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Effects of 24-Epibrassinolide on Shoot Tip Cultures Under NaCl Stress in Tomato (*Solanum lycopersicum* L.)

Emel Yilmaz-Gokdogan[a]*, Betul Burun[b]

[a]*Ministry of National Education, District Directorate of National Education, 48000, Mugla, TURKEY*
[b]*Mugla Sitki Kocman University, Faculty of Science, Department of Biology, 48000, Mugla, TURKEY*

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Corresponding Author: Emel Yilmaz-Gokdogan, E-mail: emelyilmazgokdogan@gmail.com
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ABSTRACT
The negative effects of salt stress on plants and their environment are increasing dramatically day by day, and it is crucial for plants to develop salt tolerance with various applications and biotechnological approaches. For this purpose, it is possible to improve salt tolerance in plants through different studies using controlled and uniform *in vitro* cultures, which are an alternative approach to greenhouse and pot experiments that affected by external environmental conditions. In this study, 24-epibrassinolide (24-epiBL) was used for increasing salt tolerance in *in vitro* shoot tip cultures of tomato M-28 hybrid cultivar. Shoot tips of 10-day sterile seedlings were placed in MS medium supplemented with 2 mg L^{-1} K + 0.4 mg L^{-1} NAA in a 12-day culture period, and 12-day plantlets soaked in 24-epiBL solutions (0, 1, 2 µM) were transferred to MS medium containing different concentrations of NaCl (0, 20, 40, 60, 80, 100 mM). After 20 days, the plantlets derived from *in vitro* cultures were used to assess growth (length, fresh and dry weight of plantlets) and biochemical parameters (pigment, MDA, proline, total soluble protein contents, POX and SOD enzyme activities). All growth and biochemical parameters, including pigment and total soluble protein content, were adversely impacted by salt stress (particularly at 40, 60, 80, and 100 Mm NaCl concentrations). However, MDA, proline content, as well as SOD and POX enzyme activity, increased as a results of oxidative stress at the same NaCl concentrations. As a result, NaCl responses in plant differed between various NaCl and 24-epiBL concentrations, and the different defense strategies combine multiple tolerance mechanisms. Therefore, this study, indicates that pretreatment of 24-epiBL to plantlets derived from shoot tips of the tomato M-28 hybrid cultivar played crucial role in mitigating the effects of salt stress.

Keywords: Antioxidant enzymes, 24-epibrassinolide, *In vitro* culture, Proline, Salt tolerance, Tomato

1. Introduction

Salt stress in arid and semi-arid areas is one of the most important environmental problems that can majorly limits plant productivity in our country and around the world (Osman et al. 2011; Srinieng et al. 2015). Therefore, the salinity of soils on irrigated lands decreases agricultural production, directly causing economic losses (Cristea et al. 2020; El-Sayed 2021). Salinity has impacted more than one-third of irrigated areas, and it estimated that approximately half of the world's cultivated land will be salinized by 2050 (Guo et al. 2022). Thus, soil salinity is a major abiotic constraint to crop yield and sustainable agricultural productivity (Ahmad et al. 2018).

Salinity negatively impresses plant growth by disturbing water balance, specific ion toxicity, creating an imbalance in plant nutrition, and combinations of these factors, and affecting plant physiological and biochemical processes (Loganayaki et al. 2020). Ionic effects related to NaCl stress induce damages of macromolecules and compartments of the cells such as cell membrane, cell wall, lipits, proteins, and nucleic acids as a result of nutritional disorders in leaves and meristems (Aly et al. 2012). In relation to ionic stress, the carbohydrate and protein levels vary among plants that are affected by salt stress. Additionally, proline has osmoregulatory properties and interacts with salt, drought and other stress factors (Abdel-Farid et al. 2020). The osmotic stress decreases cell expansion in the growing tissues of young plants, and also leads to stomatal closure, which helps to minimize water loss and plant damage (Rivera et el. 2022). Therefore, plants must cope with ionic and osmotic stress caused by salt stress, and plant responses must be formed against these secondary stresses. The response to ionic stress is slow, and Na^+ is either excluded from the cell or compartmentalized within the cell. A rapid response is given to osmotic stress, and external osmotic pressure is increased (Sané et al. 2021).

NaCl, which has the ability to compete with the basic ions effective in plant development and leads to the inability of plants to benefit from nutrients, shows its destructive effect as the most vital factor of salt stress, with excessive production of Reactive

Oxygene Species (ROS) (Aazami et al. 2021). The production of ROS occurs at low concentrations in chloroplast, mitochondrion and peroxisome under unstressed conditions and increases under salt stress (Ashraf 2009). ROS such as superoxide, hydrogen peroxide, hydroxyl, singlet oxygen are free radicals that highly reactive in cells (Mudgal et al. 2010). ROS can be considered both a cellular signal of different stresses and a secondary messenger involved in complex signaling pathways of stress responses (Aazami et al. 2021). Plants have non-enzymatic and enzymatic defense systems against ROS that cause damage of biomolecules e.g. proteins, lipids and nucleic acids (Koca et al. 2007). Non-enzymatic antioxidants include ascorbic acid, glutathione, proline, carotenoids, flavonoids, and tocopherol and these antioxidants play a significant role in ROS detoxification and retrograde signaling (Guo et al. 2022). It was reported that the activities of enzymatic antioxidants such catalase (CAT), superoxide dismutase (SOD), peroxidase (POX) and glutathione reductase (GR) generally increased under different abiotic stress conditions (Sairam & Tyagi 2004).

It is accepted that salt tolerance of plants is their ability to accomplish their lives on soil that has high concentrations of soluble NaCl salt (Parida & Das 2005). Salt tolerance differs depending on the genotype of plants, and it is provided via changes in physiological and metabolic events such as uptake, transport, and exclusion of salt by the roots at the cellular level (Osman et al. 2011). Tomato is considered as moderately tolerant plant to salinity, and wild tomato species exhibit higher salt tolerance than cultivated tomato (Szczepaniak & Kulpa 2012). On the other hand, the responses to salinity is variable depending upon different tomato lines or cultivars commonly used in agriculture (Zaki & Yokoi 2016). Since different environmental conditions such as temperature, light intensity, humidity and climatic factors affect the response of tomato plants to salt stress, it is important to create and maintain controlled and uniform *in vitro* conditions with reliable experimental data to respond to salt stress (Khaliluev et al. 2022). Different *in vitro* culture techniques using seeds, cotyledons and hypocotyl explants were tested on tomato M-28 hybrid cultivar under salt stress conditions (Yilmaz-Gokdogan & Burun 2015; Yilmaz-Gokdogan & Burun 2017). *In vitro* shoot tip analyses of tomato seedlings at early first-true-leaf stage is frequently used to test for salt tolerance because of high genetic stability (Cano et al. 1998; Shibli et al. 2007). Shoot tip cultures comprison to seed, cell suspension, or callus cultures can be easily propogated *in vitro* and show the nearest stress responses to the whole plant under NaCl conditions (Lokhande et al. 2011).

The use of various biomolecules has been mentioned for the improvement of salt tolerance against the destructive effects of salt stress. Brassinosteroids (BRs), as one of these biomolecules that has antistress characteristic, structurally look like animal steroidal hormones, and promote growth in plants, are new phytohormones classes (Anwar et al. 2018). BRs, when exogenously applied in micro-level (micromolar and nanomolar) concentrations, affect many developmental processes in plants (Singh et al. 2021). It is revealed that BRs lead to distinct cell responses such as stem elongation, root and leaf development, leaf bending and epinasty, formation and development of the pollen tube, reproductive development, xylem differentiation, proton-pomp activation, and regulation of gene expression (Yang et al. 2011; Ahmad et al. 2018). In addition to their roles in the development of plants, BRs confer stress tolerance on plants against different abiotic stresses such as salt, heat, cold, drought, and heavy metals (Surgun et al. 2012).

The present study was aimed to determine effects of short term exogeneous 24-epibrassinolide (24-epiBL) pretreatment against salt stress with physiological and biochemical parameters using *in vitro* shoot tip cultures in the tomato M-28 hybrid cultivar.

2. Material and Methods

2.1. Plant materials and in vitro culture

Seeds of tomato M-28 hybrid cultivar were obtained from Agrotek Seed Agriculture Industry and Commercial Limited Company, Antalya, Turkey. Seeds were sterilized with 50% dilute sodium hypochlorite (2.25% NaOCl) for 5 min, and then they were thoroughly washed with sterile deionized water three times (Yilmaz-Gokdogan & Burun 2017). Surface sterilized seeds were cultured on ½ Murashige-Skoog (½ MS) (1962) media containing 20 g L^{-1} sucrose and 7 g L^{-1} agar for germination (Murashige & Skoog 1962). *In vitro* shoot tips (5 mm) of 10-day sterile seedlings with a germination rate of 95% were transferred to MS medium supplemented with 2 mg L^{-1} Kinetin (K) + 0.4 mg L^{-1} Naphthalene Acetic Acid (NAA) for the production of the healthy plantlets (Yilmaz & Burun 2014). Plantlets derived from 12-day *in vitro* cultures soaked in 24-epiBL (0, 1, 2 µM) solutions that prepared by 70% acetone for 40 seconds, and then the plantlets were transferred to MS medium containing different NaCl concentrations (0, 20, 40, 60, 80, 100 mM) for 20 days. Cultures were maintained in culture room at temperature of 25 ± 2 ºC with 16/8 hours light/dark photoperiod using cool white florescent tubes (~45 µmol m^{-2} s^{-1}).

2.2. Determination of growth parameters

The lengths, fresh weight (FW), and dry weight (DW) of shoots and/or roots in randomly sampled plantlets were measured, and plant samples (shoots and/or roots) for dry weight determination were recorded after being oven dried at 70 ºC for 48 hours.

2.3. Determination of biochemical parameters

2.3.1. Pigment content

The pigment content (chlorophyll a, chlorophyll b, total chlorophyll, and carotenoid) of 24-epiBL non-pretreated and pretreated plantlets was extracted from fresh leaf tissue (0.05 g) by acetone (80%) and determined by a spectrophotometer following the procedure reported by Strain & Svec (1966).

2.3.2. Malondialdehyde (MDA) content

MDA content for the determination of lipid peroxidation level in cell membrane was determined using the thiobarbituric acid reaction according to Heath & Packer (1968). The leaves of plantlets (0.5 g) were homogenised in 3 ml of trichloroacetic acid (TCA), (0.1%). TCA (20%) + thiobarbituric acid (TBA), (0.5%) mixture was added to the supernatant after centrifuging at 13 000 × *g* for 10 min at room temperature and the mixture was incubated at 95 ºC in water bath for 30 min. The reaction stopped on an ice bath and the MDA concentration was calculated using the absorbance at 532 and 600 nm by spectrofotometer. The extinction coefficient (155 mM cm^{-1}) was used for the calculation of MDA concentration.

2.3.3. Proline content

For determination of proline content, the leaves of 24-epiBL pretreated plantlets under NaCl stress were extracted using the ninhydrin reagent according to Bates et al. (1973). The leaves of plantlets (0.5 g) were extracted with 10 ml aqueous sulfosalicylic acid (3%), and then samples were filtered through Whatmann (No. 2), 110 diameter filter paper. 1:1:1 solution of homogenate, ninhydrin reagent, and glacial acetic acid was incubated at 100 ºC for 1 hour for colorimetric determinations of proline. The reaction in the tubes was stopped in an iced bath and the absorbance of the fraction aspired with toluene from the liquid phase was read at 520 nm with a spectrometer. Proline content (µmol proline g^{-1} FW) was calculated using a standard calibration curve.

2.3.4. Total soluble protein content

Total soluble protein content was determined by the Bradford (1976) method using known concentrations of bovine serum albumin as a standard curve. The leaves of plantlets (0.5 g) obtained *in vitro* culture were homogenized in sodium phosphate buffer (5 mL, 0.05 M Na-P buffer, pH: 7). The extraction process was carried out at 0-4 ºC. The absorbance was read at 595 nm with a spectrophotometer after the homogenate was centrifuged at 13 000 × *g* for 15 min at 4 ºC.

2.3.5. SOD and POX enzymes activity

SOD (E.C. 1.15.1.1) activity was measured using the method described by Beauchamp & Fridovich (1971). The leaves of plantlets (0.5 g) were homogenized in Na-P buffer solution (0.05 M pH: 7.8), and then the homogenate was centrifuged at 13 000 × *g* for 15 min at 4 ºC. The supernatant was used for determining SOD activity, and the test tubes containing the reaction mixture (3 mL), were kept under white light intensity at 500 µE m^{-2} s^{-1} for 10 min. One unit of SOD activity was determined as the enzyme amount that inhibited 50% of NBT photoreduction at 560 nm wavelength and was expressed as unit SOD mg^{-1} protein.

POX (E.C. 1.11.1.7) was measured according to Chance & Maehly (1955) using guaiacol oxidation. The leaf samples of plantlets (0.5 g) were homogenized in Na-P buffer solution (0.05 M pH: 6.0), and the homogenate was centrifuged at 13 000 × *g* for 15 min at 4 ºC. After the addition of H_2O_2 to the reaction mixture, an increase in absorbance was recorded every 30 seconds at 470 nm, and POX enzyme activity was calculated as ΔA470 min^{-1} mg^{-1} protein.

2.4. Experimental design and statistical analysis

The study was set up based on a completely randomized design with a factorial arrangement in two replicates, and 15 shoot tip explants were used in each replication. The statistical analysis was subjected to one-way analysis of variance (ANOVA) using the Statistica 7 program. Data was presented as means ± standart errors, and means were compared by Tukey's HSD (Honestly Significant Differences) test, and differences with P values ≤0.05 were considered statistically significant.

3. Results and Discussion

3.1. Growth parameters

3.1.1. Length, fresh weight and dry weight of shoots

The effect of 24-epiBL pretreatment on the physiological parameters such as length, fresh weight, and dry weight of the shoots of plantlets under NaCl stress is shown in Table 1. In general, shoot length and shoot dry weight were negatively affected by the

increasing salt stress. Whereas shoot fresh weight increased at a 20 mM NaCl dose, and then decreased gradually with increasing NaCl concentration in the culture media. It was found that shoot fresh weight in 24-epiBL non-treated *in vitro* plantlets was the highest (810 mg) in the 20 mM NaCl dose by showing an inducing effect on the plant development compared to the control (0 mM NaCl) (750 mg), and increasing NaCl concentrations negatively affected shoot fresh weight, ranging from 740 mg to 180 mg (from 40 mM NaCl to 100 mM NaCl, respectively). Since tomato plants are considered moderately tolerant to salt stress, 80 mM and 100 mM NaCl concentrations caused a dramatic decrease in developmental parameters for the M-28 hybrid cultivar we used for our study. Similar results related to development of shoots and roots of plantlets were also obtained in different works (Mercado et al. 2000; Shibli et al. 2007; Abu-Khadejeh et al. 2011; Srinieng et al. 2015; Khaliluev et al. 2022). Roşca et al. (2023) stated that reducing plant development under salt conditions may be an adaptive morphological strategy to limit water loss through transpiration. Aly et al. (2012) emphasized that the decrease in growth because of high salinity is due to several factors as water and nutritional deficiency, ionic imbalance, specific ion toxicity, Na^+ and Cl^- excess might cause disorganize cell division, elongation and structure. Nutrients imbalance due to depressed uptake, shoot transport from roots and impaired internal distribution of nutritious minerals such as K^+ and Ca^{+2} can be explained the reduction in plant growth (Rashed et al. 2016). Sousa et al. (2022) also underlined that salt-induced declines in growth-related parameters primarily correlated with reduced water uptake, along with a negative interference in nutrient and ion ratios caused by the build-up of salts.

Table 1- Shoot length, fresh weight and dry weight of 24-epiBL pretreated plantlets under NaCl stress conditions

24-EpiBL (µM)	*NaCl (mM)*					
	0	***20***	***40***	***60***	***80***	***100***
			Shoot Length (cm)			
0	10.90 ± 0.43	10.25 ± 0.68	7.57 ± 0.60	7.40 ± 0.34	6.15 ± 0.45^{a}	3.33 ± 0.23^{b}
1	10.13 ± 0.42	10.98 ± 0.44	7.82 ± 0.74	7.35 ± 0.36	3.44 ± 0.31^{b}	3.89 ± 0.35^{ab}
2	9.94 ± 0.40	11.09 ± 0.51	7.45 ± 0.55	7.19 ± 0.43	4.74 ± 0.42^{ab}	4.29 ± 0.32^{a}
			Shoot Fresh Weight (mg)			
0	750 ± 30	810 ± 60^{b}	740 ± 80	520 ± 40	410 ± 40^{a}	180 ± 30^{b}
1	730 ± 30	980 ± 60^{ab}	840 ± 80	520 ± 40	210 ± 30^{b}	250 ± 40^{ab}
2	700 ± 30	1100 ± 40^{a}	660 ± 70	550 ± 40	360 ± 50^{ab}	390 ± 50^{a}
			Shoot Dry Weight (mg)			
0	31.10 ± 3.72	14.07 ± 3.18	11.07 ± 3.29^{ab}	8.30 ± 2.17	12.43 ± 0.61^{a}	2.53 ± 0.72^{b}
1	32.78 ± 4.05	24.45 ± 5.98	10.97 ± 3.84^{b}	8.34 ± 2.09	3.33 ± 1.52^{b}	3.35 ± 0.68^{b}
2	27.71 ± 3.09	26.80 ± 4.70	23.76 ± 2.76^{a}	9.36 ± 2.95	4.13 ± 0.95^{b}	7.26 ± 0.67^{a}

Values, means ± standard errors. In terms of the values in the same column, the letters show the statistical differences between 24-epiBL concentrations compared to the control at P≤0.05

When evaluating the effect of 24-epiBL pretreatment against salt stress on shoot growth, it was found that 24-epiBL used to reduce negative effect of NaCl stress improved development of plantlets. 24-EpiBL pretreatment (2 µM) increased the shoot length, fresh and dry weight of the growing plantlets at a dose of 100 mM NaCl. Again, 24-epiBL pretreatment (2 µM) caused an increase in the fresh weight of the shoots developed at 20 mM NaCl, and the increase was found to be statistically significant difference (P≤0.05) (Table 1). It is clear that salt stress negatively affects growth and development of *in vitro* plantlets and in this study, 24-epiBL pretreatment improved developmental parameters such as length, fresh weight, and dry weight of shoots under salt stress. The use of BR, especially 24-epiBL, was suggested to enhance for NaCl tolerance. Anuradha & Rao (2001; 2003) reported positive effects of 24-epiBL on parameters such as fresh weight, dry weight, and length of rice seedlings grown from seeds at salt stress condition. According to Anwar et al. (2018), plants showed very rapid responses upon BR application at very low concentrations by increasing shoot growth because of the elongation and expansion of cells under stress conditions. BRs obtained stress tolerance by enhancing the multiple plant defense systems through increasing the activities of antioxidant enzymes (APX, SOD, POD, CAT, and GR), and altering nutrient accumulation to enhance seedling growth. Ahmad et al. (2018) reported that 24-epiBL increased plant growth under salt stress by enhancing the photosynthetic efficiency and H^+-ATPase enzyme activity that is directly responsible for the activation of cell wall loosening enzymes and therefore developing growth. The present study clearly reveals that pretreatment of 24-epiBL on shoot tips is sufficient to reduce the possible negative impacts of NaCl stress.

3.1.2. Length, fresh weight and dry weight of roots

The effect of 24-epiBL pretraetment on length, fresh weight and dry weight of the plantlets roots under NaCl stress is shown in Table 2. In general, the root development process was negatively affected under NaCl stress. Root length, fresh and dry weight decreased under salt conditions. The root length was 8.13 cm in the NaCl-free control medium, and it was also determined that it was 7.88, 7.03, 6.16, 7.43, 5.35 cm from 20 mM NaCl to 100 mM NaCl. Root fresh weight and dry weight were the highest (340 mg and 31.10 mg, respectively) in the control groups and they were the lowest (30 mg and 2.53 mg, respectively) at the 100 mM NaCl stress (Table 2).

Table 2- Root length, root fresh weight and root dry weight of 24-epiBL pretreated plantlets under NaCl stress conditions

24-EpiBL (µM)	*NaCl (mM)*					
	0	***20***	***40***	***60***	***80***	***100***
			Root Length (cm)			
0	8.13 ± 0.19^{a}	7.88 ± 0.28	7.03 ± 0.36	6.16 ± 0.26	7.43 ± 0.45	5.35 ± 0.61
1	7.41 ± 0.19^{b}	8.02 ± 0.35	7.15 ± 0.35	5.97 ± 0.27	6.26 ± 0.56	6.71 ± 0.67
2	8.00 ± 0.22^{ab}	8.71 ± 0.25	7.10 ± 0.53	6.12 ± 0.29	7.24 ± 0.53	5.94 ± 0.59
			Root Fresh Weight (mg)			
0	340 ± 10^{a}	340 ± 20^{a}	210 ± 20	80 ± 8	80 ± 08	30 ± 08
1	280 ± 10^{b}	270 ± 20^{b}	180 ± 10	80 ± 9	90 ± 25	50 ± 11
2	290 ± 10^{ab}	330 ± 10^{ab}	150 ± 20	100 ± 7	80 ± 13	40 ± 06
			Root Dry Weight (mg)			
0	31.10 ± 3.72	14.07 ± 3.18	11.07 ± 3.29^{ab}	8.30 ± 2.17	12.43 ± 0.61^{a}	2.53 ± 0.72^{b}
1	32.78 ± 4.05	24.45 ± 5.98	10.97 ± 3.84^{b}	8.34 ± 2.09	3.33 ± 1.52^{b}	3.35 ± 0.68^{b}
2	27.71 ± 3.09	26.80 ± 4.70	23.76 ± 2.76^{a}	10.57 ± 3.47	4.13 ± 0.95^{b}	7.26 ± 0.67^{a}

Values, means ± standard errors. In terms of the values in the same column, the letters show the statistical differences between 24-epiBL concentrations compared to the control at P≤0.05

Excessive levels of sodium chloride in the soil near the root system may affect cell division and enzyme activity in the root tips, leading to a reduction in root length due to reduced water uptake and toxicity of sodium chloride (Abdel-Farid et al. 2020). Seth & Kendurkar (2015) reported that the reduction in root length under salt stress is mainly due to low water potential and limited cell growth in the external environment. 24-epiBL (2 µM) used against NaCl stress showed a positive effect on root length and root dry weight at 20 mM and 100 mM NaCl. In this context, root dry weight improved by 24-epiBL pretreatment at 100 mM NaCl (Table 2).

3.2. Biochemical parameters

3.2.1. Pigment content

The effect of BRs on the pigment contents of plantlets grown *in vitro* shoot tips under salt stress is summarized in Table 3. Especially pigment contents are negatively affected by increasing salinity when compared to the control (0 mM NaCl). Similarly, El-Meleigy et al. (2004) and Mohamed et al. (2011) reported that chlorophyll a and total chlorophyll contents decreased under salt stress. Photosynthesis activity is connected to photosynthetic pigments, namely chlorophyll a, chlorophyll b, and carotenoids, which are pivotal to the photosynthetic process (Bressan 2010). Additionally, salt stress leads to a reduction in pigment biosynthesis or an increase in pigment degradation. Furthermore, the disruption of the chloroplast's ultrastructure, including thylakoids, may be caused by Na^{+} toxicity or oxidative damage associated with salt stress (Aly et al. 2012).

Table 3- Chlorophyll pigment contents of 24-epiBL pretreated plantlets under NaCl stress conditions

24-EpiBL (µM)	*NaCl (mM)*					
	0	***20***	***40***	***60***	***80***	***100***
			Chlorophyll a (µg mL^{-1})			
0	5.40 ± 0.48	3.66 ± 0.14	3.22 ± 0.31	3.23 ± 0.39	2.81 ± 0.10	2.63 ± 0.017^{b}
1	3.62 ± 0.85	4.32 ± 0.51	2.05 ± 0.09	2.46 ± 0.12	3.19 ± 0.52	3.04 ± 0.005^{a}
2	3.91 ± 0.08	4.72 ± 0.24	2.18 ± 0.02	1.86 ± 0.47	1.90 ± 0.01	1.93 ± 0.011^{c}
			Chlorophyll b (µg mL^{-1})			
0	3.99 ± 0.34^{a}	1.17 ± 0.32	1.72 ± 0.226^{b}	1.44 ± 0.005^{b}	3.14 ± 0.985^{a}	3.82 ± 0.06^{b}
1	3.22 ± 0.29^{ab}	1.49 ± 0.01	2.57 ± 0.531^{ab}	2.24 ± 0.005^{a}	0.24 ± 0.026^{b}	4.82 ± 0.01^{a}
2	0.94 ± 0.02^{b}	1.59 ± 0.10	3.71 ± 0.008^{a}	1.45 ± 0.011^{b}	0.80 ± 0.005^{b}	1.13 ± 0.01^{c}
			Total Chlorophyll (µg mL^{-1})			
0	8.14 ± 0.81	5.07 ± 0.43^{ab}	5.11 ± 0.10^{b}	4.50 ± 0.58	7.02 ± 0.03^{a}	6.55 ± 0.060^{b}
1	5.55 ± 1.10	5.03 ± 0.03^{b}	5.92 ± 0.25^{a}	3.89 ± 0.65	2.77 ± 0.20^{b}	8.07 ± 0.028^{a}
2	4.77 ± 0.28	6.50 ± 0.37^{a}	5.74 ± 0.10^{ab}	3.13 ± 0.64	2.73 ± 0.01^{b}	3.13 ± 0.008^{c}
			Carotenoid (µg mL^{-1})			
0	1.07 ± 0.24	0.72 ± 0.15^{ab}	0.63 ± 0.04	0.96 ± 0.13	0.36 ± 0.003^{c}	0.54 ± 0.003
1	0.80 ± 0.29	0.32 ± 0.06^{b}	0.81 ± 0.02	0.68 ± 0.14	2.09 ± 0.083^{a}	0.66 ± 0.005
2	1.92 ± 0.30	0.78 ± 0.06^{a}	0.81 ± 0.08	0.60 ± 0.20	1.35 ± 0.003^{b}	0.93 ± 0.254

Values, means ± standard errors. In terms of the values in the same column, the letters show the statistical differences between 24-epiBL concentrations compared to the control at P≤0.05.

24-EpiBL pretreatments under NaCl stress ameliorated the negative effect of salinity. Chlorophyll a content at 100 mM NaCl, chlorophyll b content at 40, 60, and 100 mM NaCl, total chlorophyll content at 40 and 100 mM NaCl, and carotenoid content at 80 mM NaCl were improved by 24-epiBL pretreatment, and increases in these pigment contents were found statistically significant ($P \leq 0.05$). Shahid et al. (2011) reported that 24-epiBL pretreatment against NaCl stress increased chlorophyll a and chlorophyll b content. Sharma et al. (2013) underlined that enhancement of the chlorophyll content with 24-epiBL might be due to BR-mediated transcriptional and translational regulations of genes related to the synthesis of photosynthetic pigments or due to their reducing roles in chlorophyll catabolism.

3.2.2. MDA content

MDA content, which determines lipid peroxidation in cell membranes, increased under NaCl stress. MDA content ranged from 0.73 to 4.48 µmol g^{-1} FW between 0 mM-100 mM NaCl stress (Figure 1). MDA content, a decomposition product of unsaturated fatty acids called lipid peroxidation, is regarded as a biochemical marker for determining the oxidative damage in the cell and organelle membranes under salt stress conditions (Sharma et al. 2013). Lipid peroxidation changes membrane properties such as proteins, lipids, carbonhydrates, membrane permeability, fluidity and bilayer thickness, and disorders the blayer structure of the cell membrane (Ahammed et al. 2012).

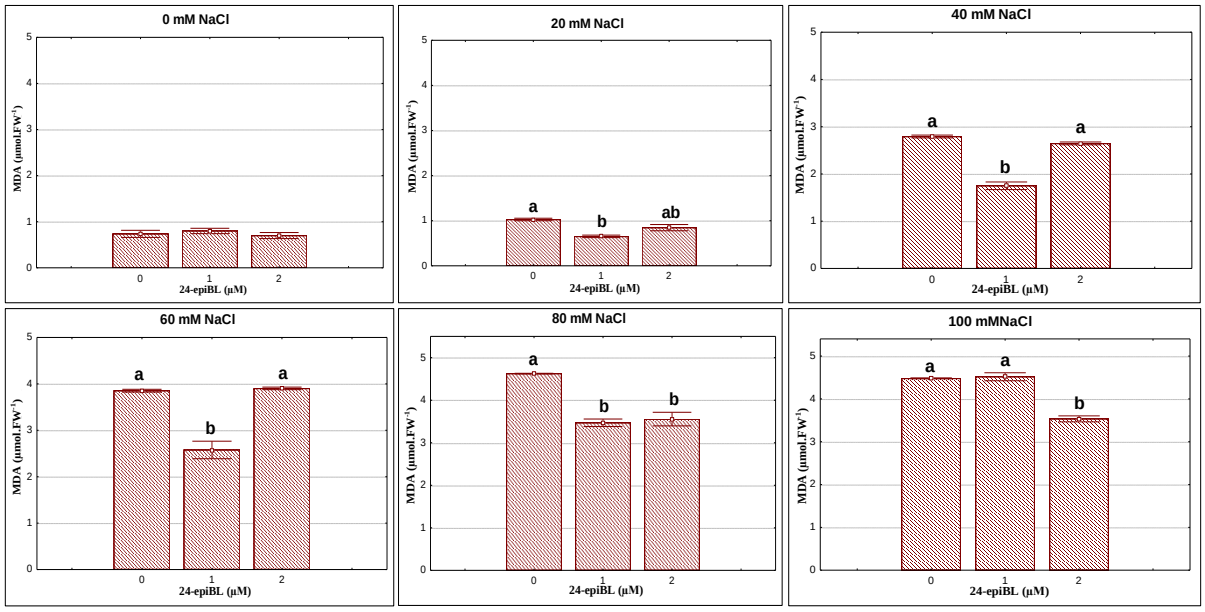

Figure 1- MDA contents (µmol g^{-1} FW) of 30 day-old plantlets grown 24-epiBL non-pretreated and pretreated *in vitro* shoot tips against to salt stress

It was found that MDA content statistically decreased with 24-epiBL pretreatment (1 µM) at 20, 40, and 60 mM NaCl, 24-epiBL pretreatments (1 µM and 2 µM) at 80 mM NaCl, and 24-epiBL pretreatment (2 µM) at 100 mM NaCl. Thus, 24-epiBL pretreatment for all NaCl concentrations ameliorated lipid peroxidation and cell membrane damage due to decreased MDA content (Figure 1). Ding et al. (2012) in eggplant, Sharma et al. (2013) in rice and Hu et al. (2016) in potato reported that 24-epiBL application against NaCl stress decreased increasing MDA content. The strong antioxidative defense system combined with other physiological differences in the plants contributes to the various salt responses between *in vitro* materials like calli or shoot tips, and whole plants (Lokhande et al. 2011). Thus, this result showed that 24-epiBL regulate lipid peroxidation in the cellular membranes and improves salt tolerance in plants.

3.2.3. Proline content

Proline is one of the most important biochemical markers accumulating under salinity conditions. Proline content increased under NaCl stress in the study. The proline content of 24-epiBL non-pretreated shoots ranged from 9.83 µmol g^{-1} FW (control) to 19.86 µmol g^{-1} FW (100 mM NaCl) (Figure 2). Proline is accumulated in various plant species under salt stress and other abiotic stress factors (Szabados & Savoure 2009). Proline, which plays a protective function against salt stress in plants, acts as a compatible osmolyte, enzyme protectant, free radical scavenger, cell redox balancer, cytosolic pH buffer, and stabilizer for subcellular structures to obtain salt tolerance (Iqbal et al. 2014). Proline acts as a store of energy that can be quickly broken down and used under NaCl stress (Woodward & Bennett 2005). Aly et al. (2012) indicated that accumulation of proline under stress conditions is due to induction in the proline biosynthesis or inhibition in the proline oxidation. Accumulation of proline play a key role on stress tolerance and proline as an osmolite contributes to the scavenging of ROS, keeping the configurations of proteins and store carbone and nitrogene resources in plants (Verbruggen & Hermans 2008). Proline maintain cellular redox

potential and serves to stabilize ultra-structural changes in cells. A higher accumulation of proline is reported to strengthen the ability of the cell to make ionic adjustments in the cell cytosol under stress conditions (Shahid et al. 2020).

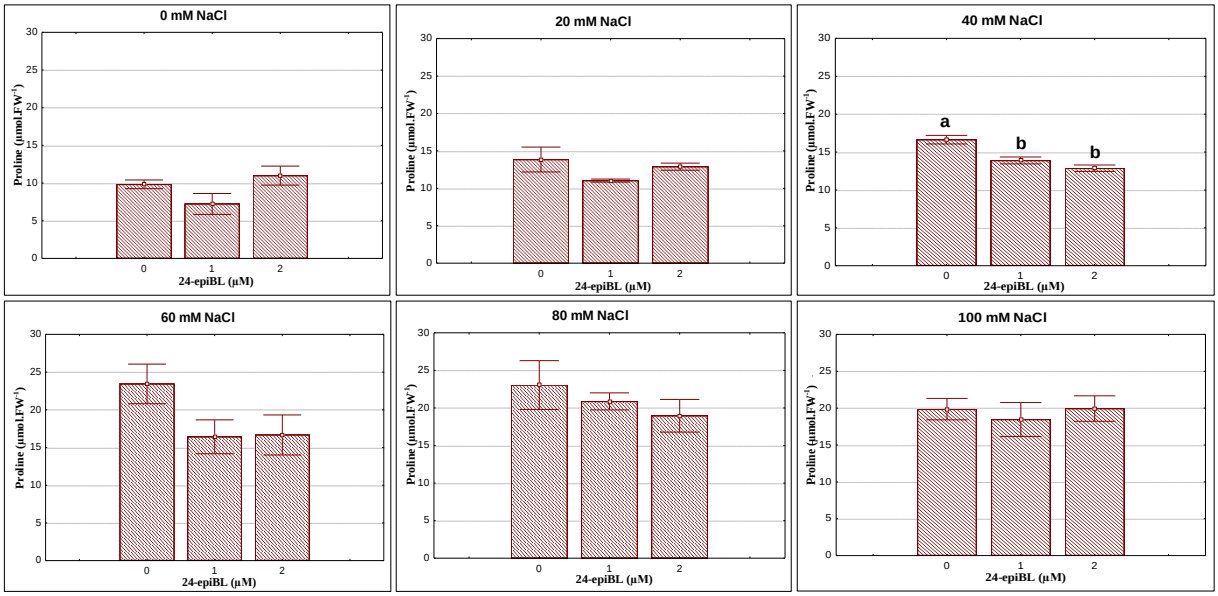

Figure 2- Proline contents (µmol g^{-1} FW) of 30 day-old plantlets grown 24-epiBL non-pretreated and pretreated *in vitro* shoot tips against to salt stress

In this study, proline content increased under salt stress. Similarly, Abu-Khadejeh et al. (2011) reported that proline content increased in the shoots of JO112 and JO992 tomato cultivars under salt stress conditions. Results from the another study showed that proline in the tomato calli increased compared with the control (Hassanein 2004; Mohamed et al. 2007; Aazami et al. 2010). It was found that this parameter decreased with 24-epiBL pretreatments. This finding was in accordance with the results of study conducted in pepper plants (Houimli et al. 2010). The decrease in proline content by 24-epiBL pretreatments (1 µM and 2 µM) was statistically significant only at a 40 mM NaCl concentration (Figure 2). As a result, 24-epiBL alleviates the negative effects of NaCl stress through different mechanisms including regulation of cytoplasmic pH and stabilization of protein, DNA, RNA, and membranes with the protective effects of proline (Sabir et al. 2012).

3.2.4. Soluble protein content

One of the osmolytes accumulated in high concentrations to maintain the osmotic balance in the cells is the soluble protein, which has at low molecular weight inside the cell. Total soluble protein content in 24-epiBL non-pretreated shoots was induced at a low NaCl concentration (20 mM NaCl), but this parameter decreased with increasing NaCl stress. Amini & Ehsanpour (2006), Shibli et al. (2007), Mohamed et al. (2011) and Abu-Khadejeh et al. (2011) reported that protein content in tomato decreased under salt stress, similar to our study findings. The decrease in the protein content under NaCl stress might be due to protein degredation, denaturation, proteolysis, decreases in the protein synthesis and free amino acids (El-Mashad & Mohamed 2012). In this study, when the effect of 24-epiBL used against salt stress was evaluated, total protein content statistically increased by 24-epiBL (2 µM) only at 40 mM NaCl concentration. Sharma et al. (2013) and Khalid & Aftab (2016) emphazised that protein content decreased under NaCl stress in rice but it increased by 24-epiBL application against to NaCl. Proteins in the plant cells may supply a nitrogen storage form of that is reutilized and may serve as an osmotic adjustment under stress conditions (Mehr 2013).

3.2.5. SOD and POX enzymes activities

In our study, SOD activity in leaves of *in vitro* plantlets was increased under salt stress conditions. SOD enzyme activity in leaves of 24-epiBL non-pretreated shoots ranged from 10.18 unit SOD mg^{-1} protein (control, 0 mM NaCl) to 41.06 unit SOD mg^{-1} protein (100 mM NaCl), (Figure 3). Various antioxidant enzymes protect the cell against reactive oxygen species that are more likely to be produced under salt stress conditions. According to Roşca et al. (2023), in tomatoes exposed to salt stress, antioxidant production and antioxidant enzymes activities can vary depending on cultivar, salt concentration, plant age, or part of the plant.

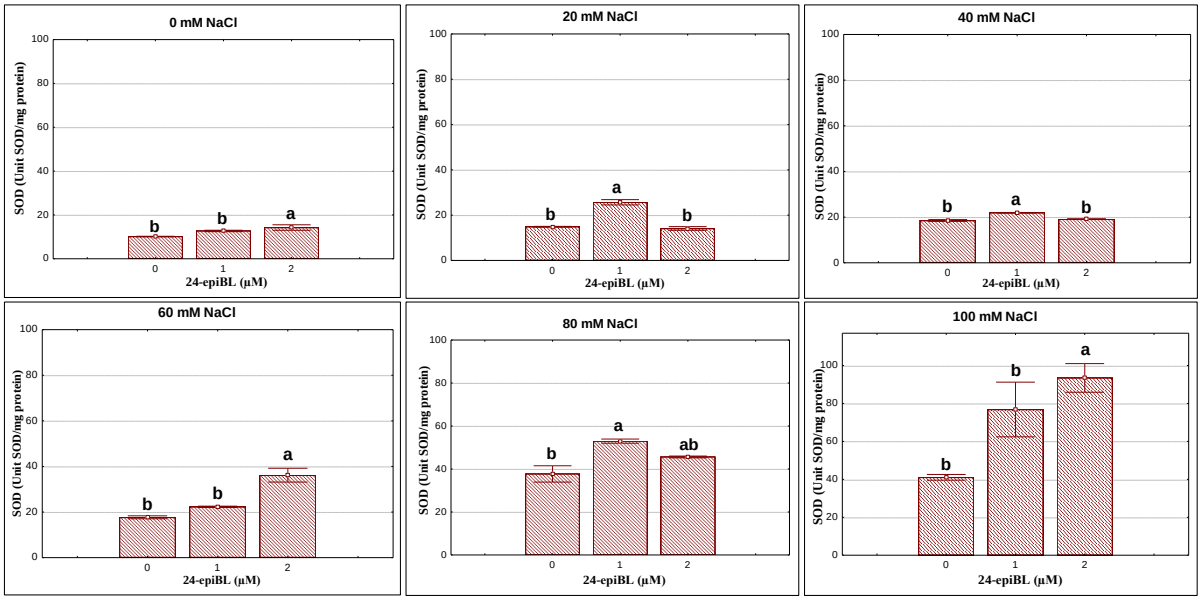

Figure 3- SOD enzyme activity (Unit SOD mg^{-1} protein) of 30 day-old plantlets grown 24-epiBL non-pretreated and pretreated *in vitro* shoot tips against to salt stress

When the effects of SOD activity according to NaCl concentrations used were statistically evaluated, 24-epiBL (1 µM) had a significant difference on SOD activity at 20, 40 and 80 mM NaCl concentrations. 24-epiBL (2 µM) had a positive effect on SOD activity at 60 and 100 mM NaCl concentrations, and this increaese was found statistically significant (Figure 3). Shahbaz et al. (2008) in wheat, Shahid et al. (2011) in pea, Ding et al. (2012) in eggplant, Sharma et al. (2013) in rice, Nafie et al. (2015) in common bean, and Upadhyaya et al. (2015) in potato reported that SOD enzyme activity increased by 24-epiBL under salt stress. ROS such as singlet oxygen, superoxide, hydrogen peroxide, hydroxyl are produced during aerobic metabolism, and ROS production under abiotic stress are much more than normal condition. However, plants generally can eliminate superoxide with the SOD activity which catalyzes the dismutation of superoxide into hydrogen peroxide and oxygen (Parida & Das 2005). BR-mediated ROS signal maintains the homeostasis which turns the activation of transcription factors that regulate stress responsive genes related to biosynthesis of SOD, POX, and CAT enzymes which enhance tolerance to different abiotic stresses by up-regulation of the antioxidant machinery system (Singh et al. 2021).

Hydrogen peroxide can be eliminated by POX (Ashraf & Harris 2004). In our study, POX enzyme activity increased under NaCl stress. POX enzyme activity in leaves of 24-epiBL non-pretreated shoots ranged from 1.65 ΔA470 min^{-1} mg^{-1} protein (control, 0 mM NaCl) to 5.12 ΔA470 min^{-1} mg^{-1} protein (100 mM NaCl) (Figure 4).

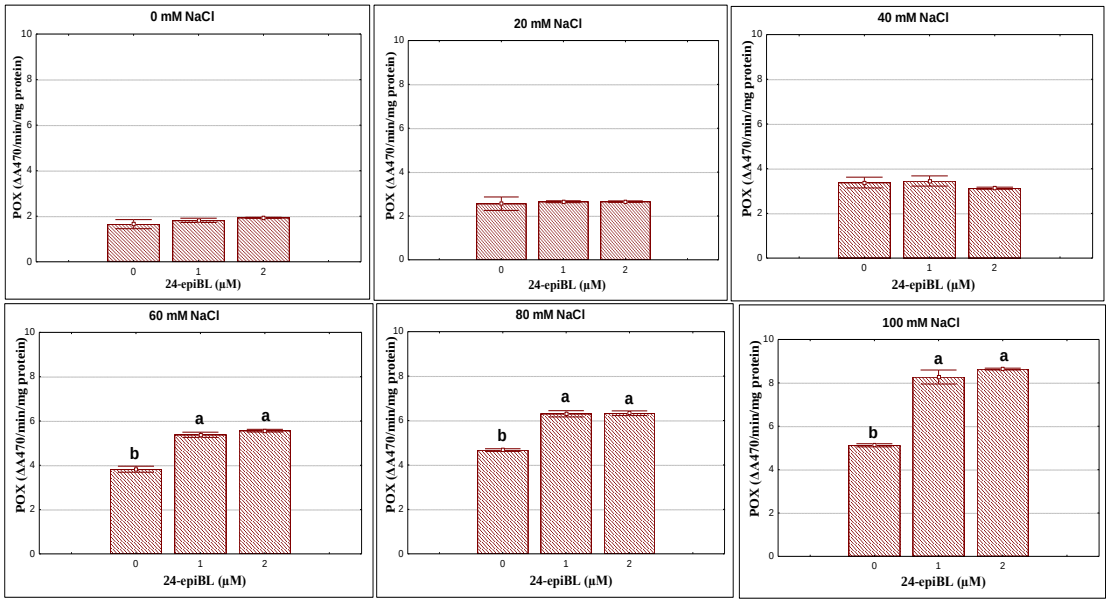

Figure 4- POX enzyme activity (ΔA470 min^{-1} mg^{-1} protein) of 30 day-old plantlets grown 24-epiBL non-pretreated and pretreated *in vitro* shoot tips againts to salt stress

As a result, POX activity was increased by 24-epiBL (1 and 2 µM) at 60, 80, and 100 mM NaCl concentrations, and it was found that the increases were statistically significant (Figure 4). The reason for the increase in the SOD and POX enzymes activity might be the possible effects of BR on expressions of the genes coding for the biosynthesis of these enzymes, which resulted in enhanced oxidation of harmful reactive substances (El-Mashad & Mohamed 2012).

4. Conclusions

Soil salinity is one of environmental problems that affect plant development and productivity, causing great economic losses. Different biotechnological approaches are used to overcome the detrimental effects of salt stress that occur in plants. Among these approaches, it is important to use these cultivars in agricultural applications, identify plant species and cultivars that have high salt tolerance, increase the salt tolerance of plants and reduce the effects of salt stress by using different substances such as proline, glisine-betain, especially BRs and 24-epiBL. As a result, in the present study, the effect of 24-epiBL against NaCl stress was first studied using *in vitro* shoot tip culture in M-28 hybrid cultivar and positive effect on salt tolerance of tomato plants was determined by growth and biochemical parameters. 2 µM 24-epiBL pretreatment showed a reformative effect on shoot and root development of plantlets under 100 mM NaCl stress. 1 µM 24-epiBL increased the pigment content in plantlets at a 100 mM NaCl dose. The MDA content, which increased under stress conditions, decreased with 1 µM 24-epiBL pretreatment at 40-80 mM NaCl dose. Osmoregulant proline content decreased with 1 µM and 2 µM 24-epiBL at 40 mM NaCl dose. 1 µM 24-epiBL at 20, 40, and 80 mM NaCl stress and 2 µM 24-epiBL at 60, 100 mM NaCl stress increased the SOD activity even more, allowing the tomato M-28 hybrid cultivar to fight the oxidative stress. POX activity increased with the pretraetment of 1 µM and 2 µM 24-epiBL at 20, 80, and 100 mM NaCl stress. In our study, 24-epiBL at both doses (1 µM and 2 µM) exogenously applied to the shoot tips showed a positive effect at modarate (40-60 mM NaCl) and high salt doses (80-100 mM NaCl). Further studies are needed to determine the effects of 24-epiBL at molecular level as well as growth and biochemical parameters by using *in vitro* cultures against salt stress in different crop species and cultivars.

Declaration of conflicting Interest

The authors declared no conflicts of interest with respect to the research, authorship and publication of this article.

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