IJNLS
INTERNATIONAL JOURNAL OF NATURE AND LIFE SCIENCES
Research article

International Journal of Nature and Life Sciences (IJNLS)
https://www.dergipark.org.tr/ijnls
e-ISSN: 2602-2397
Vol. 3(1), June 2019, pp. 20-31

Effects of Salt Stress on Morpho-Physiological and Biochemical Characters of Lentisk (*P. lentiscus* L.)

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***Received:** 27 February 2019, **Revised:** 30 April 2019, **Published Online:** 01 June 2019*

Abstract

Among abiotic stresses salinity is the most detrimental factor in limiting crop productivity. In this study, the effect of different sodium chloride (NaCl) concentrations (0, 50, 100, 150, 200, 250 mM) on growth and physiological parameters of *Pistacia lentiscus* L. seedlings raised in in vitro condition for 4 weeks was investigated. For this purpose the seeds of Lentisk were germinated in Murashige and Skoog, (1962) basal mediums containing different NaCl concentrations. The morphological, physiological and biochemical changes that occurred in the seedlings were measured and recorded after exposure to salt stress. These results show that visible leaf damage of Lentisk seedlings are affected by high salt concentrations. High salinity concentrations significantly reduce root and stem lengths, relative water content (RWC), total chlorophyll, Cl-a, Cl-b and carotenoid values after the culture periods. At 250 mM salt concentration, root and stem growth were found to be completely stopped. The parameters over the 50 mM salt concentrations caused in decrease in the activity of the antioxidant enzyme peroxidase (POD).

Key words: *P. lentiscus* L., *in vitro*, salt stress, morphology, physiology

1. Introduction

The mastic tree (*Pistacia lentiscus* L.) belongs to the family of Anacardiaceae (cashew), represented by over 600 species. This species have a strong acrid resinous smell and they can grow naturally under severe conditions in arid and semi-arid regions between latitudes of 30-45˚. It found abundantly in lowland pine forests and in sand dunes, on dry rocky slopes and on hillsides from sea-level to approximately 600 meters throughout the Mediterranean region (Di Paolo, 2015). *Pistacia*

lentiscus L., is a shrub or small tree especially in Spain, Portugal, Morocco, Italy, Greece, Turkey and including the South of France, specific to the Mediterranean region (Rhodes and Maxted, 2016).

Lentisk pistachio, like other species belonging to pistacia genus, has economic, ecological and medicinal significance. As it possesses many pharmacological properties, it has been used in traditional medicine for a variety of purposes. Various types of phytochemicals, including phenolic compounds, fatty acids, terpenoids and sterols have been isolated and identified from different components of this species (Bozorgi et al., 2013).

Biotic and abiotic stress factors constitute the majority of life conditions of *Pistacia* species growing in arid and semi-arid regions. Salinity in soil or water is one of the most damaging abiotic stress factors limiting crop (Abbaspour et al., 2012; Musyimi, 2015). Almost all physiological events and biochemical process in plants are influenced by salt stress. Physiological disorders, such as an increase in the respiration rate, ion toxicity and a decrease in photosynthesis activity, changes in plant growth, disruption in mineral transport and membrane non-stability due to the replacement of sodium ions with calcium ions develop as a result of salt stress (Yıldız et al., 2010).

Determining the tolerance of plants to salt stress under in vivo conditions can be challenging due to environmental factors such as climate, geographical difficulties, and seasonal constraints. However, the investigation of different abiotic stress factors (such as drought, salinity, low and high temperature and heavy metal stress) under in vitro conditions provides an advantage in terms of labor and cost. In this context, in vitro culture techniques can be used in woody species for their ability to withstand salinity stress in relatively short periods under controlled conditions (Shivanna et al., 2013). In recent years several studies have been reported using in vitro techniques to study salt stress on different plants and other *Pistacia* species (Chelli-Chaabouni et al., 2010; Benmahioul et al., 2009; Miljuš-Djukić et al., 2013). There are reports in the literature about the response of salt stress on the mineral elements of leaves, proline accumulation, amount of soluble sugars, stomatal frequency and morphological changes on different *Pistacia* species (Benhassaini et al., 2012; Kamiab, 2012; Asadollahi, 2013; Ben Hamed and Lefi, 2015; Cristiano, 2016). However, no information is available on morphological and physiological effects on Lentisk pistachio in *in vitro* conditions.

Salinity of soil is a serious environmental problem affecting land the globe. It has been estimated that worldwide 20% of total cultivated and 33% of irrigated agricultural lands are afflicted by high salinity. Furthermore, the salinized areas are increasing at a rate of 10% annually for various reasons, including low precipitation, high surface evaporation, weathering of native rocks, irrigation with saline water, and poor cultural practices. It has been estimated that more than 50% of the arable land would be salinized by the year 2050 (Shrivastava and Kumar, 2015). As a result, this situation emphasizes the importance of investigating the tolerance of plants to salt stress and, more importantly,

the development of salt stress tolerant cultivation.

In this study, it was aimed to determine the morpho-physiological and biochemical responses of Lentisk grown in vitro to salt stress at different concentrations.

2. Material and Methods

2.1. Plant material

The seed of Lentisk were obtained from natural populations in Çiftlikköy-Çeşme province of İzmir. The mature seeds of Lentisk were surface-sterilized by 20% NaOCl for 20 min then were rinsed with sterile distilled water and cultured for 4 weeks in a MS basal medium which containing mgl^{-1} BA and different salt concentrations (0, 50, 100, 150, 200, 250 mM) NaCl. This method was carried out by modifying the method of Kılınç et al. (2015). All cultures were kept at 25 ± 2 °C under 16 h photoperiod (40 µmol m^{-2} s^{-1}). Obtained seedlings were evaluated after 28 days of each salt treatment for morphological, physiological and biochemical analysis.

2.2. Morphological changes

Three seedlings from each treatment of salt stress were selected for the determination of morphological changes in terms of root and stem length parameters. To determine visible leaf damage, color changes were recorded from 5 randomly selected leaves from each salt stress treatment. The morphological data obtained were determined by the modified 1-6 scales of Mohanty and Ong, (2003) to state of visible leaf damage as follows: 1 = 0% no yellow leaves; 2 = 10-30% yellow leaves; 3 = 30-50% yellow leaves; 4 = 50-70% yellow leaves, 5 = most leaves were yellow; and 6 = all leaves were yellow.

2.3. Determination of RWC

Relative water content (RWC) was determined according to the method of Sairam et al., (2002). This experiment was conducted to evaluate the effect of different salt concentrations on the water content of plants. 0.5 g of fresh leaves, dipped in distilled water for 4 hour for measuring their turgid weight. Then, the samples were dried in oven for 48 h at 65 °C to measure their dry weight. The obtained data were calculated by using the following formulas.

RWC = [(fresh weight – dry weight)/ (turgid weight - dry weight)] × 100.

2.4. Determination of chlorophyll and carotenoid content

Pigment content of randomly selected leaves from each salt concentration was calculated based on the study of Arnon, (1949). For this purpose, 100 mg of fresh leaf was extracted by grinding in a mortar using 15 ml of 80 % acetone. The extract was filtered through white stripe filter paper; the

absorbance values of the resulting extraction were measured against the distilled water for total chlorophyll at 652 nm, for chlorophyll a at 663 nm, for chlorophyll b at 646 nm and for carotenoid at 470 nm. Each application repeated 3 times and the amount of pigment present in each sample was calculated according to the following equations (Lichtenthaler and Wellburn, 1983) (A: Absorbance value reading)

Total chlorophyll = (A652x27.8) /mg specimen weight

Chlorophyll a (µg/ml) = (12.21 xA663 - 2.81 xA646) /mg specimen weight

Chlorophyll b (µg/ml) = (20.13 xA646 - 5.03 xA663) /mg specimen weight

Carotenoid (µg/ml) = (1000xA470 - 3.27[Cl- a] - 104[Cl- b]/227) /mg specimen weight.

2.5. Enzyme extraction and assay

To measure an antioxidant enzyme (Peroxidase) activity, the leaves stored in the freezer was homogenized using a mortar with 0.05 M sodium phosphate buffer (pH 6.8) containing 1 mM EDTA and 2 % (w/v) PVPP. All processes were performed at 4 ºC. The enzyme extract for total protein content was determined using the method of standard bovine serum albumin (BSA) as suggested by Lowry et al. (1951). The determination of the peroxidase activity was made according to Kumar and Khan (1982). For analysis 0.5 ml enzyme extract was added to the reaction mixture containing 2 ml of 0.1 M phosphate buffer (pH 6.8), 1 ml of 0.01 M pyrogallol ($C_6H_6O_3$) and 1 ml of 0.005 M H_2O_2. The solution obtained was incubated for 5 min at 25 ºC with the addition of 2.5 N of 1 ml H_2SO_4. The purpurogall quantity was determined by measuring at 420 nm against the blank preparation. One Unit (U) refers to the change in the absorbance of 0.1-1 mg^{-1} of protein.

2.6. Statistical analysis

Measurements were recorded on the 28th day and an average of 3-5 examples were used for per experiment. The significance was determined by the analysis of variance (ANOVA) and Duncan's new multiple range tests were used to show the differences among the parameters means. Data presented in percentages were subjected to chi-square ($\chi 2$) analysis.

3. Results

3.1. Morphological changes

The morphological changes (root and stem length and visible leaf damage) are given in Table 1. As seen in Figure 1 and Table 1, the growth of the seedlings is limited by increasing the concentration of NaCl. The growth profiles of Lentisk genotypes were evaluated after a culture period (28 days) of the NaCl treatment.

Figure 1. The effects of increased NaCl concentrations on the general appearance of *P. lentiscus* L. seedlings (from left to right at 0, 50, 100, 150, 200 and 250 mM NaCl concentrations, respectively).

Table 1. The effect of increased NaCl concentrations on morphologic appearence of the *P. lentiscus* L seedlings.

Salt con. (mM)	Root lengths (cm)	Stem lengths (cm)	Visible leaf damage
0	4.42±0.07a	3.08±0.08a	1.20±0.20d
50	3.00±0.04b	2.96±0.03b	1.40±0.16d
100	1.38±0.02c	1.06±0.04c	2.60±0.13c
150	1.20±0.02d	1.00±0.03c	3.00±0.14b
200	0.64±0.02e	0.40±0.01d	4.40±0.10a
250	0.22±0.02f	0.00±0.00d	0.00±0.00e

*Data represents an average of 3 replicates per treatment after 28 days of culture. Means in a row followed by the same lowercase letters are not significantly different at $P \leq 0.05$ levels of significance according to Duncan's multiple range test.

The morphological appearance of the leaves in the control group is thick, large-sized leaves of dark green colour; however the profiles of the leaves evolve from dark green to lighter tones. In addition, most leaves yellow and leaf sizes get smaller at the 200-250 mM NaCl concentrations. Chlorosis is one of the most common symptoms of stress due to the decreasing amount of chloroplastic pigments. Chlorosis appears on both species at 100 mM and the higher NaCl concentrations; chlorosis reaches higher levels with increased NaCl concentrations. The leaf formation is strongly inhibited on the *P. lentiscus* at the highest salinity rate (250 mM) in a 28-day-culture period. Salt-stress applied seedlings

have significantly lower root, stem and leaf lengths and relative water content values when compared to the control groups. On the other hand, visible leaf damage increases depending to salt-stress applied levels for both species.

3.2. Physiological observations

The physiological changes in terms of the RWC values and the pigment contents are given in Table 2. Salinity stress has a negative effect on most of the physiological characteristics in this study, with the increase in salinity from 0 to 250 mM (Table 2). Pigment content is one of the stress symptoms in salt treated tissues. Compared with the control, a maximum inhibiting effect is recorded at high salt stress (100-250mM) for a culture period (28 days). The increase of NaCl in a medium was accompanied by a gradual decrease in total chlorophyll content for *P. lentiscus (*Table 2).

Table 2. The effect of increased NaCl concentrations on the physiological characters of the *P. lentiscus* L.

Salt con. (mM)	RWC	Total Cl	Cl-a	Cl-b	Carotenoid
0	50.44±1.46a	3.00±0.02a	2.17±0.01a	0.83±0.01a	1.02±0.04a
50	46.65±0.54b	2.77±0.01b	2.12±0.01a	0.64±0.01b	0.90±0.07b
100	44.92±0.39c	2.11±0.01c	1.26±0.02b	0.84±0.02a	0.84±0.07b
150	41.58±0.333d	1.72±0.02d	1.19±0.01c	0.53±0.02c	0.67±0.01c
200	37.35±0.33e	1.16±0.02e	0.79±0.02d	0.36±0.01d	0.50±0.01d
250	0.00±0.00f	0.00±0.00e	0.00±0.00e	0.00±0.00e	0.00±0.00e

The relevant total chlorophyll, the Cl-a, the Cl-b and the carotenoid values of the *P. lentiscus* shoots grown in in vitro conditions under NaCl stress are presented in Table 2. The control group has the highest amounts of total chlorophyll (3.007), chlorophyll-a (2.17), chlorophyll-b (0.83) and carotenoid (1.02) among all groups. The lowest chlorophyll content has measured in the 250 mM group. Salt stress increases the level of POD activity in salt treated *Pistacia lentiscus* genotype (Figure 3). The specific enzyme POD activity has increased at the maximum level in 50 mM NaCl concentration. After that it is followed by a decline in specific enzyme POD activity. POD activity is measured as 0,260 U/mg for the leaf sample taken from a stress-free condition; whereas 0,447 U/mg is measure for the 50 mM salt-applied leaf sample.

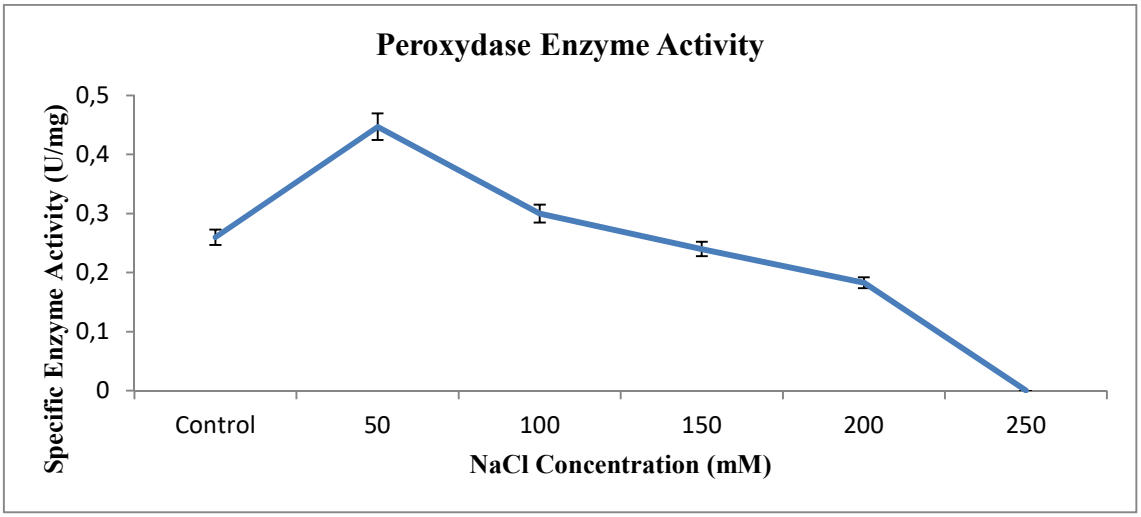

Figure 3. The effect of increased on the NaCl concentrations on the biochemical characters (POD activity) of the *P. lentiscus* L.

4. Discussion

Salinity stress experiments were performed under in vitro conditions to observe the effects of salinity on shoot growth and proliferation for *Pistacia lentiscus*. Observations were taken from this genotype exposed to different salt concentrations in in vitro conditions at the end of a culture period and, consequently, it has been determined that *P. lentiscus* genotype shows different tolerances at the morphological and the physiological levels when salt stress is induced by the application of high NaCl concentrations. The morphological results obtained from this current study are similar to findings in the literature reported on other *Pistacia* species such as: shortened shoot length, reduced leaf area and decreased leaf numbers, lightened leaf colors, yellowing and necrosis in leaves, and drought and termination of plants (Rezaeyan et al., 2009; Benmahioul et al., 2009; Chelli Chaabouni, 2010; Benhassaini et al., 2012). There has not been any report concerning morphological change especially relating to the VLD (Visible leaf damage) of the plant leaves upon the increasing salinity for the *P. lentiscus* L. seedlings.

The early stage of development usually represents the most salt sensitive phase in woody plants. For this reason young seedlings represent a valuable material to detect early salt responses (Ben Hassaini et al., 2012). Leaf growth inhibition can appear to also be a result of the inhibition of the symplastic xylem loading of calcium in the root by salt (Batool et al., 2014). Alterations in visible leaf damage are an important indicator for screening salt tolerance for both genotypes. Several researchers have shown that generally the RWC, the total chlorophyll and the carotenoid contents of leaves decrease during salt stress. Among these, Ben Hamed and Lefi, (2015) observed in their study the NaCl decreases the total chlorophyll content in control seedlings for both of the *P. vera* L. and the *P. atlantica*

Defs. In another case study concerning the *P. atlantica* Desf. subsp. *atlantica*, it has been reported that increasing salt applied concentrations (100-400 mM NaCl and $CaCl_2$), shows an increased effect on the relative water content for the first three days of the culture, but then gradually this decreases by the end of the tenth day. Increased salt concentrations also cause stress on the plantlets by reducing the growth of roots and shoots (Ben Hassaini et al., 2012). In addition, in another study that investigated the impact of saline on the growth and the development of the *P. vera* L. seedling, the total biomass is reduced in the shoots that emerge on the 30th and 60th days, while no differences for relative leaf water content is reported (Hokmabadi et al., 2005). Total chlorophyll amount is reduced due to ion accumulation and disruptions in stomata functionalities in mediums that include higher salt concentrations. Consequently, decreasing photosynthesis activity results in difficulties in plant development. A decrease in chlorophyll amounts has been determined to be due to the disruption in the metabolic process under salt stress (Topaloğlu, 2010). Whereas Ferguson et al. (2002), investigates the in vivo impacts of SO_4^{-2}, Cl^-, and Boron in pistachio rootstocks; Ranjbar et al. (2002) investigates NaCl and $CaCl_2$ salt combinations in the *P. khinjuk* and the *P. mutica* plants. These studies both report a decrease in the chlorophyll content of the leaves. In the study by Adish et al. (2010) regarding the Badami- Zarand rootstock of the *P. vera* L, it has been reported that there are reductions, such as the root-shoot length, the number of leaves, the fresh/dry weights of the shoots, and the rate of photosynthesis in the plant under salt stress. The reduction in the amount of chlorophyll is a result of the adverse effects of salt on membrane stability (Ashraf & Bhatti, 2000). In this regard, our findings show that the negative effects of NaCl application on photosynthesis are comparable with the results of other researchers. Several studies have been reported in the literature concerning the effects of salinity on the antioxidant systems in various plants. The majority of these studies reveal that NaCl saline increases peroxidase activities (Diego et al., 2003; Swapna, 2003; M'barek et al., 2007; Kumar et al., 2009; Kahrizi et al., 2012; Çelik and Atak, 2012; Weisany et al., 2012). However, there have been only a limited number of studies on the *Pistacia* genus. Tavallali et al. (2008) investigates the antioxidant enzyme activities of the *P. vera* L. "Badami" seedlings which were induced by NaCl stress using zinc. The researchers also determined a significant increase in the levels of superoxide dismutase, catalase and ascorbate peroxidase. Feng, (2011) compares one-year-old *P. chinensis* and *Hovenia dulcis* seedlings induced by saline stress. He identifies an increase in the POD activities of these plants in the beginning, but then, there is a reduction later. The present study investigates responses of peroxidase, one of the antioxidant enzymes, toward various NaCl levels, and it has been determined that this represents parallel behavior to occurring NaCl concentrations. In this regard, our findings comply with the results of experiments performed by Tavallali et al. (2008) and Feng, (2011).

There has been only a single study investigating the physiological and anatomical responses of

the pistachio against salt stress in vivo. In their study investigating leaf, stem and fruit anatomies of two cultivars (Ohadi, KaleGochi) of the *P. vera* L. Zarinkamar and Farjady, (2011) report that whereas salt stress decreases the concentration of trichomes, it increases the stoma concentration. Moreover, they have reported that salt stress increased the leaf cuticle thickness, the density of palisade cells and leaf width. Our study which has been conducted to investigate the effects of NaCl stress on *P. lentiscus* L, has concluded that as saline concentration increases, it causes morphological and physiological harm to plants, adversely affects the growth and the development of plants, and develops a cellular defense mechanism.

In brief, the present study will be useful in elucidating the tolerance mechanisms of the *Pistacia* genus against saline stress, which can aid survival in arid and in semi-arid climate conditions. This study will also be helpful for researchers in providing guidelines for carrying out a detailed study on other *Pistacia* species.

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