the leaf blade decreased in two plants. Our observations indicated that pretreatment with 24-epibrassinolide caused improved the above-mentioned attributes. Therefore, it is clear that 24-epibrassinolide provide protection against a salt stress by increasing the activity of some antioxidative enzymes resulted in mitigation of oxidative stress caused by salinity in pepper.

## Introduction

Salt stress in soil is one of the major stresses especially in arid regions and can severely reduced plant growth and productivity. Salts have adverse effects on growth, yield of many crops (Apel & Hirt, 2004). Seedling growth, and fruit set affected by high salt concentration, leading to diminished yield. In addition, chlorophyll a, b and carotenoids content, the chemical constituents and nutrients decreased in plants (Sairam & Tyagi, 2004). High concentrations of salts in soil inhibit growth by causing ion toxicity and water deficits, and affecting mineral nutrition (Waheed *et al.*, 2006). Many stress-related genes and the list of antioxidant enzymes, hormones, and metabolites present in plants have been recorded (Cao *et al.*, 2005). Reactive oxygenspecies (ROS) produced in oxidative stresses was modifying cellular components and cause damage to lipid membranes, proteins and nucleic acids (Tuna *et al.*, 2008). Potentially of plants to detoxify, reactive oxygen has been related to the stress tolerance of plants (Apel & Hirt, 2004). Scavenging these ROS in plants, by antioxidant defense system comprises various enzymes, non-enzymatic metabolites, and different types of phytohormones among these, brassinosteriods (BRs) (Tuna *et al.,* 2008). These compounds, when applied to plants, improve their yield and make them more resistant to stresses. Brassinosteroids (BRs) are steroidal sixth group of phytohormones with significant growth promoting potential, and are essential for many processes in plant growth and development (Sasse, 2003). Application of brassinosteroid has ameliorates the adverse effects of salt stress on seed germination and growth and root elongation (Amzallag & Goloubinoff, 2003). BRs play important roles in various physiologic processes, including the induction of cellular responses such as stem elongation, leaf epinasty, induction of ethylene biosynthesis, regulation of gene expression and photosynthesis. Several studies have shown that BRs alter the antioxidants in plants under stress conditions (Yin *et al.,* 2008).

Vardhini & Rao (2002) observed that application of BRs significantly increases contents of reducing sugar, sucrose, protein content and polysaccharides in tomato plant.

The spraying of maize shoots with BRs increased total N, P, K, Ca and Mg concentration as. BRs are playing a vital role in the regulation of ion uptake (Kerrit, 2005).

Hot and sweet pepper (*Capsicum annuum*) is one of the most important crops, mainly grown in semi-arid soilsas a summer crop in Egypt it considered as salt sensitive crop. It is an important agricultural crop, because of its economic importance and for the nutritional value of its fruits (Lycoskoufis *et al.,* 2005).

## Material and Methods

**Plant material and experimental conditions:** The seeds of two varieties of pepper [C1: Hot pepper *(Capsicum annuum* var. *frutescens* (L.) Kuntze & C2: Sweet pepper (*Capsicum annuum* var. *baccatum* (L.) Kuntze)] obtained from the Ministry of Agriculture, Agriculture Research Centre, and Giza, Egypt. Seeds of pepper were surface sterilized for 1 min in ethanol 70% (v/v), 20 min in 5% (v/v) sodium hypochlorite, rinsed with bi-distilled water, sown in pots and germinated in a glasshouse. After the second true leaves appeared, uniforms pepper seedlings were irrigated with salt water (4 g L-1 NaCl) for one week after 20 days from sowing and were sprayed twice with a foliar application 24-epibrassinolide (EBR) at different concentrations (0.1-0.5-1.0 mg L-1). The treated plants were grouping in three replicas for each treatment. Samples were collected on the 45 days after salt and 24-epibrassinolide treatments application to analyze the following parameters.

**Plant growth:** The plants with various treatments were up-rooted, washed with distilled water and then the length of stem, freshand dry weight of stems and roots were determined. The samples were oven dried at 70°C for 72 h.

**Biochemical analysis**

**Estimation of carotenoid:** Carotenoids were determined using spectrophotometric method described by (Metzner *et al.,*1965).

**Determination of proline content:** The proline content was determined using the method recorded by (Bates *et al.,*1973). Proline extracted from 100 mg of fresh leaves samples with 2 mL of 40% methanol. 1 mL of a mixture of glacial acetic acid and orthophosphoric acid (6 M) (3: 2, v/v) and 25 mg of ninhydrin mixed with 1 mL of the extract. After 1 h incubation at 100°C, the reaction terminated by putting the tubes in ice bath, 5 mL toluene added. The absorbance of the upper phase was spectrophotometrically determined at 520 nm.

**Determination of total anthocyanins content:** Fresh samples were homogeneous with 12 mL of 1% (w/v) HCl in methanol for 2 days at 3 to 5°C with continuous shaking. The samples were measuring at 530 and 657 nm and anthocyanin concentrations calculated by means of (Mancinelli *et al.,*1975).

**Measurement of minerals concentration:** The concentration and total uptake of macronutrients (Nitrogen N, Phosphorus P, and Potassium K) and Sodium Na) in Pepper, plants were determined using the standard method described by (Cottenie *et al.,* 1982).

**Extraction and assay of enzymatic antioxidant:** Isozymes were estimating in plants by using polyacrylamide gel electrophoresis (Native–PAGE). Three isozymes [esterase (EST)(EST, E.C.3.1.1.1), peroxidase (POD) (EC 1.11.1.7), and superoxide dismutase (SOD) (EC 1.15.1.1)] estimating from the plant samples according to (Stegmann *et al.,*1985). After electrophoresis, the gels stained according to their enzyme system, incubated at 37$^{o}$C in a dark room for complete staining, the staining gels were carried out according to (Jonathan & Wendel, 1990; Graham *et al.,* 1964) for esterase, peroxidase and superoxide dismutase, respectively.

**RAPD-PCR: PCR-RAPD analysis:** DNA was extracting according to (El-Fiky *et al.,* 2002). Ten random primers used; however, only 5 primers gave reproducible results, PCR reactions were conducted according to (Williams *et al.,* 1990).Each RAPD-PCR marker was named by the primer used and DNA fragment size in base pairs (bp). RAPD patterns were scored for each plant and genetic distances were calculated by using RAPD distance software package, version 1.04, (Armstrong *et al.,*1994). The nucleotide sequences and GC ratios of 5 primers used in RAPD-PCR were represented in (Table 1).

**Gel analysis:** All gels resulted from protein and isozyme electrophoresis was scanned using Gel Doc-2001 Bio-Rad system. The densitometry scanning of the bands was performance on three directions. Each band is resigning by its length, width and intensity.

**Table 1. The nucleotide sequences and GC ratios of 5 primers used in RAPD-PCR analysis.**

| Description | Sequence | % GC |
|---|---|---|
| OP B4 | 5-GGACTGGAGT-3 | 60 |
| OP B5 | 5-TGCGCCCTTC -3 | 70 |
| OP B9 | 5- TGGGGGACTC -3 | 70 |
| OP B11 | 5- GTAGACCCGT -3 | 60 |
| OP B14 | 5- AGCATGGCTC-3 | 70 |

**Leaf anatomical characterization:** Thin cross section of fresh leaves were taken and kept in formalin acetic acid (FAA) medium for anatomical study in Plant Department, Faculty of Sciences, Ain Shams University as reported method by (Johnson, 1940).

**Data analysis:** The obtained data were analyzing statistically by Anon., 2006. The statistical analysis model used was two ways analysis of variance with interaction at H.S.D. 5%.

**Results and Discussion**

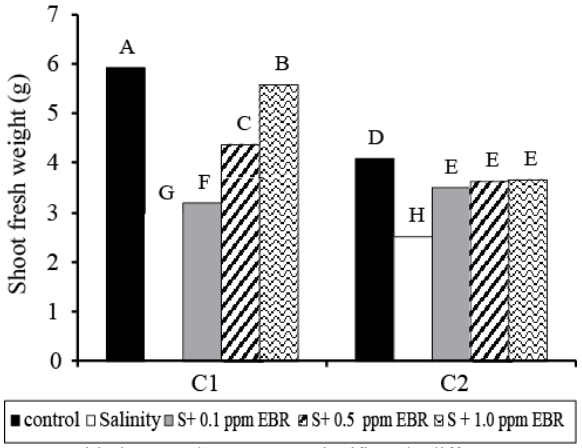
**Growth parameters:** Effect of salt treatments on C1 and C2 growth parameters were represented at (Fig. 1-5). Salt stress (4 g L-1) caused a significant decrease in fresh, dry weights of shoot and roots and shoot length of pepper plants as compared to the control.It clear that salinity affected more significantly the growth parameters of C1 than that of C2.

The data were shown that the foliar applications of EBR plus NaCl of plants alleviated the reduction of the fresh and dry weights of plants produced by NaCl-stress. The most significant increase in fresh and dry weights of plants at salinity and EBR 1.0 ppm treatment in varaity1 (hot pepper) and in variety 2 (sweet pepper) as compared with plants treated with salt alone.

Salt stress has a depressing effect on growth, yield of many crops by affecting water absorption and other biochemical processes. In addition, seedling growth, flowering production and fruit were affected by high salt concentration, leading to decreased in yield (Apel & Hirt, 2004).

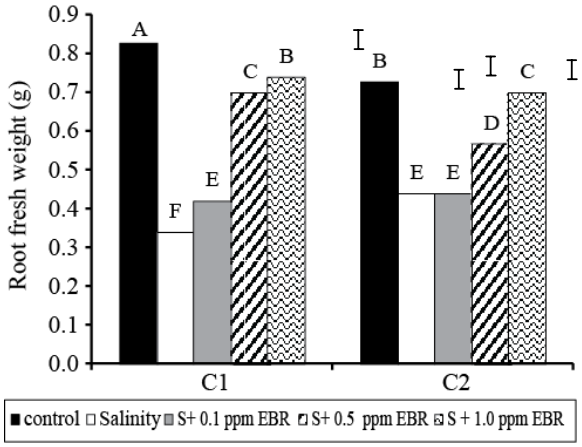
Plants hormone EBR have a capacity to alleviate the effects of a biotic stresses. The increasing in growth of stressed pepper plants with 24-epibrassinolide treatments was attributed to that application of 24- epibrassinolide, stimulate cell elongation (Sumalee, 2011; Muhammad & Hussain, 2012).

Ozdemir *et al.,* (2004) he reported that epibrassinolide promoted stem elongation of peppers. In addition, salt stress affecting both cell division and cell enlargement in the shoots of rice .The promotion of growth by BR under salt stress conditions was associated with enhanced levels of nucleic acids and proteins (Anuradha & Rao, 2001).



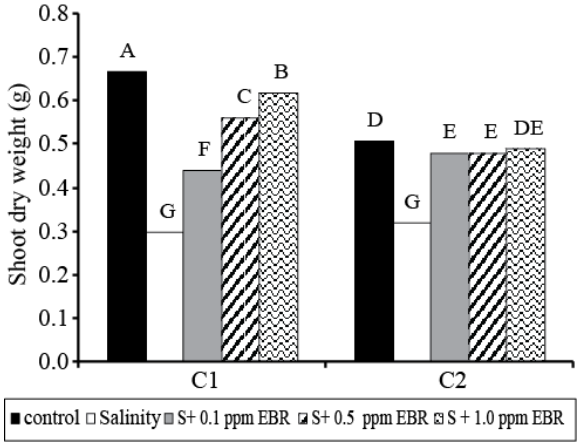

Means with the same letter are not significantly different.

Fig. 1. Effect of salt stress and EBR on fresh weights of shoot in two varieties of *Capsicum annuum* (C1: hot pepper, C2: sweet pepper). (HSD 5% =0.16202).



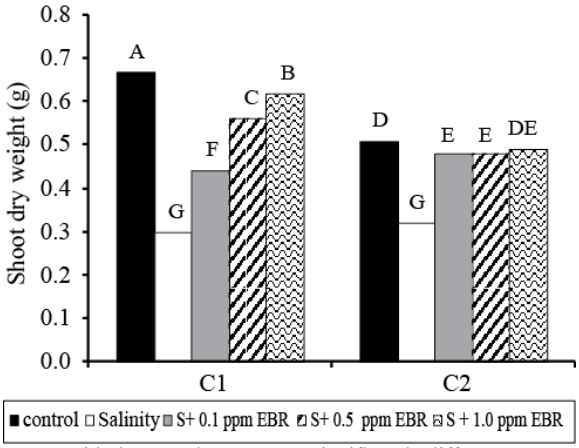

Means with the same letter are not significantly different.

Fig. 2. Effect of salt stress and EBR on fresh weights of roots in two varieties of *Capsicum annuum* (C1: hot pepper, C2: sweet pepper). (HSD 5% = 0.0289).



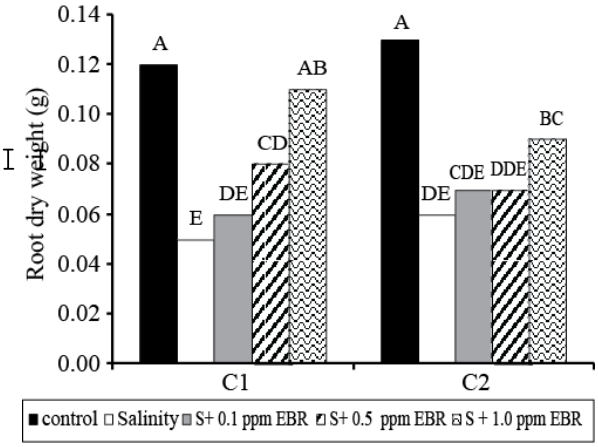

Means with the same letter are not significantly different.

Fig. 3. Effect of salt stress and EBR on shoots length in two varieties of *Capsicum annuum* (C1: hot pepper, C2: sweet pepper). (HSD 5% =0.2891).



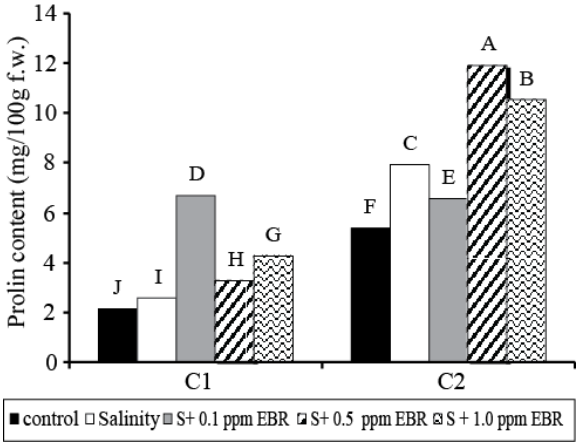

Means with the same letter are not significantly different.

Fig. 4. Effect of salt stress and EBR on dry weights of shoot in two varieties of *Capsicum annuum* (C1: hot pepper, C2: sweet pepper). (HSD 5% =0.0289).



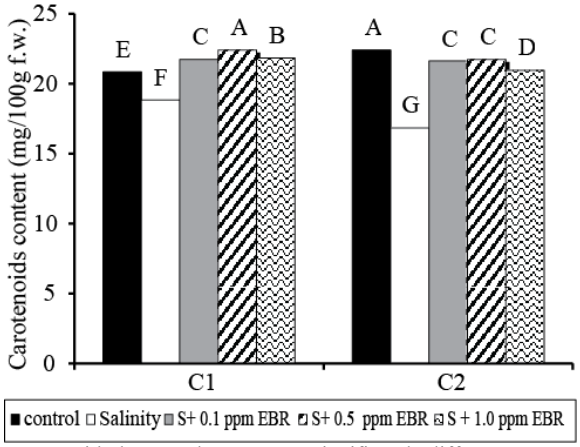

Means with the same letter are not significantly different.

Fig. 5. Effect of salt stress and EBR on dry weights of root in two varieties of *Capsicum annuum (*C1: hot pepper, C2: sweet pepper). (HSD 5% = 0.0289).



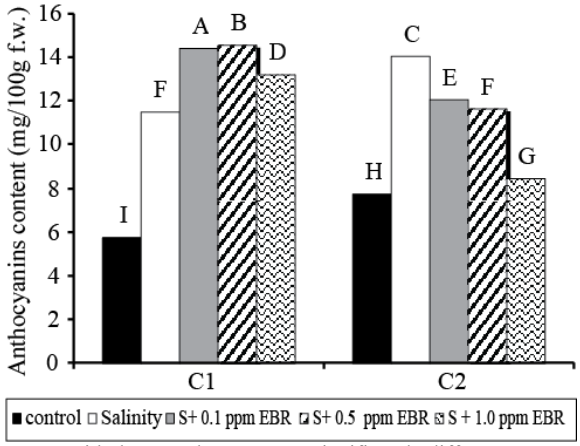

Means with the same letter are not significantly different.

Fig. 6. Effect of salt stress and EBR on Proline content (mg/100gm f.w.) in two varieties of *Capsicum annuum* (C1: hot pepper, C2: sweet pepper). (HSD 5% =0.0955).



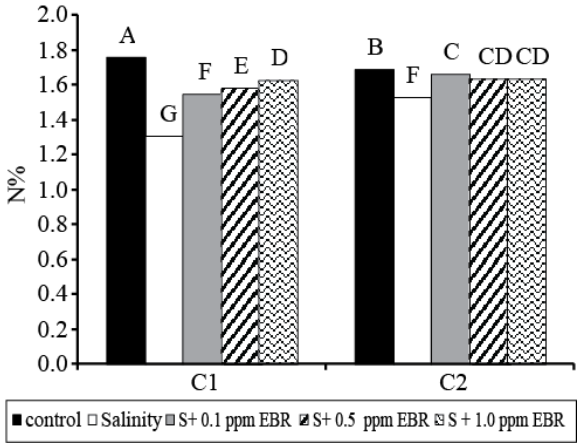

Means with the same letter are not significantly different.

Fig. 7. Effect of salt stress and EBR on Carotenoids content (mg/100gm f.w.) in two varieties of *Capsicum annuum* (C1: hot pepper, C2: sweet pepper). (HSD 5% =0.0289).



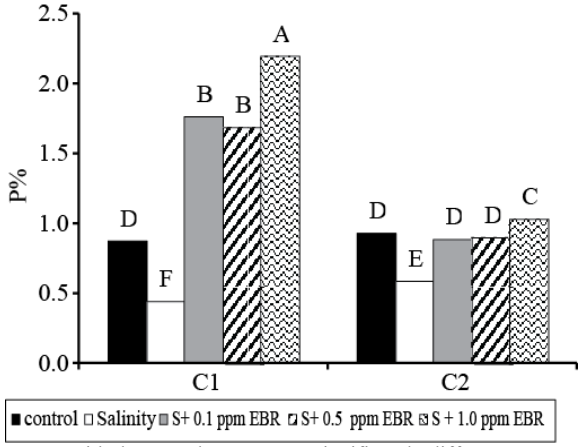

Means with the same letter are not significantly different.

Fig. 8. Effect of salt stress and EBR on Anthocyanins (mg/100gm f.w.) in two varieties of *Capsicum annuum* (C1: hot pepper, C2: sweet pepper). (HSD 5% =0.0289).



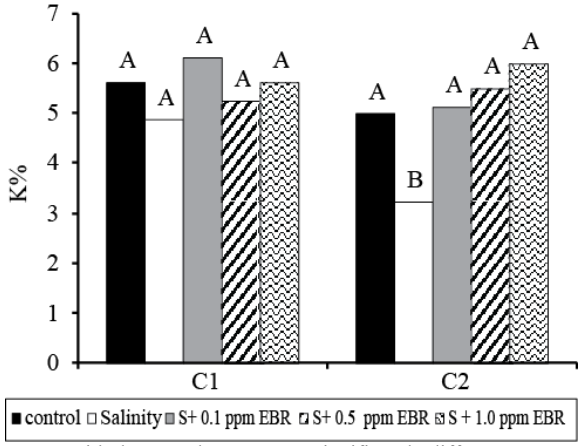

Means with the same letter are not significantly different.

Fig. 9. Effect of salt stress and EBR on Nitrogen percentage in two varieties of *Capsicum annuum* (C1: hot pepper, C2: sweet pepper). (HSD 5% =0.0289).



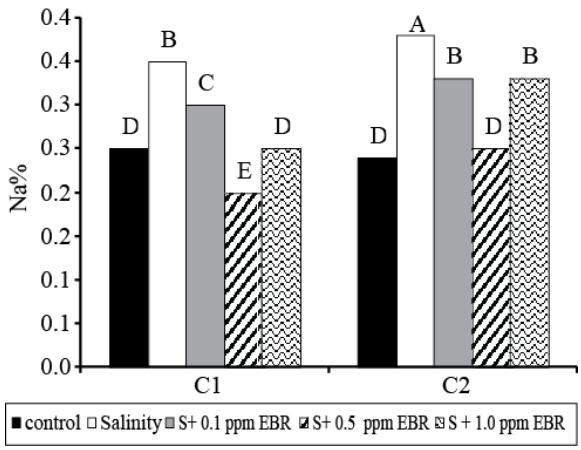

Means with the same letter are not significantly different.

Fig. 10. Effect of salt stress and EBR on Phosphorus percentage in two varieties of *Capsicum annuum* (C1: hot pepper, C2: sweet pepper). (HSD 5% = 0.0955).




Means with the same letter are not significantly different.

Fig. 11. Effect of salt stress and EBR on Potassium percentage in two varieties of *Capsicum annuum* (C1: hot pepper, C2: sweet pepper). (HSD 5% = 0. 1.2965).




Means with the same letter are not significantly different.

Fig. 12. Effect of salt stress and EBR on Sodium percentage in two varieties of *Capsicum annuum* (C1: hot pepper, C2: sweet pepper). (HSD 5% =0.0289).

**Proline content:** The proline accumulation is an adaptive response to salt-tolerance and it contributes to water status of plants under salt stress and act as free radical scavenger. Increased proline content was observed with salinity treatment on proline content are shown in (Fig. 6). Proline content increased in pepper plants in response to NaCl and EBR treatments compared to control treatments. The most significant increase in proline content achieved at salinity and EBR 0.1-ppm treatments in variety 1 hot pepper 6.69 mg/100g. f.w. in compared to control 2.19. Acumulation of nitrogen-containing substances including proline plays a role in osmotic adjustment, protection of cellular macromolecules from damage and storage of nitrogen and scavenging of free radicals (Cicek & Cakirlar, 2002; Irfan, 2011). Increased proline under salt stress has an action on lowering the generation of free radicals lead to reduction in the lipid peroxidation linked membrane damage resulting in their stabilization (Cao *et al.,* 2005; Yaşar & Samsunlu, 2012).

**Carotenoid contents:** Photosynthetic pigments in pepper leaves were significantly decreasing with salinity stress (Fig. 7). The salt stress caused high significant decrease in carotenoid contents in leaves of pepper plants as compared with control. The data showed that the application of EBR enhanced the carotenoid content in the tested plants and this increment was greater at plants treated with salinity and EBR 0.5 ppm as compared with plants treated with salt alone.

The reduction in carotenoid contents in plants under salt stress may be due to enhancing the activity of chlorophyll degrading enzyme chlorophylls (Mishra & Sharma, 1994). Moreover, the decrease in chlorophyll content may attribute to increased activity of chlorophyll-degrading enzyme chlorophyllase (Reddy &Vora, 1986). However, the application of EBR exhibited increased in carotenoid content in the plants grown with salinity stress. EBR increase the total chlorophyll contents and photosynthetic rate in *Brassica juncea*. In addition, EBR was protected pigment-protein complexes resulting in decreased degradation of chlorophyll (Fariduddin *et al.,* 2009).

**Anthocyanins content:** In this study, the effects of both NaCl and EBR treatments on anthocyanin contents in the two varieties of pepper plants presented (Fig. 8). Anthocyanin contents were highly significant decreasing in treated-plants with salt. There was significant increase in anthocyanin contents in plants treated with salt stress and EBR as comparing to the control.

Anthocyanins allow the plant to develop resistance to environmental stresses (Steyn *et al.,* 2002). The production of anthocyanin increased in salinity and EBR treatments, because anthocyanin either act as a hydroxyl radical scavenger (Cooper, 2001), or as a solute that inhibits lipid per oxidation and stimulates the activities of antioxidant enzymes which actas defense system in plants in response to a biotic events (Hassanein *et al.,* 2005). In addition, anthocyanin is an important flavonoid as a modulator of salinity stress and plays an important role in the prevention of stress-induced oxidative damage in plants (Eryılmaz, 2006). Peng *et al.,* (2011) suggested that EBR affects JA-induced anthocyanin accumulation by regulating the anthocyanin biosynthesis genes.

**Minerals percentage:** The data presented in (Fig. 9-12) recorded the effects of foliar application of EBR plus NaCl on minerals percentage on two varieties of pepper. The salinity was associated with decreasing the concentrations of N, P and K percentage in two varieties of pepper. Plants treated with EBR improved the content and uptake of the nutrients (N, P and K) as compared with untreated plants. On the other hand, the interaction between salinity levels and EBR treatments decreased in Na+ percentage compared with salinity stressed plants.

Salinity decreasing the mineral nutrients in several vegetable crops (Yildirim *et al.,* 2006). (Gemea *et al.,* 1996) found that Na, K concentrations decreased in leaves and roots with salinity. In addition, the reduction in P content under saline conditions attribute to that Na salts raised the pH of the soil, and in turn reduced the availability of P to the plant. The increase in Na concentration in plants with the salinity may be attributed to the ability of plants to use Na to maintain an osmotic potential gradient between the plant tissues and the external solution (Sivritepe *et al.,* 2003).

Mona & Ibrahim, (2011) stated that concentrations the total uptake of N, P and K significantly increased over the control when plants were sprayed with EBR concentrations.

**Isoenzymes expression:** The induction of new isozymes and the change in the isoenzymes profiles play an important role in the cellular defense against oxidative stress, caused by salt stress. The multiple isoforms of enzymes is one of the primary control mechanisms of cellular metabolism in plants. The data recorded the response of some antioxidant isozymes: Superoxid dismutase (SOD), Peroxidase (POD), and Esterase(EST) in both pepper varieties treated with EBR and salt (Tables 2 & 3, Fig. 13).

**Peroxidase activity (POX):** Electrophoreses profiles of peroxidase isozyme generally showed seven activity bands in hot and sweet pepper treated with EBR and salt.

Expression of the elevated peroxidase isoenzyme can be explained that the total number bands were increasing not only under salted stressed conditions, but also due to application of EBR plus salt.This in cerement was proportional to increase in EBR concentration as compared to control. Band No.3 was present in all treatments expect at control of sweet pepper and 5 bands were present in some treatments and absent in the others (polymorphic bands). Moreover, bands No. 1 and 7 weresa unique bands, which characterize the plants treated with Salt ++EBR 1.0-0.5 respectively. Peroxidase in pepper leaves increased because of treating plants with EBR plus salt particularly by using the high concentration of EBR (1.0ppm) as compared with that of the control. In addition, exposed to NaCl stress and BR caused enhancements in density of the POD isozyme bands (Tables 2 & 3), (Fig. 13).

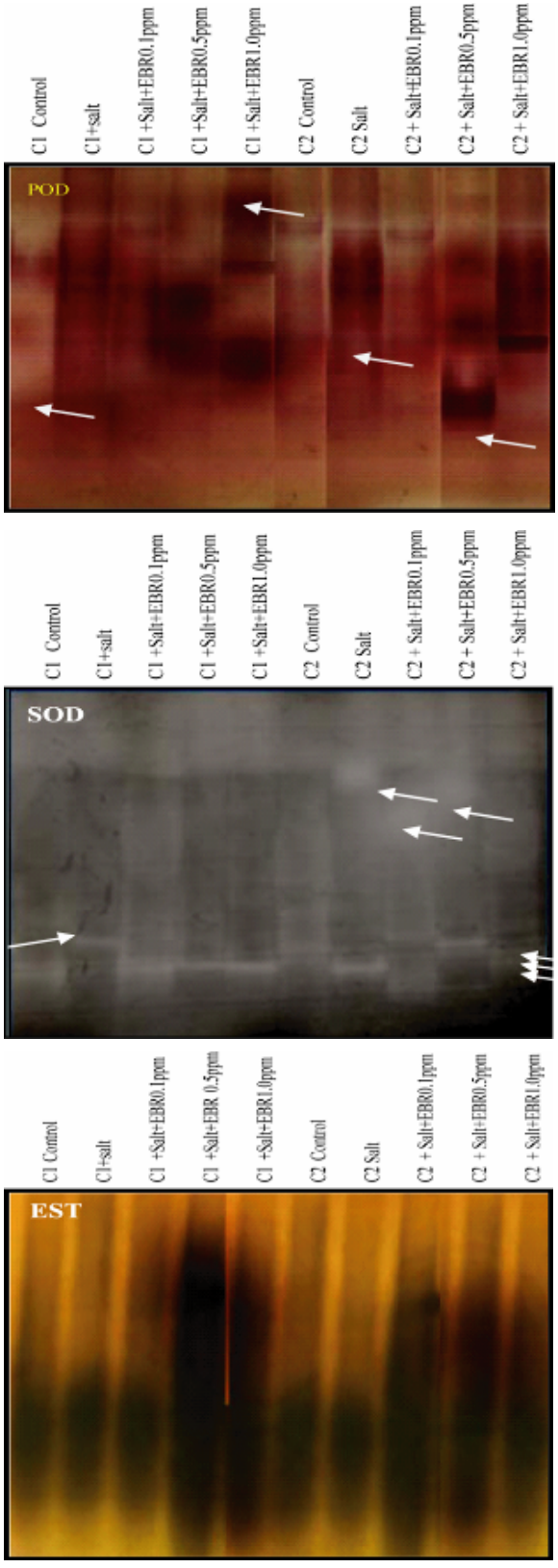



Fig. 13. Effect of salinity and Epibrassinolide on Zymogram of three enzymes, SOD superoxide dismutase, POD peroxidase and EST Esterase on two varieties of *Capsicum annuum* (C1: hot pepper, C2: sweet pepper).

**Table 2. The presence (+) and absence (-) of bands in three isozymes, SOD superoxide dismutase, POD peroxidase and EST Esterase at the effect of salinity and Epibrassinolide on two varieties of *Capsicum annuum* (C1: hot pepper, C2: sweet pepper).**

| SOD | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 |
|---|---|---|---|---|---|---|---|---|---|---|
| 1 | - | - | - | - | - | - | + | - | + | - |
| 2 | - | - | - | - | - | - | - | + | - | - |
| 3 | - | + | - | - | - | - | - | - | - | + |
| 4 | + | + | + | + | + | + | + | + | + | + |
| 5 | + | - | - | - | - | - | - | + | + | + |
| **Total** | **2** | **2** | **1** | **1** | **1** | **1** | **2** | **3** | **3** | **3** |
| POD | | | | | | | | | | |
| 1 | - | - | - | - | + | - | - | - | - | - |
| 2 | - | - | + | - | - | + | + | + | + | + |
| 3 | + | + | + | + | + | - | + | + | + | + |
| 4 | - | + | + | + | + | - | + | - | + | + |
| 5 | - | - | - | + | + | - | + | + | + | + |
| 6 | + | + | - | + | + | + | - | + | - | + |
| 7 | - | - | - | - | - | - | - | - | + | - |
| **Total** | **2** | **3** | **3** | **4** | **5** | **2** | **4** | **4** | **5** | **5** |
| EST | | | | | | | | | | |
| 1 | - | - | + | + | + | - | - | + | + | + |
| 2 | - | - | - | + | + | - | - | - | + | + |
| 3 | - | - | - | + | + | - | - | + | + | + |
| 4 | - | - | - | + | + | - | - | + | + | + |
| 5 | + | + | + | + | + | + | + | + | + | + |
| 6 | + | + | + | + | + | + | + | + | + | + |
| 7 | + | + | + | + | + | + | + | + | + | + |
| **Total** | **3** | **3** | **4** | **7** | **7** | **3** | **3** | **6** | **7** | **7** |

**C1: Hot**
Lane 1 = control, Lane 2 = SA, Lane 3 = SA+ BR 0.1ppm
Lane 4 = SA+ BR 0.5ppm, Lane 5 = SA+ BR 1.0ppm
**C2: Sweet**
Lane 6 = control, Lane 7 = SA, Lane 8 = SA+BR 0.1ppm
Lane 9 = SA+BR 0.5ppm, Lane 10 = SA+BR1.0ppm

**Esterase (EST) Isoenzyme:** The total numbers of bands in profiles of esterase isozyme were 7 activity bands in hot and sweet pepper treated with EBR plus salt (Tables 2 & 3), (Fig. 13). Esterase isozymes showed differences in density and number of bands between control and treated sample. Under control condition, electrophoretic patterns were showed the appearance of three bands while, under salt stressed and increase in the BR concentration appearance of 7 isoform of EST bands. Esterase electrophoretic patterns illustrated that there are three bands appeared in all treatments (common bands). The other four bands were present in some treatments and absent in the others (polymorphic). The application of EBR plus salt increase Esterase (EST) activity in treated plants, as compared with control. In addition, treating the plants with salt (NaCl) alone not affected the enzyme activity comparing with the controls.

**Superoxide dismutase (SOD):** The profiles of Superoxide dismutase (SOD) isozyme were five activity bands in hot and sweet pepper treated with EBR plus salt (Tables 2 & 3), (Fig. 13). Band No. 4 was appeared in all

treatments (common bands). The other four bands were present in some treatments and absent in the others (polymorphic). The application of with EBR plus salt increases Superoxide dismutase (SOD) activity in sweet pepper as compared with those of controls and the other cultivar of plant. These results indicated that BR increased the accumulation of the antioxidant isozyme.

In this research, generally the activity of enzymes studied (POD, SOD, EST) increased when treated with EBR. Exogenous applied 24-EBL enhanced SOD, POD, and EST activities in salt stressed pepper plants. BRs can induce the expression of some antioxidant genes and were increased the activities of antioxidant enzymes in plants under oxidative stress (Cao *et al.,* 2005).

**Table 3. Number and types of bands as well as the percentage of the total polymorphism generated by three enzymes Superoxid Dismutase, Peroxidase and Esterase.**

| Isozymes | Monomorphic Bands | Polymorphic | | Total bands | Polymorphic % |
|---|---|---|---|---|---|
| | | Unique | Non Unique | | |
| SOD | 1 | 1 | 3 | 5 | 80% |
| POD | - | 3 | 4 | 7 | 100% |
| EST | 3 | - | 4 | 7 | 58% |

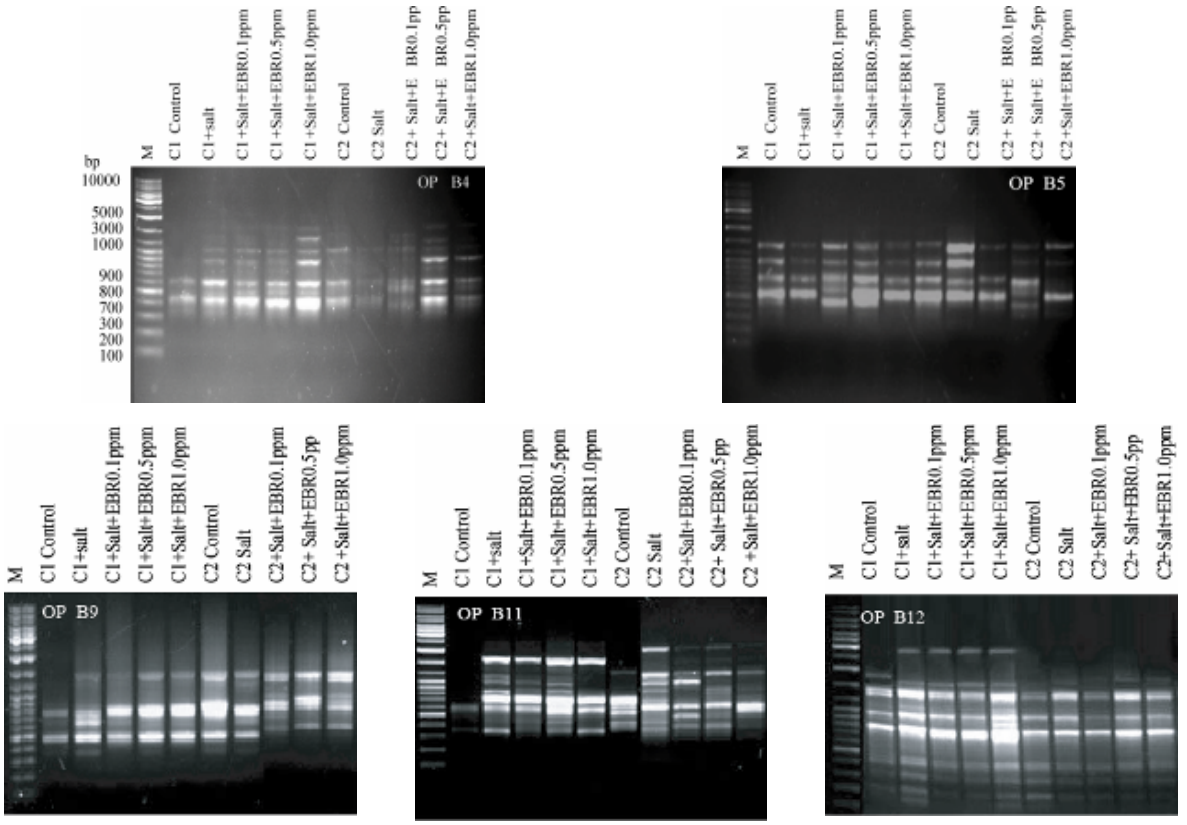



Fig. 14. Effect of salinity and Epibrassinolide on RAPD-PCR polymorphism of DNA using OPB-4, OPB-5, OPB-9, OPB-11 and OPB-19 primers on two varieties of *Capsicum annuum* (C1: hot pepper, C2: sweet pepper) M = molecular marker M: 1Kb DNA ladder.

The increase in the activity of these enzymes may be due to the effects of EBR on expression of biosynthetic genes of these enzymes that resulted in increased oxidation of harmful compounds (Cao *et al.,* 2005).

Wang *et al.,* (2004) found that the profile pattern of peroxidase isoenzyme modified during salt stress conditions; this may be due to its ability to tolerate salt stress that cause some shift in gene expression. Özdemir *et al.,* (2004) showed that BR treatment increased antioxidant enzyme activities in soybean plants under stress conditions. Mohamed, (2005) stated that the enhancement of the esterase isozyme bands in shoots of maize plants similar pattern observed in roots under salt stressed.

Generally, the induction of new isozymes and the change in the isozymeprofile patterns considered to play an important role in the cellular defense against oxidative stress (Wang *et al.,* 2004; Mukhtar *et al.,* 2013).

**RAPD analysis:** In the present experiment, RAPD analysis evaluate the variability in *Capsicum annuum* genomes challenged with salt and EBR treatments, thereby to detect the molecular changes associated with the presence of EBR priming in *Capsicum annuum* leaves.

**Table 4. Molecular weight base pairs of amplified DNA fragment that produced by using RAPD analysis with two primers OPB-4 and OPB-5.**

| DNA marker | Size (bp) | Hot pepper | | | | | Sweet pepper | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 |
| **OPB-4** | | | | | | | | | | | |
| AF01 | 1550 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 1 | 1 |
| AF02 | 1380 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 |
| AF03 | 1275 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 1 | 1 | 0 |
| AF04 | 1100 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 1 | 0 |
| AF05 | 1000 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| AF06 | 830 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| AF07 | 750 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 |
| AF08 | 600 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| AF09 | 530 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 |
| AF10 | 500 | 0 | 1 | 1 | 1 | 1 | 0 | 0 | 0 | 1 | 1 |
| AF11 | 420 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| AF12 | 380 | 1 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| **Total** | | **4** | **6** | **5** | **6** | **6** | **4** | **4** | **8** | **9** | **6** |
| **OPB-5** | | | | | | | | | | | |
| AF13 | 1350 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| AF14 | 1030 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 1 |
| AF15 | 710 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 |
| AF16 | 700 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 1 |
| AF17 | 600 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| AF18 | 520 | 1 | 1 | 0 | 1 | 1 | 1 | 1 | 0 | 1 | 0 |
| AF19 | 430 | 1 | 0 | 1 | 1 | 0 | 1 | 1 | 1 | 1 | 1 |
| AF20 | 330 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 1 |
| **Total** | | **5** | **4** | **5** | **5** | **4** | **5** | **5** | **3** | **6** | **5** |

**C1: Hot**

Lane 1 = control **C1,** Lane 2 = salt **C1,** Lane 3 = salt + EBR 0.1ppm **C1,** Lane 4 = salt + EBR 0.5ppm **C1,** Lane 5 = salt + EBR 1.0ppm **C1**

**C2: Sweet**

Lane 6 = control **C2,** Lane 7 = salt **C2,** Lane 8 = salt + EBR 0.1ppm **C2,** Lane 9 = salt + EBR 0.5ppm **C2,** Lane 10 = salt + EBR 1.0ppm **C2**

In the present work, five-mer primers are used. The results obtained refer to that four primers of them (B4, B9, B11, B14) could efficiently align genomic DNA of *Capsicum annuum*, while primer B5 gave poor reproducibility. 54 bands were amplified between the sweet and hot pepper under different treatments using five primers (Tables 4 & 5, Fig. 14). Monomorphic and polymorphic bands are present in all individuals. The mean percentage of polymorphic bands was 76%, with molecular sizes ranging from 100 to 1700 pb (Table 6, Fig. 14). Thirteen bands of the 54 commonly detected in all the samples, so it could be the specific genus bands of *Capsicum annuum*.

The varieties-specific markers were represented in (Table 7), they differed among the different treatments (12 markers) were appeared in the sweet pepper with treatment salt+ EBR 0.1ppm exhibited five specific fragments and with treatment salt + EBR 0.5ppm showed two specific markers. In addition, the control of hot pepper and treatment salt + EBR 0.1ppm showed two specific markers respectively while the rest treatments did not show any specific bands. The results showed that the using of RAPD analysis to characterize each variety with the appearance of specific markers and produce informative bands that distinguished the two varieties. The expressions of many genes induction by stress, involved directly in stress tolerance and regulation of gene expression and signal transduction (Zhou *et al.*, 2010).

The expression of stress-responsive genes is important for the plants' ability to grow under different environmental stress conditions (Chinnusamy *et al.*, 2007). Malik *et al.*, (2000) they revealed that RAPD technique has a potential to find DNA-based polymorphisms between the drought resistant and drought-susceptible genotypes of the same varieties. Abdel-Bary *et al.*, (2005) recorded eight positive and negative RAPD markers for salinity tolerance in maize.

**Table 5. Molecular weight base pairs of amplified DNA fragment that produced by using RAPD analysis with three primers OPB-9 , OPB-11 and OPB-14.**

| DNA marker | Size (bp) | Hot pepper | | | | | Sweet pepper | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 |
| **OPB-9** | | | | | | | | | | | |
| AF21 | 1460 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| AF22 | 1220 | 0 | 0 | 0 | 0 | 1 | 1 | 1 | 1 | 1 | 1 |
| AF23 | 1090 | 0 | 0 | 0 | 1 | 0 | 0 | 1 | 1 | 1 | 1 |
| AF24 | 900 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 1 | 1 |
| AF25 | 850 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 1 | 1 |
| AF26 | 780 | 0 | 0 | 1 | 1 | 1 | 0 | 0 | 0 | 0 | 1 |
| AF27 | 750 | 1 | 1 | 0 | 0 | 0 | 1 | 1 | 1 | 1 | 0 |
| AF28 | 700 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| AF29 | 650 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 0 | 0 | 0 |
| AF30 | 580 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 |
| AF31 | 470 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| AF32 | 420 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| AF33 | 350 | 1 | 1 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 |
| **Total** | | **6** | **6** | **6** | **6** | **6** | **7** | **7** | **8** | **8** | **8** |
| **OPB-11** | | | | | | | | | | | |
| AF34 | 1550 | 0 | 1 | 1 | 1 | 1 | 0 | 1 | 1 | 1 | 1 |
| AF35 | 1330 | 0 | 1 | 1 | 1 | 1 | 0 | 1 | 1 | 1 | 1 |
| AF36 | 1180 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 1 | 0 | 1 |
| AF37 | 1100 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 |
| AF38 | 1050 | 0 | 1 | 1 | 1 | 0 | 0 | 1 | 1 | 1 | 0 |
| AF39 | 970 | 0 | 1 | 1 | 1 | 0 | 0 | 1 | 1 | 1 | 1 |
| AF40 | 820 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 0 | 0 |
| AF41 | 710 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| AF42 | 580 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| AF43 | 420 | 1 | 1 | 1 | 1 | 0 | 1 | 1 | 1 | 1 | 1 |
| AF44 | 350 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 0 | 1 | 1 |
| **Total** | | **4** | **9** | **9** | **9** | **6** | **5** | **9** | **8** | **9** | **8** |
| **OPB-14** | | | | | | | | | | | |
| AF45 | 1700 | 1 | 1 | 1 | 1 | 0 | 0 | 0 | 0 | 0 | 0 |
| AF46 | 1600 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 1 | 1 | 1 |
| AF47 | 1500 | 1 | 1 | 1 | 1 | 0 | 0 | 0 | 0 | 0 | 0 |
| AF48 | 1300 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| AF49 | 900 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| AF50 | 700 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| AF51 | 400 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| AF52 | 300 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| AF53 | 200 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 0 |
| AF54 | 100 | 1 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 |
| **Total** | | **9** | **8** | **8** | **9** | **6** | **6** | **7** | **7** | **6** | **6** |
| Total AF (55) | | 28 | 36 | 33 | 35 | 28 | 27 | 32 | 34 | 38 | 33 |

**AF** =Amplified fragments, **Shaded box** = Isolate-specific marker

**C1: Hot**

Lane 1 = control **C1,** Lane 2 = SalT**+C1,** Lane3= salt+ EBR 0.1ppm **C1,** Lane 4= salt+ EBR 0.5ppm **C1,** Lane 5= salt+ EBR 1.0ppm **C1**

**C2: Sweet**

Lane 6= control **C2,** Lane 7= salt **C2,** Lane 8= salt + EBR 0.1ppm **C2,** Lane 9= salt + EBR 0.5ppm **C2,** Lane 10= salt +EBR 1.0ppm **C2**

**Table 6. RAPD analysis from the DNAs of *Capsicum annum* 5 random primers.**

| Primer code | Total amplified fragments | Length range (bp) | Mono-morphic | Polymorphic fragments | % of polymorphism |
|---|---|---|---|---|---|
| OP-B4 | 12 | 380-1550 | 3 | 9 | 75 |
| OP-B5 | 8 | 330-1350 | 1 | 7 | 88 |
| OP-B9 | 13 | 350-1460 | 2 | 11 | 85 |
| OP-B11 | 11 | 350-1550 | 2 | 9 | 82 |
| OP-B14 | 10 | 200-1700 | 5 | 5 | 50 |
| **Total** | **54** | | **13** | **41** | |

**Table 7. Genotypes-specific markers of *Capsicum annum* as revealed by RAPD analysis.**

| Primer code No. | Samples No. | +/- marker type | RAPD markers and their molecular sizes in base pairs |
|---|---|---|---|
| OP-B4 | (8),1,9 | (+),-,+ | (1380, 530), 1000, 750 |
| OP-B5 | (8),3 | (+,-)+ | (710, 700), 600 |
| OP-B9 | 1,8,3 | -,+,+ | 1460, 580, 420 |
| OP-B11 | 9,5 | +,- | 1100, 420 |
| OP-B14 | (1,2,3,4)<br>(7,8,9,10) | +,+ | (1700, 1500)<br>(1600) |

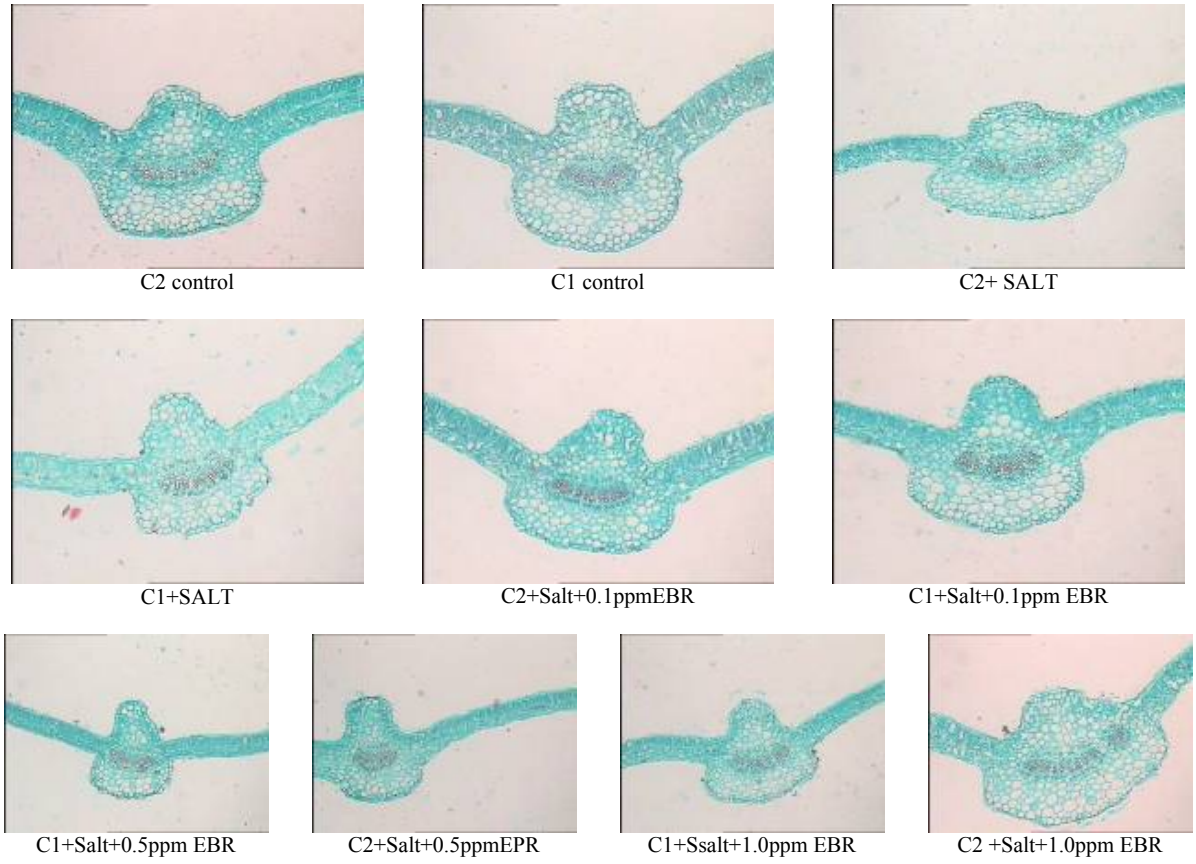



Fig. 15. Effect of NaCl and EBR at anatomical feature on two varieties of *Capsicum annuum* (C1: hot pepper, C2: sweet pepper).

Thus, the different primers have different performances for evaluation of genetic polymorphism. The extensive polymorphism detected among hot, sweet pepper of salt and EBR treatments elevated the degree of change occurring in DNA sequences. The results of RAPD-PCR indicated the existence of differences in RAPD fragments. The quantitative polymorphism obtained might be due to the changes of some regions of the nucleotide sequences aligned by arbitrary primers.

These promotive may be due to effects of EBR or due to the enhancement of annealing between primers and DNA templates by activation the recognition of sequences and / or activation of Tag polymerase activity by the steroidal hormones **(**Clark & James, 1991).

**Table 8. Effect of NaCl and EBR on two varieties of *Capsicum annuum* (C1: hot pepper, C2: sweet pepper) on anatomical feature.**

| Treatment | Control | | Salf | | Salt+0.1 ppm EBR | | Salt+.05 ppm EBR | | Salt+1.0 ppm EBR | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| **Varieties** | **C1** | **C2** | **C1** | **C2** | **C1** | **C2** | **C1** | **C2** | **C1** | **C2** | **HSD 5%** |
| Number of stomata | 8 AB | 9 A | 2E | 3 DE | 4 CDE | 6 BC | 4 CDE | 5 CD | 5 CD | 4 CDE | 3.8913 |
| Width of mesophyll | 152.87 B | 160.07 A | 86.87 I | 125.45 F | 130.11 E | 139.01 D | 98.98 H | 145.13 C | 109.2 G8 | 158.77 A | 2.8913 |
| Width of vascular bandle | 159.18 F | 224.84 B | 137.01 H | 157.48 F | 238.87 A | 201.42 C | 44.23 G | 168.74 E | 1.29 D | 169.82 E | 28913 |

Mean with the same letter are not significantly different
C1: Hot pepper
C2: Sweet pepper

In addition, the action of EBR were achieved by enhancing the activity level of free radical scavenging enzymes could reduce the incidence of DNA damage, explaining the appearance of new DNA in BR treatment **(**Clark & James, 1991;Larson, 1997).

**Leaf anatomy:** In this study salt stress affect the anatomy of sweet and hot pepper plants .It's obvious from the results that ,thickness of mesophyll width, dimensions of main vascular bundle and number of stomata were decreased at salt stress in comparison with the control (Table 8, Fig. 15). On the other hand these anatomical characters increased as increasing in concentrations 24-epibrassinolide (EBR). In addition, salinity and EBR treatments tended to increase the thickness of messophyll, dimensions of vascular bundle (width) and thickness of palisade and spongy tissues as compared with plants treated with salt alone.

The physiological basis of these anatomical changes because of osmotic effect and the difficulty of water uptake from the saline soil, serve to give better protection to a plant against the salt stress. Hussein *et al.,* (2010) reported that the salinity decreased length and width of parenchyma tissues, the vascular bundles, and diameter of xylem vessels, and thickness of the mesophyll of leaf blade. Reduction in these anatomical characters by salinity may be due to that salinity may have an inhibition effect on the activity of the initial cells forming the leaf blade with regard to cell division and enlargement (Hussein *et al.,*2010).

Furthermore, the diameter of vascular bundle in the leaf blade decreased in two plants as result in lowering the accumulation of necessary water required for photosynthesis under salinity. The inhibition effects of salinity on leaf structure may be due to inhibition the growth of vascular elements, an inhibition of the procambial activity and decrease in size of mesophyll cells (Reinhardt & Rost, 1995).

In addition, the increasing in salinity leads to a decreasing in the development of the xylem (Pimmongkol *et al.,* 2002). The high salt levels reduced the cambial activity in *Populuseuphratica.* NaCl contributed to lowered stomatal conductance that leads to decreased CO2 to leaf mesophyll cells (Shahid *et al.,* 2011**)**. The increasing in width of vascular bundle of sweet and hot pepper plants after treated with EBR (Boyer, 2001).

The present study revealed that EBR was effective in ameliorating the oxidative salt stress ascompared to untreated control plants and thus make the plant improved in terms of physiological, biochemical, genetics and anatomical parameters.

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