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Determination of the Effect of Harpin Protein on NaCl Salt Stress in Pistachio (*Pistacia vera* L.) Seeds

Selçuk BİNİCİ[1]*, Civan ÇELİK[2], Fatma YILDIRIM[1], Adnan N. YILDIRIM[1]

[1]Isparta Uygulamalı Bilimler Üniversitesi, Ziraat Fakültesi, Bahçe Bitkileri Bölümü, Isparta
[2]Isparta Uygulamalı Bilimler Üniversitesi, Ziraat Fakültesi, Bahçe Bitkileri Bölümü, Isparta
Selçuk BİNİCİ: ORCID NO: 0000-0002-2373-3990
Civan ÇELİK: ORCID NO: 0000-0002-1696-5902
Fatma YILDIRIM: ORCID NO: 0000-0001-7304-9647
Adnan Nurhan YILDIRIM: ORCID NO: 0000-0003-2526-040X

**Corresponding author: d.selcukbinici@gmail.com*

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Pistacia vera, Seedlings, Harpin protein, Salt stress, Antioxidant enzyme activity, Morphological development

Abstract: Drought and salinity are among the most important abiotic stress factors. Although there is a growing interest to the sustainability of fruit growing in arid and salty areas, there are not enough studies. Therefore, this study was carried out for the determination of the effects of harpin protein at different salt concentrations in Pistachio (*Pistacia vera* L.) seedlings. At the end of the treatment, there was a 10% decrease in plant height, 43% in root length. Superoxide dismutase, catalase and ascorbate peroxidase antioxidant enzyme activities increased by 56.65%, 410.36% and 343.48%, respectively. In addition, the amount of chlorophyll a, chlorophyll b, and chlorophyll a+b (respectively, 39.31%, 34.16%, and 36.12%) was decrease. In addition, it was determined that harpin applications protected the chlorophyll content related to photosynthesis, increased the diameter of the stem and root and decreased the stress enzyme activities. Thus, applications of harpin protein may be beneficial to increase tolerance to drought/salinity stresses in pistachio plants, especially in the early stages of seedling development. These findings may pave the way for future research on stress management in sustainable fruit growing in arid and semi-arid areas.

Antepfıstığı (*Pistacia vera* L.) Çöğürlerinde Harpin Proteininin NaCl Tuz Stresi Üzerine Etkisinin Belirlenmesi

Anahtar Kelimeler
Pistacia vera, Çöğür, Harpin proteini, Tuz stresi, Antioksidan enzim aktivitesi, Morfolojik gelişme

Öz: Tarımsal üretimi ve verimliliği sınırlandıran en önemli abiyotik stres faktörlerin başında kuraklık ve tuzluluk gelmektedir. Kurak ve tuzlu alanlarda meyve yetiştiriciliğinin sürdürülebilirliği konusuna ilgi bulunmakla birlikte yeterli çalışma bulunmamaktadır. Bu nedenle bu çalışma, Antepfıstığı (*Pistacia vera* L.) çöğürlerinde harpin proteinin farklı tuz konsantrasyonlarındaki etkilerinin belirlenmesi amacıyla yürütülmüştür. Uygulama sonunda tuz konsantrasyonun artmasıyla birlikte bitki boyunda %10, kök uzunluğunda %43, yaş bitki ağırlığında yaklaşık olarak %40 oranında azalma meydana gelmiştir. Süperoksit dismutaz, katalaz ve askorbat peroksidaz antioksidan enzim aktiviteleri ise sırasıyla, %60, %410 ve %345 oranında artmıştır. Ayrıca klorofil a miktarı %39, klorofil b miktarı %34 ve klorofil a+b miktarı %36 oranında azalmıştır. Bununla birlikte harpin uygulamalarının fotosentezle ilişkili olan klorofil içeriğini koruduğu, gövde ve kök çapını artırdığı ve stres enzim aktivitelerini düşürdüğü saptanmıştır. Sonuçlar Antepfıstığı bitkilerinde özellikle çöğür gelişiminin hassas olduğu ilk zamanlarında kuraklık/tuzluluk streslerine toleransı arttırmak için harpin proteini uygulamalarının yararlı olabileceğini göstermiştir. Bu bulgular, kurak ve yarı kurak alanlarda sürdürülebilir meyve yetiştiriciliğinde stres yönetimi konusunda gelecekteki araştırmaların önünü açacaktır.

1. INTRODUCTION

Salinity is the second most important abiotic stress factors limiting agricultural production and productivity after drought in the world. About 20% of the cultivated lands in the world and about half of the irrigated lands are affected by salinity [1]. Salinization occurs in 1-1.5 million hectares of land in the world every year and causes about 12 billion dollars of income loss [2]. Salinity is generally defined by the concentration of soluble salts in the soil. Accordingly, 100-150 mg (0.10-0.15%) water-soluble salt per 100 g of soil is acceptable limit values, and when it exceeds 150 mg, the growth and development of many plant species is prevented. When the salt percentage rises to 0.65%, almost all cultivated plants cannot survive in these soils [3]. Salinization is an important issue particularly in arid and semi-arid climates due to insufficient precipitation as well as high evaporation. In addition, unconscious agricultural irrigation, insufficient drainage and high ground water play an important role in soil salinization [4]. In Turkey, which has arid and semi-arid climate, salinity and alkalinity problems are experienced in approximately 1.5 million hectares of irrigable agricultural lands (comprising 32.5% of irrigated lands). Especially in the Southeastern Anatolia Region, which has arid and semi-arid climate, irrigated areas have increased with the activation of the GAP project, but salinization has occurred in many areas as a result of unconscious irrigation. For example, as a result of wrong irrigation in the last 25-30 years in Şanlıurfa Harran Plain, saline areas have increased three times [2]. Again, the ground water, which is also a cause of salinization in the south of Şanlıurfa, rose up to 0.5 m, and therefore drying up in the orchards began [5].

Pistachio, namely *Pistacia vera* L., belongs to the genus Pistacia of the Anacardiaceae family of the Terebinthales order. The seeds of pistachio, which is one of the hard-shelled fruit types, are very rich in terms of unsaturated fatty acids, antioxidants, phenolic substances, vitamins and minerals [6]. Since its fruits can be stored for a long time, it has a strategic and economic value in the nutrition of human beings since ancient times. At the same time, it has ecological importance as it can be easily grown in barren and poor soils. It is grown in arid and semi-arid regions around the world [1]. *P. vera* can survive in areas with annual precipitation below 200 mm and withstand drought better than olive, almond, and fig species, which are considered typical xerophyte plants. It also tolerates salinity [1-7].

Turkey is one of the gene centers of pistachio, has suitable ecological conditions and is one of the first three countries (Iran, Turkey and USA) producing pistachios in the world. As a matter of fact, 474.004 tons of pistachios are produced in the world and Turkey ranks second with a production amount of 296.376 tons [8]. Quality and efficient pistachio production in our country is carried out in the Southeastern Anatolia Region, with the highest amount in the provinces of Sanliurfa and Gaziantep. Pistachio is propagated by grafting. The most widely used rootstock is *P. vera* seedlings [9].

It is known that high salt in the soil decreases production and causes some physiological issues in plants. It has been reported by different researchers that, high salt concentrations cause disruption of ion balance in cells, ion toxicity and osmotic stress, and produce reactive oxygen species (ROS) that damage DNA, lipids and proteins [10-11-12-13-14-15-16]. Therefore, molecular and enzymatic reactions developed by plants against biotic and abiotic stress conditions have recently been the focus of attention of researchers [11-13-17-18-19-20-21]. Plants perform some enzymatic and non-enzymatic mechanisms to avoid the harmful effects of ROS during biotic/abiotic stress [11]. One of these mechanisms is antioxidant enzyme activities. Antioxidant enzymes (superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX) etc.) abolish the harmful effects of H_2O_2 accumulated in the cell during stress by catalyzing H_2O and O_2 [20]. As a result, several biochemical reactions take place in the cell in terms of tolerance levels of plants under oxidative stress. Much research is needed to fully understand the mechanism of these reactions.

In recent years, researchers have focused on different amino acid and hormone applications in order to reduce the effect of stress in plants [22-23-24-25-26-27-28-29-30-31]. One of these amino acids is the harpin protein. Harpin is known as an acidic, glycine-rich, protease sensitive and heat resistant protein that is encoded by the hrpN gene of *Erwinia amylovora bacterium*, and also increases disease resistance in plants [32]. In addition, the harpin protein evokes a natural defense mechanism in plants [33]. Harpins activate the defense mechanism in plants by stimulating the expression of defense-related genes in the plant cell [34-35-36]. Indeed, Dong et al. [37] and Zhang et al. [38] reported that harpin increased drought tolerance by activating abscisic acid (ABA) signaling in *Arabidopsis*, and hrf1, the gene coding for harpin, provides tolerance against drought stress in rice. Almas [39] reported that antioxidant enzyme activities increase during drought stress in cotton, but plant activators reduce the level of oxidative damage caused by drought stress. Many studies have been conducted to determine the effect of harpin protein on yield and quality [40-41-42]. However, as a result of the literature review, it was seen that there are a limited number of studies examining the effects of harpin protein on plant growth and antioxidant enzyme activities under abiotic stress conditions.

The early stages of seedling development in arid and semi-arid soils is a critical stage when plants are exposed to a series of abiotic stresses. Plants exposed to salt stress are adversely affected by reduced growth and development. In this study, it was aimed to determine the morphological, biochemical and enzymatic activity levels of the plants with the salt stress relieving effect of different levels of salt stress and harpin protein applications in the seedlings obtained from the seeds of the *P. vera* L. species.

2. MATERIAL AND METHOD

This study was carried out in the glass greenhouse of Isparta University of Applied Sciences (ISUBÜ), Faculty of Agriculture, Department of Horticulture, in the vegetation period of 2021.

2.1. Material and Growing Conditions

In order to obtain the seeds, the seeds of the Siirt variety of pistachio were folded in boxes containing perlite, in a cold storage for 120 days at +4 ºC and 90-95% humidity. The seeds removed from the stratification were planted in 32x13 cm plastic tubes containing peat + perlite at a ratio of 1:1 and transferred to the side ventilated glass greenhouse. Maintenance procedures were applied to the emerging seedlings.

2.2. Salt Stress and Harpin Applications

When the seedlings were about two months old (03.08.2021), the applications were started. In order to create different salt stress in the experiment, three different concentrations of NaCl salt (40 mM, 80 mM and 160 mM) were given to the seedlings with irrigation water every five days according to the previously determined potted field capacity. In the experiment, the commercial dose of harpin protein (Messenger TM) was sprayed on the leaves 3 times, 15 days, 30 days and 45 days after salt applications. The experiment was terminated on the 50th day (22.09.2021). The experiment was set up according to the randomized plot design with 3 replications and 4 plants in each replication.

The irrigation water used in the experiment was evaluated as 1st class irrigation water [43]. Temperature and humidity in the greenhouse were measured with the HOBO UX100-003 Recorder. Temperature and air relative humidity values are given in Figure 1 and Figure 2, respectively.

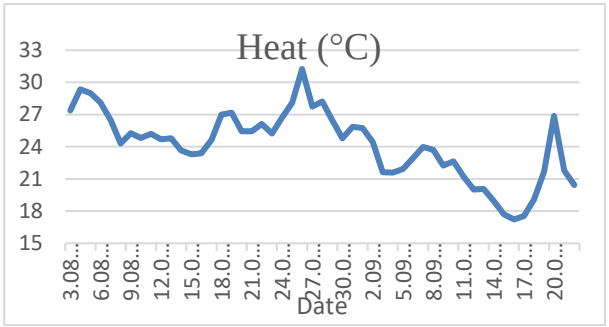

Figure 1. Daily average temperature values measured in the greenhouse of 2021 in August-September

In the study, seedling length (cm) and seedling stem diameter (mm) were measured as morphological growth parameters before and after the application. In addition, at the end of the application, seedlings were removed and plant fresh weight (g), stem fresh weight (g), root length (cm), root fresh weight (g), root dry weight (g), root diameter (cm), shoot number (piece/plant) and leaf area (mm2), leaf circumference (mm), leaf width (mm) and leaf length (mm) were measured.

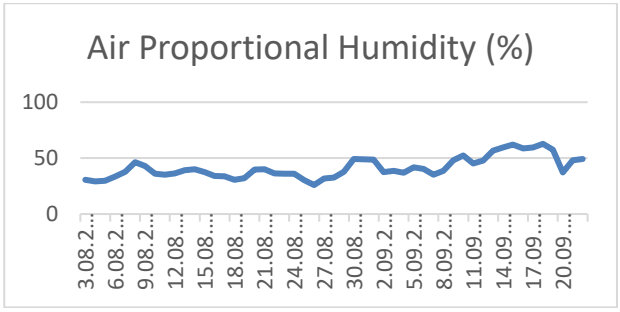

Figure 2. Daily average air relative humidity values measured in the greenhouse of 2021 in August-September

As physiological measurements, leaf stomatal conductivity (mmol/m2/s) and leaf temperature (°C) were measured with a porometer (Delta-T, Porometer-AP4) device before and after the application. As biochemical analyzes, chlorophyll content and APX, CAT and SOD antioxidant enzyme activity analyzes were performed in leaf samples taken before and after the application.

Chlorophyll content was determined according to the method developed by Zhang and Huang [44]. Results are expressed as mg/g.

Ascorbate peroxidase (APX) enzyme activity, APX enzyme activity Nakano et al. [45] according to the method specified. Accordingly, 4 g samples were weighed and analyzes were carried out in line with the researchers' practices. Results are expressed as mol/min/g protein.

Superoxide dismutase (SOD) enzyme activity, SOD enzyme activity Jiang et al. [46] was carried out according to the method specified. Accordingly, 10 g samples were weighed and analyzes were carried out in line with the researchers' practices. Results are expressed as U/mg protein.

Catalase (CAT) enzyme activity, CAT enzyme activity Beers et al. [47] according to the method specified. Accordingly, 10 g samples were weighed and analyzes were carried out in line with the researchers' practices. Results are expressed as U/mg protein.

The obtained data were subjected to one-way variance analysis method in MINTAB 17 statistical program. The resulting differences were determined according to the Tukey multiple comparison test, and the differences between the averages were shown with the help of different letters.

3. RESULT AND DISCUSSION

3.1. Morphological Development Characteristics

At the beginning of the first reaction given to salinity in plants is the slowdown and interruption of growth depending on the severity and duration of stress [48-49-50-51-52-53-54-55]. Harpin protein (Hpa 1) produced by bacterial blight induces a vegetative growth-promoting response by activating the ethylene signaling pathway,

increasing the rate of photosynthesis and increasing EXPANSIN (EXP) gene expression levels [42].

In the study, the mean values of seedling length and seedling stem diameter obtained from pistachio seedlings treated with salt stress and harpin protein, and statistical analysis results are presented in Table 1. There were no statistically significant differences ($p \leq 0.05$) between the treatments in terms of seedling length and seedling stem diameter (Table 1). At the end of the application, the longest plant height was measured in the control application (26.36 cm) and the shortest plant height was measured in the 160 mM NaCl application (23.66 cm). The largest diameter value in terms of seed stem diameter was determined in 40 mM NaCl+Harpin application (3.86 mm), followed by 80 mM NaCl+Harpin (3.57 mm) and 160 mM NaCl+Harpin (3.43 mm) applications., The seedling length and stem diameter change rates increased in all applications compared to the pre-application values. These increase rates in plant height and stem diameter varied according to the applications, and no significant correlation was observed. However, relatively low rates of change (9.28% and 28.73%, respectively) were detected at high salt concentration (160 mM) relative to the control. As a matter of fact, Ashraf et al. [56] stated that salt application reduced the plant height between 13.0% and 36.5%. On the other hand, 80 mM NaCl application in the study, relatively high change rates (28.73% and 52.25%, respectively) were detected in both seedling length and seedling stem diameter compared to the control, and it was observed that the growth continued relatively. These results indicate that pistachio tolerates salinity. This can be explained by the fact that the growth and development of plants are not adversely affected by maintaining the transport of mineral substances with water even under a certain degree of osmotic stress. Similar results were also found in a study on quince and pear rootstocks [55]. In addition, the application of 40 mM NaCl+Harpin had a relatively increasing effect on the stem diameter.

Table 1. The effects of salt stress and harpin protein treatments on seedling length (cm) and seedling stem diameter (cm) in pistachio seedlings

Treatments	Seedling Length (cm)			Seedling Stem Diameter (cm)		
	Before Treatment	After Treatment	Change (%)	Before Treatment	After Treatment	Change (%)
Control	21.53±2.31	26.36±2.81	+22.43	2.27±0.38	3.29±0.23	+44.93
40 mM NaCl	20.61±1.99	25.48±4.36	+23.63	2.39±0.12	3.21±0.14	+34.31
40 mM NaCl+Harpin	24.41±2.68	25.04±1.99	+2.58	2.45±0.35	3.86±0.30	+57.55
80 mM NaCl	19.11±0.96	24.60±1.62	+28.73	2.22±0.17	3.38±0.32	+52.25
80 mM NaCl+Harpin	23.17±3.49	26.16±3.92	+12.90	2.62±0.25	3.57±0.46	+36.26
160 mM NaCl	21.65±3.57	23.66±1.54	+9.28	2.48±0.12	3.17±0.09	+27.82
160 mM NaCl+Harpin	20.69±2.10	24.61±0.91	+18.94	2.37±0.29	3.43±0.10	+44.72

*The difference between the means with different letters is significant at the $p \leq 0.05$ level.

The average values of plant fresh weight, stem fresh weight and shoot number and variance analysis results obtained from pistachio seedlings treated with salt stress and harpin protein are shown in Table 2. In the measurements made at the end of the experiment, there were statistically significant differences ($p \leq 0.05$) between the treatments in terms of plant fresh weight, stem fresh weight and shoot number. It was determined that plant and stem weight decreased with salt stress. The highest plant weight was obtained in the control application and the lowest in the application of 160 mM NaCl (12.51 g and 7.73 g, respectively) and it was determined that this application decreased the fresh plant weight by 38% compared to the control. In stem weight, the highest value was obtained in the application of 160 mM NaCl+Harpin (8.58 g), while the lowest value was determined in the application of 80 mM NaCl and 160 mM NaCl (4.04 g and 4.18 g, respectively). Salt applications reduced the stem weight by approximately 50% compared to the control. In a study conducted on pistachio, it was reported that the dry weight of shoots decreased significantly with the increase in salinity [7]. However, in this study, values close to control were obtained in harpin applications in terms of these properties. This may indicate that plants treated with harpin protein continue to absorb water from the soil and accumulate dry matter (Table 2). As a matter of fact, the stem diameters of the plants to which the harpin was applied were also found to be higher than the control (Table 1). In a study conducted on pepper, while harpin applications did not increase plant height compared to control, it increased the stem diameter relatively [57]. When the number of shoots was examined, there was a significant difference between the control and other treatments and the number of shoots decreased by half (Table 2). Harpin applications did not have an effect on the number of shoots.

Table 2. The effects of salt stress and harpin protein treatments on plant fresh weight (g), stem fresh weight (g) and shoot number (piece/plant) in pistachio seedlings

Treatments	Plant Fresh Weight (g)	Stem Fresh Weight (g)	Shoot Number (piece/plant)
Control	12.51±0.70a	7.55±0.71ab	3.23±0.25a
40 mM NaCl	8.96±1.50ab	4.90±0.88bc	1.50±0.50b
40 mM NaCl+Harpin	11.05±2.20ab	7.37±1.35ab	1.16±0.28b
80 mM NaCl	8.06±0.83ab	4.04±0.92c	1.50±0.50b
80 mM NaCl+Harpin	8.14±2.26ab	5.14±1.43bc	1.33±0.57b
160 mM NaCl	7.73±2.03b	4.18±0.61c	1.16±0.28b
160 mM NaCl+Harpin	11.67±0.53ab	8.58±0.63a	1.41±0.38b

*The difference between the means with different letters is significant at the $p \leq 0.05$ level.

Average values of root fresh weight, root dry weight, root diameter and root length, and variance analysis results obtained from pistachio seedlings treated with salt stress and harpin protein are given in Table 3. Although decreases were detected in the wet and dry root weights with the effect of salt concentration in the study, it was determined that the difference between the applications was not statistically significant ($p \leq 0.05$). It was determined that the applications had significant effects on root diameter and root length, and statistically significant differences emerged ($p \leq 0.05$). The highest root diameter was obtained in 40 mM NaCl+Harpin, control and 80 mM NaCl+Harpin applications (6.19 cm, 6.17 cm and 5.62 cm, respectively). The lowest root diameter was found in 80 mM NaCl and 160 mM NaCl applications (4.38 cm and 4.49 cm, respectively) and these applications reduced the root diameter by approximately 28%. It has also been reported in other studies that salt stress reduces root growth [58-59-60 61-62-63]. However, in the study the root diameter promoting effect of harpin applications under salt stress was determined. In terms of root length, a statistically significant difference was observed only between the control and 160 mM NaCl application. However, relatively lower values were obtained in salt + harpin applications compared to the control application.

Table 3. The effects of salt stress and harpin protein treatments on root fresh weight, root dry weight, root diameter, and root length in pistachio seedlings

Uygulama	Root Fresh Weight (g)	Root Dry Weight (g)	Root Diameter (cm)	Root Length (cm)
Kontrol	4.18±0.57	2.28±0.27	6.17±0.10a	39.86±1.03a
40 mM NaCl	3.77±0.64	1.90±0.35	5.15±0.25bc	37.65±0.81a
40 mM NaCl+Harpin	3.67±0.98	2.09±0.11	6.19±0.49a	33.85±1.23ab
80 mM NaCl	3.63±0.60	1.73±0.23	4.38±0.35c	29.40±1.25ab
80 mM NaCl+Harpin	2.99±0.83	1.46±0.43	5.62±0.41ab	35.48±2.37ab
160 mM NaCl	2.54±0.44	1.76±1.0	4.49±0.27c	22.73±2.13b
160 mM NaCl+Harpin	3.42±0.68	1.56±0.60	5.02±0.04bc	36.38±13.58ab

*The difference between the means with different letters is significant at the $p \leq 0.05$ level.

The average values of leaf area, circumference, length and width obtained from pistachio seedlings that were treated with salt stress and harpin protein, and the variance analysis results are given in Table 4. In the measurements made at the end of the experiment, statistically significant differences ($p \leq 0.05$) were found between the applications in terms of leaf area, circumference, length and width (Table 4). Accordingly, it was determined that as the salt concentration increased, the leaf area decreased up to 51%. The highest leaf area was obtained from the control group (1071.50 mm2), and the lowest leaf area was obtained from 80 mM NaCl, 160 mM NaCl and 160 mM NaCl+Harpin applications (534.72 mm2, 515.90 mm2 and 534.80 mm2, respectively). Again, it was determined that there was a 27%, 23% and 40% reduction in leaf circumference, length and width, respectively, compared to the control. The highest leaf circumference, length and width were measured from the control group (122.80 mm2, 46.91 mm and 32.74mm, respectively). The lowest values were determined in 160 mM NaCl and 160 mM NaCl+Harpin applications. These results were similar to the findings of other studies on pistachio seedlings [7]. Also in the study, harpin applications at 40 mM and 80 mM salt doses had a healing effect on the leaf area. These results show that harpin has the potential to improve plant growth and salt tolerance by activating the plant defense mechanism. Indeed, harpin may exert its effect on plant growth and defense response through activation of transcription factors [64].

Table 4. The effects of salt stress and harpin protein applications on leaf area, girth, length, and width in pistachio seedlings

Treatments	Leaf Area (mm^2)	Leaf Girth (mm)	Leaf Length (mm)	Leaf Width (mm)
Control	1071.50±35.30a	122.80±3.13a	46.91±2.78a	32.74±1.64a
40 mM NaCl	747.08±13.39c	117.17±1.16b	45.62±3.26a	29.52±1.05ab
40 mM NaCl+Harpin	888.00±48.20b	117.62±0.66ab	45.48±3.15a	25.96±1.95bc
80 mM NaCl	534.72±13.29d	92.20±1.86c	43.60±2.30a	22.53±1.03cd
80 mM NaCl+Harpin	870.34±13.66b	117.28±0.46b	45.44±1.40a	29.72±2.04ab
160 mM NaCl	515.90±19.70d	89.86±2.92c	35.94±2.29b	19.83±1.50d
160 mM NaCl+Harpin	534.80±32.40d	89.80±1.20c	35.90±1.20b	22.67±1.89cd

*The difference between the means with different letters is significant at the $p \leq 0.05$ level.

3.2. Physiological Features

Average values and variance analysis results of leaf stomatal conductivity and leaf temperature obtained from pistachio seedlings treated with salt stress and harpin protein are given in Table 5. In the study, the differences between control and other salt and harpin applications at the end of the application in terms of stomatal conductivity were statistically significant ($p \leq 0.05$). The highest stomatal conductivity was measured in the control application (348.7 $mmol/m^2/s$) and the lowest in 160 mM NaCl (67.80 $mmol/m^2/s$) application. At the end of the experiment, only 5.17% decrease was observed in stomatal conductivity in the control group, while a significant decrease occurred in the combinations applied salt and harpin, varying between 40.48% (40 mM NaCl+Harpin) and 80.06% (160 mM NaCl) (Table 5). As the salt concentration increased, the stomatal conductivity gradually decreased. It has also been reported in many studies that salinity reduces stomatal conductivity [55-65-66]. The effect of harpin applications on stomatal conductivity was not significant. Romero-Arondo et al. [67] found that salt stress caused significant decreases in stomatal conductivity. However, they reported that combined applications with plant activators had higher stomatal conductivities. In this

study, higher values were obtained in combinations of 80 mM NaCl and 160 mM NaCl doses with harpin.

There was no statistical difference between the applications in terms of leaf temperature both before and after the application, and values at similar levels were obtained (Table 5). However, the leaf temperature, which was approximately 33.5 ^{0}C before the application, decreased by approximately 14% at the end of the experiment and decreased to 28.5 ^{0}C. As seen in Figure 1, it is thought that there is a decrease in the recorded greenhouse temperature before (29°C) and after the application (20°C), thus affecting the decrease in leaf temperature.

Table 5. The effects of salt stress and harpin protein treatments on leaf stomatal conductivity, and leaf temperature in pistachio seedlings

Treatments	Stomatal Conductivity (mmol/m^2/s)			Leaf Temperature (^{0}C)		
	Before Treatment	After Treatment	Change (%)	Before Treatment	After Treatment	Change (%)
Control	367.7±100.40	348.7±25.3a	-5.17	33.700±0.890	28.54±0.20	-15.31
40 mM NaCl	500.5±53.70	188.7±78.5b	-62.30	33.447±0.731	28.61±1.57	-14.46
40 mM NaCl+Harpin	303.1±168.80	180.4±53.2b	-40.48	33.533±0.635	28.25±0.35	-15.75
80 mM NaCl	454.6±42.70	123.1±18.9bc	-72.92	33.827±0.270	28.92±0.57	-14.51
80 mM NaCl+Harpin	440.2±118.80	140.83±2.8bc	-68.01	33.593±0.277	28.57±0.46	-14.95
160 mM NaCl	340.1±67.20	67.80±12.8c	-80.06	33.393±0.643	28.81±1.44	-13.72
160 mM NaCl+Harpin	390.3±45.70	84.6±32.7bc	-78.32	33.807±0.580	29.14±1.52	-13.80

*The difference between the means with different letters is significant at the p ≤0.05 level.

3.3. Biochemical Properties

Decreases in the amount of chlorophyll, which plays an important role in photosynthesis, affect growth and development of plant negatively by reducing carbohydrate synthesis. One of the most important parameters seen in plants under drought and salt stress is the decrease in total chlorophyll content. Indeed, it has been reported that chlorophyll degradation is an indicator of salt stress [68]. In the study, the mean values and variance analysis results of chlorophyll a, b, and a+b values obtained from pistachio seedlings treated with salt stress and harpin protein are given in Table 6. There were statistically significant differences between the treatments at the end of the experiment in terms of chlorophyll a, b and a+b contents (p≤0.05). Accordingly, while control and salt+harpin treatments were in the same statistical group, chlorophyll contents decreased significantly in only salt applied groups. Again, as the salt concentration increased, the chlorophyll a, b and a+b values gradually decreased. While the lowest chlorophyll

a contents were determined in the application of 160 mM NaCl (11.70 mg/g), the highest was determined in the control application (21.81 mg/g) (Table 6). At the end of the experiment, 160 mM NaCl application decreased chlorophyll a content by 39.31%, chlorophyll b content by 34.16% and chlorophyll a+b ratio by 36.12%. These results were in aggrement to the literature results reporting that salinity reduces chlorophyll content [55-69-70-71-72-73]. However, in this study, it was concluded that the application of harpin under salt stress preserved the chlorophyll contents. As a matter of fact, in a study conducted on pepper, it was reported that B. cinrea disease + harpin applications preserved chlorophyll content compared to only infected plants [74]. These results may show that harpin, an amino acid, plays a role in chlorophyll synthesis and degradation mechanisms. Indeed, in a previous study, it was stated that harpin modulates cell wall modifications, systemic resistance and gene expression related to the photosynthesis system [64].

It has also been reported in many studies that salt stress has negative effects on plants and that enzymatic and non-enzymatic antioxidants take part in the plant defense mechanism [69-70-71-72-73-75]. In the study, the mean values and variance analysis results of the antioxidant enzyme activities (APX, SOD and CAT) obtained from the pistachio seedlings treated with salt stress and harpin protein are given in Table 7. Before the application to the data obtained, variable results were obtained between the applications in the APX and CAT enzyme activities and were found to be statistically different (p≤0.05). However, these differences were not found to be significant. It is thought that this situation is caused by the differences in the current physiological mechanisms of the plants in the applications. However, statistically significant differences were found between the treatments in terms of all enzyme activities (APX, SOD and CAT) at the end of the experiment (p≤0.05) (Table 7). The lowest APX, SOD and CAT activities were in the control group (10.84 mol/min/g protein, 1.00 U/mg protein and 7.89 U/mg protein, respectively), and the highest was 160 mM NaCl application (49.37 mol/min/g protein, 2.71 U/mg protein and 27.28 U/mg protein, respectively). At the end of the experiment, an increase of 41.70% was observed in the APX enzyme activity in the control application, while an increase was determined between 103.77% (40 mM NaCl+Harpin) and 343.48% (80 mM NaCl) in applications. While there was an increase in SOD enzyme activity in 80 mM NaCl (8.97%) and 160 mM NaCl (56.65%) applications, decreases were observed in 40 mM NaCl and other salt + harpin combinations with control application. The highest decrease was found in the control group with 39.76%. The least decrease was 2.96% in 160 mM NaCl+Harpin application. Again, an increase of 4.50% was observed in the CAT enzyme activity in the control application, while an increase was observed between 67.22% (40 mM NaCl) and 410.36% (80 mM NaCl) in applications (Table 7). It has been determined that antioxidant enzyme activities increase with salt stress and the results of previous studies support our findings [69-70-71-72-73-77-78]. In the study, in general, harpin applications decreased antioxidant enzyme activities under salt stress. Especially, this decrease was more

pronounced in APX and SOD activities. As a matter of fact, while the SOD enzyme activity increased in 80 mM NaCl and 160 mM NaCl applications, there was a decrease in the harpin combinations of these applications. Similarly, Zhou et al. [76] reported that tomato seedlings treated with harpin protein under drought stress had lower CAT, SOD and APX antioxidant enzyme activities.

Table 6. The effects of salt stress and harpin protein treatments on chlorophyll contents

Treatments	Chlorophyll a (mg/g)			Chlorophyll b (mg/g)			Chlorophyll a+b (mg/g)		
	Before Treatment	After Treatment	Change (%)	Before Treatment	After Treatment	Change (%)	Before Treatment	After Treatment	Change (%)
Control	16.21±6.32	21.81±1.49a	+34.59	27.46±10.24	35.35±246a	+28.73	43.67±16.56	57.17±3.95a	+30.91
40 mM NaCl	17.72±4.65	17.74±0.95b	+0.11	30.02±6.99	28.56±0.775b	-4.83	47.74±11.65	46.31±1.63b	-3.00
40 mM NaCl+Harpin	18.34±2.14	21.21±0.20a	+15.67	30.59±3.59	34.36±0.383a	+12.33	48.93±5.73	55.58±0.58a	+13.59
80 mM NaCl	19.41±0.92	14.96±0.88c	-22.91	32.702±0.98	25.06±0.246c	-23.34	52.11±1.90	40.03±1.10c	-23.18
80 mM NaCl+Harpin	17.53±2.83	20.96±0.86a	+19.57	29.25±4.91	34.07±1.355a	+16.49	46.78±7.74	55.03±2.22a	+17.64
160 mM NaCl	19.29±3.71	11.70±0.68d	-39.31	31.07±6.02	20.45±0.449d	-34.16	50.36±9.73	32.17±0.99d	-36.12
160 mM NaCl+Harpin	18.55±6.47	21.25±0.52a	+14.57	29.93±10.71	34.30±0.180a	+14.63	48.48±17.18	55.56±0.58a	+14.60

*The difference between the means with different letters is significant at the p ≤0.05 level.

Table 7. The effects of salt stress and harpin protein treatments on APX, SOD and CAT enzyme activity

Treatments	APX (mol/min/g protein)			SOD (U/mg protein)			CAT (U/mg protein)		
	Before Treatment	After Treatment	Change (%)	Before Treatment	After Treatment	Change (%)	Before Treatment	After Treatment	Change (%)
Control	7.65±1.01d	10.84±0.47d	+41.70	1.66±0.06	1.00±0.08d	-39.76	7.55±0.00a	7.89±1.59d	+4.50
40 mM NaCl	15.43±0.37bc	42.71±9.74ab	+176.80	1.62±0.13	1.51±0.07bc	-6.79	7.23±0.55a	12.09±0.60cd	+67.22
40 mM NaCl+Harpin	11.67±1.53cd	23.78±1.92c	+103.77	1.67±0.16	1.41±0.08c	-15.57	4.75±0.99ab	10.05±0.71cd	+111.58
80 mM NaCl	7.29±1.63d	32.33±2.09bc	+343.48	1.56±0.20	1.70±0.05b	+8.97	3.57±1.87b	18.22±2.00b	+410.36
80 mM NaCl+Harpin	19.82±4.44a	25.63±2.02c	+29.32	1.77±0.07	1.51±0.06bc	-14.69	6.94±1.04a	13.16±2.16c	+89.63
160 mM NaCl	17.59±1.47b	49.37±5.98a	+180.67	1.73±0.14	2.71±0.15a	+56.65	6.91±1.10a	27.28±1.46a	+294.79
160 mM NaCl+Harpin	15.88±0.03bc	35.91±1.16bc	+126.13	1.69±0.03	1.64±0.03b	-2.96	6.30±1.08ab	20.19±1.75b	+220.48

*The difference between the means with different letters is significant at the p ≤0.05 level.

4. CONCLUSİON

Salt stress is one of the important abiotic stress factors in the world and in our country. Due to effects such as drought, wrong irrigation and fertilization programs, soluble salts cannot be washed in the soil and excessive salt accumulation occurs. This negatively affects plantyield and quality. Pistachio, constituting the plant material of this study, is the typical fruit type of Southeastern Anatolia Region and it tolerates drought and salinity. However, in addition to the arid and semi-arid climate of the Southeastern Anatolia Region, serious problems arise in the region such as the gradual salinization of many areas as a result of faulty irrigations. With the effect of increasing population and drought, researchers have made various applications in recent years to obtain plants resistant to biotic and abiotic stress conditions or to reduce the harmful effects of salt. This study was carried out in order to find an alternative way to these problems. For this, salt applications were applied to the pistachio seedlings for two months with different intensity and harpin applications were made in order to eliminate the effects of salt. According to the findings of the study, it is possible to say that the harpin protein in pistachio seedlings reduces the effect of salt stress relatively. This can be explained by the Hpa1-mediated regulation of plant growth and related physiological-molecular responses [42]. Indeed, in tomato, harpin has been shown to modulate the defense response and plant growth-related gene expression (via ethylene and ABA) by regulating the activity of the SlERF5 (ethylene-response factor 5) transcription factor [64]. In this study, it was determined that harpin applications protected the chlorophyll content related to photosynthesis, increased the diameter of the stem and root and decreased the stress enzyme activities. In addition, it can be said that the seedling rootstock used in the study can be used in salty soils and can tolerate salt up to a certain severity (40 mM NaCl and 80 mM NaCl). As a result, it has been demonstrated that harpin protein can be used to increase tolerance to drought/salinity stresses, especially in the early stages of seedling development in pistachio plants. These findings may pave the way for future research on stress management in sustainable fruit growing in arid and semi-arid areas.

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