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Investigation on the relationship between salinity stress and epibrassinolide in spinach (*Spinacia oleracea L. cv. Matador*) seedlings

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Abstract

Soil salinity is a very important abiotic stress condition that affects plant growth and crop yield. Photosynthetic activity, fresh weight, total protein amount decrease due to salinity condition. Brassinosteroids (BR) are a new group of hormones in the steroidal structure which is involved in the plant hormone group. BRs play an important role in various physiological processes. BR shave a curative effect on the plants exposed to environmental stress. In this study, the effect on seedling development was examined by spraying 24-epibrassinolide (eBL), the active form of brassinosteroids, on the seedlings exposed to salt stress. For this purpose, seedlings are divided into three groups such as Hoagland, Hoagland+NaCl, Hoagland+NaCl+eBL. As a result of preliminary experiments, 150 mM NaCl as the salt concentration reducing seedling growth and 10-9 M eBL which promotes growth by reducing this inhibition were determined as the appropriate concentration. This study; biochemical analysis of spinach seedlings exposed to salt stress and applied to eBL showed the curative effect of eBL on salt toxicity.

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1. Introduction

Scientists strongly indicate that the world will be affected by the agricultural drought which may arise as a result of the effects of global warming. Based on both global warming and poor use of farmland, serious problems such as drought and salination come to existence.

Both in growing conditions and in natural environments, plants often are faced with stress. Plants, as a whole or some parts of them, are resistant to stress. Stress factors which limit the growth and development of the plant and even cause the death of plant are divided into two as biotic and abiotic. Biotic stress factors consist of pathogens including viruses, bacteria and fungi, insects, and herbivores [1-3]. Abiotic stress factors are drought, temperature, salinity, lack of nutrients, heavy metals and the radiation [4-6]. As a consequence of the salt stress; growth and development of the plants are inhibited depending on the osmotic and ion stress [7]. Although the root system is directly exposed to salt, leaf growth is more sensitive to salt stress than root growth [8].

Tolerance mechanisms developed by the plants to be able to fight with salt stress contain the synthesis of compounds that are compatible, change in the photosynthetic road and in the membrane structure, the increase in the activity of the antioxidant enzymes, and the induction of plant hormones [7].

Salinity shows its primary effect on plants by creating osmotic and ion stress. The significant secondary effects that NaCl causes are as follows: The synthesis of reactive oxygen species (ROT) which damage the DNA, protein, chlorophyll, and the function of the membrane; inhibition of photosynthesis; metabolic toxicity; inhibition of K+ intake and cell death [9].

It is possible to make a variety of applications in order to stimulate the growth and development of plant under stress conditions. One of these conditions is the application of plant hormone. Brassinosteroid which is considered as resistance providers to the plants against different abiotic stress factors induces growth as well as various development processes such as seed germination, rhizogenesis, flowering, senescence, abscission, and ripening [10].

Spinach is a vegetable which is rich in vitamin and mineral and important for human health.

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2. Materials and Methods

2.1. Plant material; applications of salt (nacl) and 24- epibrassinolide (24-ebl)

In this research, Spinacia oleracea L. cv. Matador was used widely grown in Turkey as plant material and eBL (Sigma- E1641) is provided from Sigma-Aldrich. eBL was used after being dissolved in absolute ethanol. Seedlings grown in plant growth chamber, in which there is a 15-hour photoperiod under a light intensity of 6000 lux at 15 ± 2 °C, were watered on alternate days with 400-500 ml distilled water for the first 7 days. Growing seedlings were irrigated on alternate days from 8th day to 15th day (including 15th day) with Hoagland Nutrient Solution.

On the 16th day, seedlings are divided into three groups to investigate whether 10-9 M eBL solution which is applied by spraying to all of the plant (except the root) has an ameliorative effect against salt stress. The first group: Control group grown in Hoagland nutrient solution; the second group: The experimental group grown in Hoagland nutrient solution (salty Hoagland) containing 150 mM NaCl; the third group: It is the experimental group grown in salty Hoagland nutrient solution and applied eBL.

Whereas eBL is applied by spraying to all the parts of the seedlings in the 3. group, other groups are sprayed only with distilled water.

2.2. Measurement of fresh weight, determination of the difference

The fresh stem weights of the plants were measured on the 15th day. Afterwards, hormone application was made during 30 days to the plants transferred into the water culture. At the end of the 45th day, the fresh weights were measured again, and the differences were calculated.

2.3. Determination of total chlorophyll amount

After 45 days seedlings were harvested, their stems were extracted with 90% acetone and centrifuged at 3000 g at +4 °C for 10 minutes after waiting in the dark at +4 °C for 24 hours. The absorbance was measured at wavelengths of 630 nm, 645 nm, and 665 nm using spectrophotometer. Total chlorophyll content was determined in μg/g fresh weight [11].

2.4. Determination of total soluble protein amounts

Samples were homogenized in pairs with 0.05 M sodium phosphate buffer (pH 7.8) containing 2% (w/v) polyvinylpolypyrrolidone (PVPP) and 1 mM EDTA in cold press by using liquid nitrogen. The protein concentration of the supernatant was determined by a Bradford protein assay [12]. Bovine Serum Albumin (BSA) was used as a standard. The protein concentrations were determined in µg protein /g fresh weight.

2.5. Determination of proxidase (POD) enzyme activity

The peroxidase activity was determined as described by previously to determine the peroxidase activity, the method of the Birecka et al. [13] was used. The absorbance was measured at the wavelength of 470 nm. Using microplate spectrophotometer BioTek Epoch2. Enzyme activity was determined in ΔA/g. fresh weight.x min.

2.6. Determination of superoxide dismutase (SOD) enzyme activity

Superoxide dismutase activity was determined as described by Beauchamp and Fridovich [14]. The activity is defined by measuring the ability of SOD to inhibit the reduction of nitro blue tetrazolium (NBT) photochemically. Reaction mixture contains 50 mM phosphate buffer (pH 7.8), 33 μM NBT, 10 mM L-methionine, 0.66 mM EDTA-Na2, 0.0033 mM riboflavin. After the supernatant was diluted and the mixture left for 20 minutes under 300 µmol m-2s-1 light intensity, the absorbance of the reaction mixture was measured at 560 nm. The amount of protein (mg) to inhibit reducing with light 50% was defined as an enzyme unit for SOD and accordingly SOD activities of stem samples were determined.

2.7. Measurement of membrane permeability changes

Before being dried, 45-day seedlings were washed with tap water and distilled water, respectively. Samples were placed into distilled water in pairs as 0.1 g sample/10 ml distilled water. First, the samples stood at 40 °C for 30 minutes and their EC (Electrical Conductivity) was measured in conductivity measuring device (C1). Then, they stood in the water bath at 100 °C for 10 minutes before measuring EC again (C2). The obtained values were expressed in terms of mS/cm by being put into the formula below [15]. (MSI: Membrane Stability Index).

MSI: [1-(C1/C2)] x100

2.8. Statistical analysis

Analyzing data obtained using One-Way Analysis of Variance (One–way ANOVA) in Statistical Package for Social Sciences (SPSS for Windows 10.0) program, the differences between the averages were found as $P<0.05$ with the Duncan Multiple Comparison Test.

3. Results

3.1. Shoot fresh weight

As it is understood from Figure 1 and Figure 2, the development of seedlings exposed to salt stress was decelerated significantly and their fresh weights were decreased at a rate of 60%. When compared with the eBL unimplemented group, the fresh weights of seedlings exposed to 10^{-9} M eBL were increased at a rate of 35%.

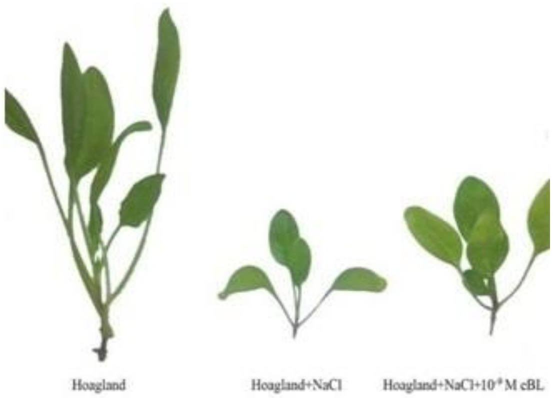

Figure 1. Shoot development of spinach seedlings which were grown in Hoagland solution with and without salt and applied with 10^{-9} M eBL for 30 days.

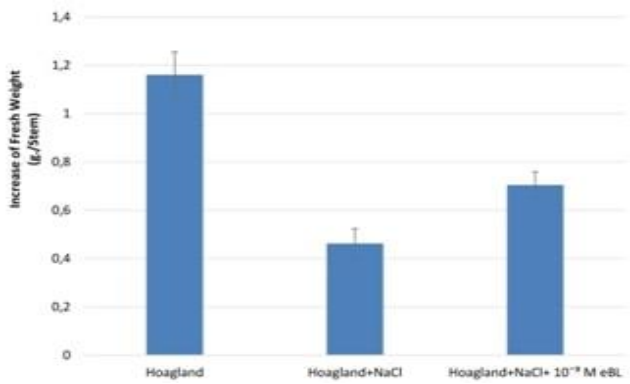

Figure 2. Increase of fresh weight of spinach seedlings which were grown in Hoagland solution with and without salt and applied with 10^{-9} M eBL for 30 days ($P<0.05$).

3.2. Total chlorophyll content

The total chlorophyll content of the seedlings used in this study was specified to determine their photosynthetic activities. Seedlings which were grown in Hoagland+NaCl solution and whose development was affected due to toxic effects were harvested on the 45th day and total chlorophyll content of all groups was identified (Figure 3).

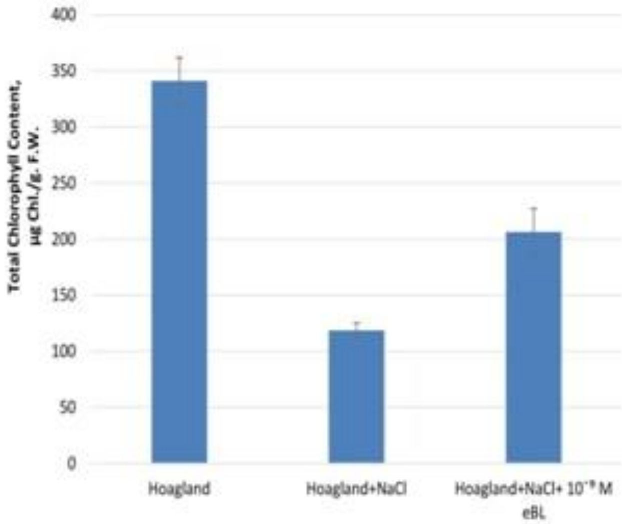

Figure 3. Total chlorophyll content of spinach seedlings which were grown in Hoagland solution with and without salt and applied with 10^{-9} M eBL for 30 days ($P<0.05$).

As seen in Figure 3, when Hoagland solutions containing NaCl and non- containing NaCl were compared with each other, chlorophyll amount of those NaCl containing

Hoagland solution declined 3 times. This phenomenon reveals that 150 mM NaCl application causes stress in spinach seedlings. Comparing total chlorophyll amount of Hoagland+NaCl plant group with total chlorophyll amount of Hoagland + NaCl + 10^{-9} M eBL plant group, an increase at a rate of 74% was detected in the seedlings with eBL applied. This result shows that eBL stimulates photosynthesis by increasing chlorophyll amount of and thus, salt stress affects toxic effect in a remedial way.

3.3. Total protein amount

Since it is known that total soluble proteins disintegrate in plants subjected to the salt stress, total protein amounts of the 45-day seedlings were measured and it was noticed that there is a significant difference ($P<0.05$) between the groups (Table 1).

Table 1. Total protein amounts of spinach seedlings which were grown in Hoagland solution with and without salt and applied with 10^{-9} M eBL for 30 days (P<0.05).

Treated Seedlings	Total Protein Amount, µg Protein/g.F.W.
Hoagland	0.0109±0.00097
Hoagland+NaCl	0.0034±0.00016
Hoagland+NaCl+10^{-9} M eBL	0.0060±0.00011

Table 2. SOD activity of spinach seedlings which were grown in Hoagland solution with and without salt and applied with 10^{-9} M eBL for 30 days (P<0.05).

Treated Seedlings	Superoxide Dismutase (SOD) Activity, %
Hoagland	170.4332±0.3468
Hoagland+NaCl	188.0439±2.6872
Hoagland+NaCl+10^{-9} M eBL	430.3297±5.4647

When seedlings grown in NaCl containing and non-containing Hoagland solutions were compared, it is defined that total protein amounts of the ones in Hoagland solution with NaCl decrease at least 3 times. This phenomenon revealed that 150 mM NaCl application inhibits growth in spinach seedlings. In contrast, Hoagland+NaCl+10^{-9} M eBL application increased the total protein amount at a rate of 43%. These findings align with literature in which it is expressed that eBL application during salt stress causes synthesis of new proteins [16].

3.4. Antioxidant enzyme activities

It is identified that there occur major changes in peroxidase (POD) activities of various seedlings exposed to salt stress [16, 17]. POD activity in seedlings exposed to salt stress was determined in 45-day spinach seedlings. The changes in peroxidase activity were shown in Figure 4.

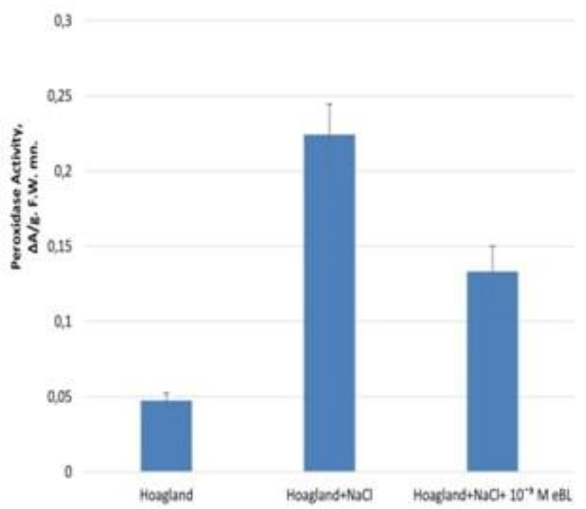

Figure 4. Peroxidase activity of spinach seedlings which were grown in Hoagland solution with and without salt and applied with 10^{-9} M eBL for 30 days (P<0.05).

From Figure 4 it can be seen that the POD activity of the seedlings grown in NaCl containing Hoagland solution increased approximately 5 times more than control (Hoagland). This phenomenon revealed that 150 mM NaCl application enhances enzyme activity due to the stress in spinach seedlings. Comparing POD activity of Hoagland+NaCl plant group with POD activity of Hoagland+NaCl+10^{-9} M eBL plant group, an increase of 1.7 times was detected in the eBL applied seedlings. This situation aligns with literature in which it is mentioned the remedial effect of the eBL application during the salt stress [16, 17].

There occur significant changes in superoxide dismutase (SOD) activities of the various plants exposed to salt stress [16]. The SOD activities of 45-day spinach seedlings were also identified (Table 2).

Compared to the seedlings which were not exposed to salt stress, in seedlings exposed to salt stress the SOD activity showed only 10% increase. However, this ratio increased at a rate of 56% for the spinach seedlings grown in solutions containing NaCl+eBL compared to eBL unimplemented seedlings. When cross-checked with current literature data, this result indicates that eBL shows a stress relieving effect [18].

3.5. Membrane stability index

In order to investigate the changes in membrane permeability, the values obtained from measuring leaked molten substance amount of spinach seedlings to the incubation environment and defined in terms of membrane stability index (MSI) were given in Table 3.

Table 3. Change of the membrane permeability of spinach seedlings which were grown in Hoagland solution with and without salt and applied with 10^{-9} M eBL for 30 days (*P*<0.05).

Treated Seedlings	Membrane Permeability, mS/cm
Hoagland	170.4332±0.3468
Hoagland+NaCl	188.0439±2.6872
Hoagland+NaCl+10^{-9} M eBL	430.3297±5.4647

As can be seen in Table 3, the conductivity of the seedlings grown in Hoagland+NaCl solution was 17% higher than the seedlings grown in Hoagland solution.

Hoagland+NaCl+10^{-9}M eBL solution decreased 6.11% compared with the ones grown in Hoagland+NaCl solution. The highest leaked substance amount resulting from permeability change occurred in the plants in salt stress condition. Applying eBL to these seedlings partially decreased the leaked substance amount and had a remedial effect on the impact of salt stress which disrupts the membrane integrity.

The aim of this study is to investigate the impact of eBL on the plant's resistance which was grown in salty lands because of the increased drought as a result of global warming and unconscious irrigation.

4. Discussion

The aim of this study was to investigate whether there is any remedial effect of eBL application on the development of spinach seedlings which are exposed to toxic effects of salt (NaCl) stress and have an important place in the food chain. Hence, epibrassinolide (eBL) was applied by spraying to spinach seedlings grown in Hoagland solution containing salt (NaCl) and the development of the seedlings was observed. The obtained data showed that the development of seedlings exposed to 150 mM NaCl (salt) stress decelerated and their fresh weights decreased by 60%. 10-9 M eBL application to seedlings that cannot develop under toxic effect due to the salt stress, caused their fresh weight to increase at a rate of 35%. This result showed that 10-9 M eBL application had a remedial effect on the seedling whose development decelerated under stress (Figure 2 and made the fresh weight of stems increase. With the increase of salt amount in the medium, the Na+/K+ balance and the osmotic balance shifted, the usable water content became less due to the decreasing osmotic potential, development rate and photosynthesis rate decelerated [19].

In this study, it was determined that chlorophyll content in seedlings exposed to the salt stress generated a decrease up to 3-times than control. Increasing Na+ causes degradation of D1 protein which is located in the reaction center of PSII. It is reported that the real target of NaCl in thylakoid membrane is PSII [20]. Applying eBL to the seedlings whose chlorophyll contents decreased when they were exposed to the salt stress, increased the chlorophyll content at a rate of 74%. In fact, there is study expressing that the chlorophyll content increases in seedlings to which brassinosteroid is applied [21].

One of the structural decompositions that stress factor created indirectly is the degenerative events affecting protein amount. From being exposed to the abiotic stress on, plants produce proteins without tryptophan starting the expression the genes related to stress in order to deal with this factor [22]. In this study, statistically significant differences were determined in total protein amounts of spinach seedlings exposed to salt stress compared to the control. A 3-times decrease was identified in the total protein amounts of seedlings exposed to the salt stress than the ones grown in Hoagland solution (Figure 4, 5). This situation explains the cause of the deceleration in the growth of the seedlings exposed to the salt stress. On the other hand, eBL application to these seedlings developed in stress condition provided an increase of 43% in the protein content of these seedlings. In addition, the activities of antioxidant systems were decreased under the influence of environmental stress factors (salinity, drought, high or low temperature, UV, ozone, etc.) and these conditions cause ROS to accumulate by inducing its synthesis [23]. In this study, when the differences between the POD activities of spinach seedlings grown in Hoagland and Hoagland+150 mM NaCl were statistically examined, significant differences between the groups were identified (P<0.05). 5 times increase was identified in POD activities of the seedlings exposed to the salt stress (Figure 4). In fact, in many plants such as Ceratophyllum demersum L., Brassica juncea L. Czern. (mustard), Triticum aestivum L. (wheat), Vigna mungo L. (black gram) and Phaseolus vulgaris L. (beans) an increase in POD enzyme activity and gene expression were observed under stress conditions and these increases were associated with stress defense [24, 25]. On the other hand, applying eBL to these seedlings developed under stress conditions caused approximately 1.7 times decrease in POD activity. This result shows that eBL reduces the negative effect of the stress. The increase in POD activity is found to be greater than the decrease in the chlorophyll and protein contents.

In a study conducted with Zea mays (corn) seedlings grown under the salt stress, it is expressed that 28-homoBL shows a remedial effect on salinity stress increasing the activity of superoxide dismutase (SOD), one of the antioxidant enzymes, and decreasing the lipid peroxidation [18]. Indeed, what's more, in this study eBL application showed a remedial effect of stress by decreasing the POD activity and increasing the SOD activity 65%, and thus, it indicated similarities with the findings of Arora et al. [18]. On the other hand, they stated that there is no difference in SOD activity of cucumber plant under the salt stress condition [26]. Whereas the relationship of CAT and SOD enzymes with salinity tolerance in wheat and increase of SOD activity with salt [27] were highlighted, in another study conducted with hordeum it was stated that there is no relationship between antioxidant enzyme and salt tolerance [28].

ROS that arise from oxidative stress caused by the salt stress led to changes also in membrane permeability [29]. In this study, it was seen that salt stress caused damage in the membrane of spinach stem cells. An increase of 17% in electrical conductivity is a sign of the fact that plants are under stress and a sign of loss of vitality (Table 3). In many conducted types of research, plants were subjected to the salt stress and thus, membrane permeability was disrupted [30, 31]. In this study, the eBL application to the stems of spinach seedlings exposed to the salt stress caused a 6% decrease in membrane permeability of these seedlings. So that it was identified that the ionic imbalance in seedlings due to the salinity was tolerated by the plant by means of eBL. The results we have obtained are parallel to the findings of Çoban [32] and Çoban and Baydar [33] showing the decrease of membrane permeability with the eBL application. Thus, being adversely affected by the stress of the plant which is inhibited. The disruption of the membrane structure was interpreted as the impairment of the ion gradient and the partial failure of the ATP-H+ pump to function. Thus, the production of ATP required for photosynthesis and respiratory events gradually decreases, and in the long-term stress condition, the process results in cellular death. There is finding supporting these results [34]. A negative correlation is present between the decreasing chlorophyll and protein contents as a result of the increased membrane permeability in spinach plant stems under stress condition.

In conclusion, then, salt stress may create morphological, physiological and biochemical damage and adversely affect the efficiency of the product. This study, carried out in salt stress conditions of salt stress, reveals that the plant increases its resistance to the stress condition and has a remedial effect by increasing chlorophyll and protein content, decreasing the peroxidase activity, and providing membrane integrity. Even plants to have their molecular defense mechanisms to reduce or inhibit the adverse effects, while stress adversely affects plant growth and development, it causes the plant organs to die. Hence, it is crucial that mechanisms associated with stress must be found out and stress-tolerant species and variants must be developed.

5. Conclusion

In order to minimize the negative effects of global warming, scientists have been working hard in recent years. In this study, spinach seedlings, which is one of the most consumed foodstuffs, was exposed to salt stress.

The newly discovered plant hormone (epibrassinolide) was applied to these seedlings that growth in NaCl Hoagland solution. The concentration of 10^{-9} M eBL was found to be the alleviating effect in this study.

As a result of these applications, morphological differences were observed; peroxidase and superoxide dismutase enzyme activities alongside total chlorophyll, total protein amount, and changes in membrane permeability were identified as stress parameters in all parts of the plant at the 45th day which is the day that the salt application shows its effect. The results were supported by biochemical analyzes.

At the end of this study, the effect of spinach seedlings exposed to salt stress and treated with 10^{-9} M eBL on salt toxicity was shown. This information is very important in the field of agriculture.

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