A Primary Study: Investigation of the *In vitro* Salt Stress Effects on Development in *Thymus Cilicicus* Boiss. & Bal

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Abstract: Since plants are soil-dependent organisms, they are directly exposed to biotic and abiotic stress factors. Of these factors, salinity has a direct effect on the growth and development of plants, and salinity at increasing rates can be critical for the viability of plant species. The current study aimed to investigate the effects of increasing *in vitro* salinity on the growth of endemic *Thymus cilicicus* Boiss. & Bal.. In this context, it has been observed that increasing concentrations of salinity suppress growth, and this study is a preliminary study to investigate the effects of salinity stress at the molecular level in the future.

Keywords: Abiotic stress, *In vitro* development, Salinity, Thyme.

INTRODUCTION

The growth and development conditions are under the control of the environmental factors in plant species. Changes in environmental factors can affect growth and development and reveal some stress situations. In general, stress is the factor that suppresses plant growth and development and reduces plant yield by causing metabolic and physiological disorders. The defenses of plants that can be exposed to more than one stress factor, depending on their species, appear as natural selection in the evolutionary process. Depending on different stress conditions, plants have also developed very different defense mechanisms. Stress factors are classified as biotic and abiotic [1, 2]. The biotic stress factors; some pathogens host various factors such as microorganisms (fungus, bacteria, virus, etc.), animals (insect, nematode, herbivores, rodents, etc.), allelopathy and parasitic plants, the abiotic stress factors; it includes one or more of many factors such as low or high temperature, drought, salinity, chemicals, radiation, toxins, wind, UV, pH changes, soil infertility (hardness), wastes, heavy metals, oxidative stress [3, 4].

Since plants are soil-dependent organisms, they had to develop more diverse defense mechanisms against abiotic stress factors in the evolutionary process. The overcoming of the abiotic stress factors is usually provided by strengthening the plant root. Because for a living thing that is dependent on the soil and feeds from it, the strong root part will ensure its survival under these stress conditions. Stress resilience is the ability of an organism to continue its activities under adverse conditions. As a living being can be resistant to stress as a whole; A certain organ may be resistant, while other organs may be susceptible. Factors affecting this resistance; the duration of the stress, its severity, the tissue-organ to which it is exposed, and the period of its arrival at the time of stress. Plants provide stress resistance either with the substances they synthesize or by activating some tolerance mechanisms [5-7].

The salinity is an abiotic stress factor that is common especially in arid-, semi-arid regions and can hinder plant growth. Soluble salts can be easily taken up by plants. Depending on the type and amount of salt compounds entering the plant, it can be harmful to the plant when it exceeds a certain concentration. The soil salinity caused by salts in the soil or formed as a result of irrigation has two effects on plants: First, it is the total salt effect or osmotic effect, which prevents plants from taking water from the soil solution, and the second is the toxic ion effect, which affects some physiological events in plants. Excess amounts of exchangeable sodium in the soil can adversely affect plant growth, as it causes problems such as water permeability and decreased aeration [8, 9].

Salinity can also reduce plant transpiration and respiration, as well as water uptake and root growth. As a result, hormonal balance is destroyed,

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photosynthesis decreases, protein synthesis decreases as a result of decreased nitrate intake, and the plant may be adversely affected. Since this affects the fresh and dry weight of the plant, it reduces the number of flowers and causes a decrease in yield. These negative effects of salinity may vary depending on factors such as the duration of exposure to salinity, the plant variety and salinity tolerance of the plant, the development period of the plant, and the ion concentration in the environment [10, 11]. In this context, the present study aimed to examine the effects of increasing salt concentrations on the *in vitro* growth of the endemic *T. cilicicus* and this primary study is the basis for a transcriptomic and protemic-based study to be carried out in the future.

MATERIALS AND METHODS

Plant Material and Culture İnitiation

The healthy shoots of *T. cilicicus* were collected from the natural habitat located within the Southwest part of Turkey (Muğla-Köyceğiz, C2 square, 36°59'28.63”N, 28°38'49.07”E). The shoots were surface sterilized via the modified methods developed by Ozudogru *et al*. [12] and Kaya *et al*. [13]. The shoots (~1cm in size) were soaked with 70% ethyl alcohol for five minutes, then they were treated with 4% commercial bleach for fifteen minutes and they were rinsed with sterile distilled water. After surface sterilization, the cut shoot tips (~1 mm in size) were transferred to the previously optimized MS nutrient medium [14-16] supplemented with 1 mgL^{-1} kinetin. Samples subcultured at four-week periods were micropropagated under standard culture conditions.

Salt Treatments

In the related study, 4 different parameters were applied as 0 (control), 25 mM, 50 mM and 100 mM for salt applications. For each application, salt was added to the previously optimized nutrient medium at the above-mentioned concentrations. Each parameter was repeated at least three times and 10 shoot tips were used in each replicate.

Data Analysis

The data of the shoot tips transferred to the control and experimental petri dishes were recorded by measuring the regeneration rate, the number of stems formed per shoot tip and stem length values. Shoots with at least two leaves from each shoot tip were considered regenerated. The analysis of the obtained data was carried out using the IBM SPSS statics V18.0 program. A value of $p<0.05$ was considered statistically significant.

RESULTS

In the control group specimens, shoot tip regeneration showed a positive development, while at least two stems were developed per shoot and all stems that developed from the shoot tip had also a very healthy and productive appearance (Figure **1A**-**B**). The measured stem lengths are between 0.2 and 1.8 cm and the calculated average stem length is ~0.31 cm. The shoot-forming capacity (SFC) index calculated based on the percentage of regeneration and the number of stems formed per shoot is 1.6 (Table **1**). In the nutrient medium containing 25 mM NaCl, an average of 2.21 stems were obtained per shoot, while the lengths of these stems were measured between 0.1 and 1.1 cm and the calculated average stem length is ~0.78 cm. The SFC index calculated based on the percentage of regeneration and the number of stems formed per shoot is 2.1 (Table **1**). When compared to the control group, there is no negative situation in the development of plants treated with 25 mM salt (Figure **1C**-**D**). In the shoot tip explants grown in the medium containing 50 mM NaCl, development of up to 4 stems per shoot tip was observed and the measured stem lengths were between 0.2 and 1.1 cm and the calculated average stem length is ~0.62 cm. The SFC index calculated based on the percentage of regeneration and the number of stems formed per shoot is 2 (Table **1**). No improvement was observed in some of the shoot tips exposed to salt application at this concentration. However, curling in the leaves of the shoots, shortening of the stem length and hyperhydricity-like formations were observed in parts (Figure **1E**-**F**). Finally, although up to 3 stems per shoot were obtained in the shoot tips transferred to the nutrient medium containing 100 mM salt, which is the highest concentration in our study, shoot growth was recorded as quite low in some of the experimental results repeated three times. The lengths of the stems formed per shoot in this developing individual were calculated as 0.1 cm and 0.6 cm and the calculated average stem length is ~0.55 cm. The SFC index calculated based on the percentage of regeneration and the number of stems formed per shoot is 1.2 (Table **1**). In this nutrient medium, where the concentration was doubled, growth and development were significantly suppressed, the leaf color turned brown, and some leaves had necrosis (Figure **1G**-**I**).

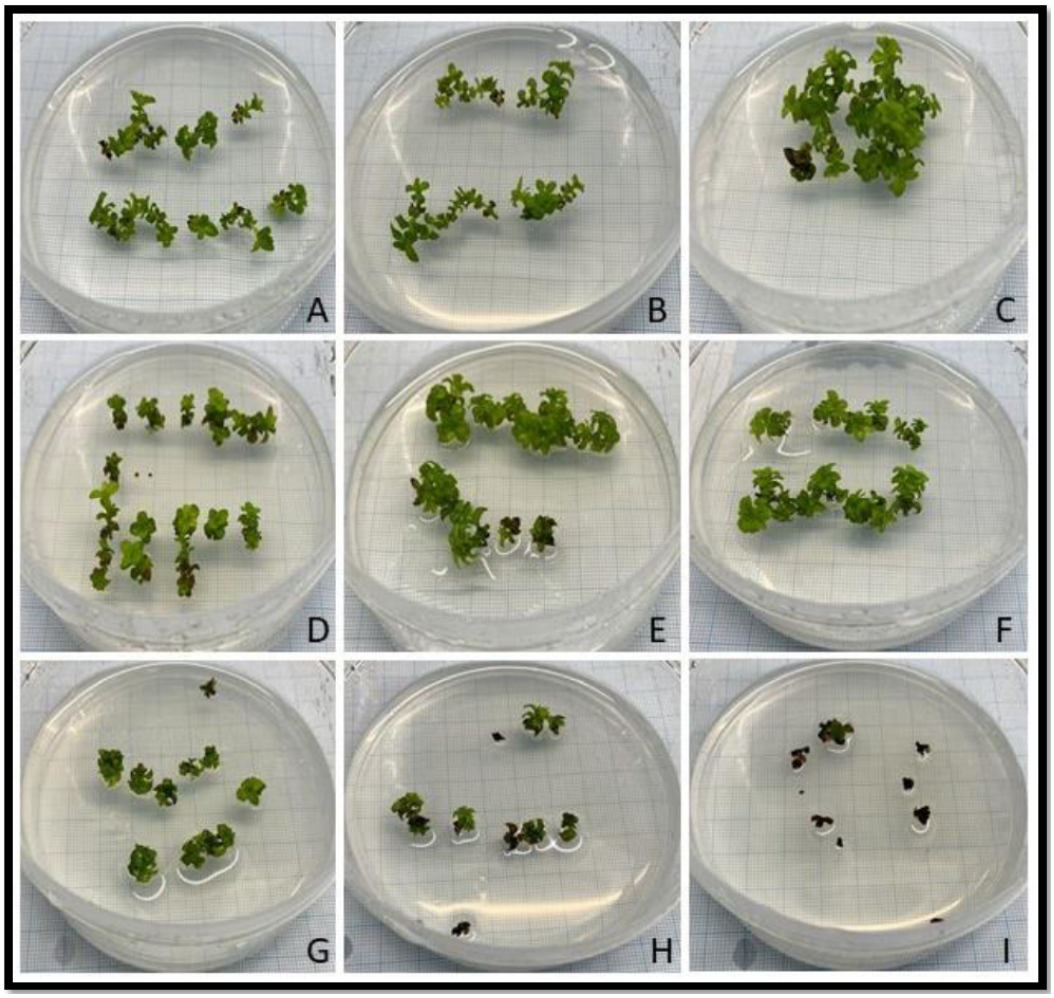

Figure 1: *In vitro* regeneration of *T. cilicicus* shoot-tips on MS nutrient medium containing different salt concentrations. A-B, control groups; C-D, 25 mM NaCl; E-F, 50 mM NaCl; G-I, 100 mM NaCl.

Table 1: ***In vitro* Shoot Development of *T. Cilicicus* of Control Group and After Treatments of Different Salt Concentrations. Each Column was Statistically Evaluated Within Itself**

Treatments	Regeneration Percentage (% ± SE*)	Mean Shoot Number (No ± SE)	Mean Shoot Length (cm ± SE)	Shoot-forming Capacity (SFC) Index***
Control Group	$100 \pm 0.0^{a**}$	1.6 ± 0.63^{B}	0.31 ± 0.19^{c}	1.6
25 mM NaCl	100 ± 0.0^{a}	2.1 ± 1.10^{A}	0.78 ± 0.29^{a}	2.1
50 mM NaCl	96.7 ± 5.8^{ab}	2.07 ± 0.88^{A}	0.62 ± 0.35^{ab}	2.0
100 mM Nacl	80 ± 17.3^{b}	1.63 ± 1.11^{B}	0.55 ± 0.26^{b}	1.3

*Percentage values statistically analyzed by a non-parametric test, the post hoc multiple comparisons test [17].
**Statistical analysis performed by ANOVA, followed by LSD test at $P \leq 0.05$.
***SFC = (average no of shoots per regenerating explant) × (% of regenerating explant) / 100 [18].

DISCUSSION

Decreased growth is a common phenomenon in many plants exposed to salt stress, often associated with a reduction in their photosynthetic capacity. The reduction in photosynthesis under salt stress is mainly associated with partial stomatal closure and/or non-stomatal restriction involving carbon reduction functions of CO_2 assimilation. The proteomic results have significantly increased the understanding of photosynthetic functions underlying salt stress response and salt tolerance. These salt-response proteins involve the regulation of light reaction, CO_2 assimilation, and other photosynthesis-related functions [19, 20].

In this study, physiological effects of increasing salt concentrations such as growth, development and shoot formation of the *in vitro* grown *T. cilicicus* was evaluated by using a nutrient medium containing three different concentrations of salt. In the results obtained, it was seen that the plant was tolerant up to 50 mM and even gained a better development, regeneration and shoot formation potential compared to the control group. There are numerous examples in the literature regarding the growth, development, regeneration and shoot formation capacities of nutrient media components, especially in *in vitro* studies [12, 15, 21-24]. This study is the first study on salt tolerance of thyme plant.

There are studies in the literature examining the effects of *in vitro* salt stress on plant growth. For example, in a study on *Eucalyptus camaldulensis*, they added NaCl and abscisic acid to the growth and development medium and found that the plant adapted to salinity up to 100 mM concentration [25]. In another study, the effects of chitosan on antioxidant activity and essential oil components were evaluated in *Carum copticum* L. plant under salt stress *in vitro*. It has been observed that 20 mgL^{-1} chitosan is effective in increasing the phenolic components of the plant [26]. In our study, the effects of increasing salt concentrations on the development, shoot formation and shoot length of *in vitro* growing *T. cilicicus* plant were evaluated.

CONCLUSIONS

In the current study, when compared to the control group, the best regeneration of *in vitro* grown *T. cilicicus* plant at 25 mM concentration, the number of stems per shoot and the SFC indices calculated based on these values were obtained. However, it was observed that the plant gave close results at salinity up to 50 mM and was not intolerant to increased salt concentrations afterwards. This study is a preliminary study of a prospective molecular-based study, and the data obtained will be used for a study to be conducted in the near future.

CONFLICT OF INTEREST

The authors declare no potential conflict of interest.

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