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ARAŞTIRMA MAKALESİ **RESEARCH ARTICLE**

Impact of tryptophan on physiological and histological responses of pepper plants under salt stress

Tuz stresi altındaki biber bitkilerinin fizyolojik ve histolojik yanıtlarına triptofan'ın etkisi

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ABSTRACT

Salt stress is a significant abiotic stressor adversely affecting plant functions. Tryptophan, a precursor to IAA, plays a crucial role in plant growth under such stress. This study investigated the effects of different tryptophan doses (L-tr) on young pepper plants subjected to salt stress. Preceding salinity stress, three L-tr doses were administered to plant roots, excluding control and salt applications. Subsequently, all plants except controls received 150 mM salinity (NaCl) during irrigation. The experiment concluded after 35 days post-salinity treatment. Significant variations were noted in histological (cortex and cell diameters, xylem and midrib diameters, epidermis thickness) and physiological (leaf water content, membrane permeability, chlorophyll and carotenoid concentrations, total phenolic content) parameters among treatments. Evaluation of these parameters revealed L-tr's positive impact on osmotic regulation. L-tr applications increased xylem, midrib, and cortical cell diameter, cortex, and epidermis thickness compared to salt stress, while cortical cell number also decreased. Particularly, NaCl + L-tr 100 µM treatment exhibited a 25% increase in xylem conduit diameter, a 30% increase in midrib diameter, and a significant 15% increase in epidermis thickness compared to controls. Overall, NaCl + L-tr 100 µM treatment displayed values closest to controls across various parameters. This study suggests the potential utility of L-tr in mitigating salinity stress in pepper plants.

ÖZET

Tuz stresi bitki fonksiyonlarını olumsuz yönde etkileyen önemli bir abiyotik stres faktörüdür. İndol-3-asetik asit (IAA) öncüsü olan triptofan, tuz stresi altında bitki büyümesinde önemli bir rol oynamaktadır. Bu çalışma, tuz stresine maruz kalan genç biber bitkilerinde farklı triptofan dozlarının (L-tr) etkilerini ortaya koymayı amaçlamıştır. Tuzluluk stresi öncesinde bitki köklerine kontrol ve tuz uygulamaları hariç olmak üzere 3 triptofan dozu uygulanmıştır. Daha sonra kontrol dışındaki tüm bitkilere sulama sırasında 150 mM tuz stresi (NaCl) uygulanmıştır. Çalışma, tuz stresi uygulaması başladıktan 35 gün sonra sona erdirilmiştir. Yapılan ölçümler arasında histolojik (korteks ve hücre çapları, ksilem ve orta damar çapları, epidermis kalınlığı) ve fizyolojik (yaprak su içeriği, membran geçirgenliği, klorofil ve karotenoid konsantrasyonları, toplam fenolik içerik) parametrelerde önemli farklılıklar kaydedilmiştir. Bu parametrelerin değerlendirilmesi triptofanın (L-tr) ozmotik düzenleme üzerindeki olumlu etkisini ortaya çıkarmıştır. L-tr uygulamaları tuz stresine göre ksilem, midrip, korteks hücre çapları ile epidermis ve korteks kalınlığını artırırken, korteks hücre sayısını azaltmıştır. Özellikle, NaCl + L-tr 100 µM uygulaması, kontrole kıyasla ksilem kanal çapında %25'lik bir artış, midrip çapında %30'luk bir artış ve epidermis kalınlığında %15'lik önemli bir artış göstermiştir. Genel olarak, NaCl + L-tr 100 µM uygulaması, çeşitli parametrelerde kontrollere en yakın değerleri sergilemiştir. Bu çalışma, biber bitkilerinde tuzluluk stresinin azaltılmasında L-tr'nin potansiyel faydasını ortaya koymaktadır.

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INTRODUCTION

Salinity, a significant abiotic stressor, is known to limit both yield and quality in crop production, particularly in arid and semi-arid regions. Approximately 20% of agricultural land and 50% of cultivated land globally are adversely affected by salt stress (Islam et al., 2019). Common practices for sustainable agricultural management, such as removing excess salt from the soil, reducing irrigation water salinity, or developing salt-tolerant plants, are labor-intensive and challenging for each plant species (Cantrell & Linderman, 2001). Therefore, recent studies have explored the external application of certain substances to plants as a means of alleviating salt stress (Hancı & Tuncer, 2020; Aras et al., 2020).

First discovered by the British chemist Frederick Gowland Hopkins in 1901, L-tryptophan (3-indolyl alanine) (L-tr) is an essential amino acid for plants, animals, humans, and some bacteria (Frankenberger & Arshad, 1991). It serves as a biologically active precursor molecule of auxin, and when applied externally, it increases the level of auxin in plant tissues. L-tryptophan can be applied in various ways, including foliar spray, seed priming, and soil application. When applied to the soil, L-tr is either directly absorbed by plants or metabolized into a toxin and then absorbed by plant roots (Mustafa et al., 2018). Numerous researchers have reported positive effects of L-tr application on the germination and growth performance of various plants (Antony et al., 2017; Mustafa et al., 2018). When applied externally, it can enhance a plant's stress tolerance (Hancı & Tuncer, 2020).

Pepper (*Capsicum annuum* L.), a member of the Solanaceae family, is widely used in studies on plant responses to various abiotic stresses, including salinity (Altunlu, 2020), drought (Khazaei & Estaji, 2020), heat (Gisbert-Mullor et al., 2021), and heavy metals (Mokarram et al., 2020). Pepper is moderately salt-sensitive (Ayers & Westcot, 1985), and a significant decrease in yield occurs when the salinity level in the water reaches EC 4.4 dS m-1 (De Pascale et al., 2003). The primary objective in enhancing the development of plants grown in a salty environment is to restore the disturbed osmotic balance (Altunlu, 2020). According to Li et al. (2015), tryptophan, known as a natural, cost-effective, and environmentally safe substance, could be externally applied to plants to increase their tolerance to water deficit conditions. While morphological and physiological studies on the effects of tryptophan in plants under salt stress conditions are available in the literature (Hancı & Tuncer, 2020; Kahveci et al., 2021; Coban, 2023), there is a need for studies to determine its effects on histological features. This study aims to investigate the effects of different concentrations of tryptophan applied to young pepper plants exposed to salt stress on certain physiological and histological properties.

MATERIALS and METHODS

The research was conducted within a controlled greenhouse environment at Yozgat Bozok University Faculty of Agriculture in 2021. In the study, Pepper (*Capsicum annuum* L.) cv., which is moderately resistant to salinity (Emirzeoğlu & Basak, 2020), Çetinel seedlings (3-5 leaves) were used. Seedlings were planted in 2-liter pots filled with peat:perlite mixture (1:1, v:v). The greenhouse maintained a temperature range of approximately 26-30 °C during the day and 18-22 °C at night, with a relative humidity of 50-70%. The experiment employed a randomized plot design with 3 replications and 15 plants per application 150 mM NaCl (Chartzoulakis and Klapaki, 2000; Yılmaz et al., 2004; Siddikee et al., 2011) applications started 6 days after planting the seedlings (5-6 true leaves), salt was applied at each irrigation and the experiment was terminated after 35 days Tryptophan (L-tr) doses of 25, 50, and 100 µM were applied to the plant rhizosphere one day prior to salinity stress, excluding control and salt (NaCl) treatment. Irrigation was done every 2 days with 20% drainage and Hoagland nutrient solution (Hoagland & Arnon, 1950) was applied once a week. The electrical conductivity (EC) values of control and salinity were 0.59 and 11.7 dS m^{-1}, respectively.

Measurements

Upon completion of the experiment, stem diameter was measured in millimeters using a digital caliper, and the length of the plants from the root collar to the growth tip was measured in centimeters using a tape measure. The Leaf Relative Water Content (LRWC) method, developed by Smart and Bingham in 1974, was employed. Individual fresh weights (FW) of matured young leaves were obtained for each repetition. Turgor weights (TW) were measured 6 hours after the leaves were floated in distilled water. Leaf samples were stored at 72 °C for 48 hours, and their dry weights (DW) were recorded. The LRWC was calculated using the formula:

LRWC (%) = [(FW – DW) / (TW – DW)] x 100. **Eq.1**

Membrane permeability (MP) was calculated using an electrical conductivity meter. Leaf samples (1 cm x 1 cm) were placed in 10 mL of distilled water and shaken for 24 hours at room temperature (25 °C). The initial electrical conductivity (EC1) was recorded. Subsequently, the samples were autoclaved for 20 minutes at 120 °C before the second measurement (EC2) was taken. Membrane permeability was calculated as (EC1/EC2 x 100) (Lutts et al., 1996).

To measure carotenoid and chlorophyll (a, b, and a+b) amounts, 0.25 g leaf samples from three plants in each experimental unit were obtained. The samples were extracted with 80% acetone before being filtered. Measurements at 645, 663, and 450 nm were taken to determine chlorophyll and carotenoid contents in the spectrophotometer. Data were computed using formulas described by Güneş et al., (2007).

For the determination of total phenolics, methanol was used for extraction. The total phenolic content was measured at 765 absorbance according to Singleton and Rossi (1965), using the Folin-Ciocalteu reagent.

Histological studies

Leaf samples, preserved in 70% ethanol, underwent histological analysis. Toluidine blue at a 5% concentration was used to stain the cortex, and 1% phloroglucinol in 20% hydrochloric acid was used for xylem observations. Stained samples were visualized using a light microscope (Olympus CX21) with a digital camera (Camera 5) at 4x, 10x, and 40x magnifications. Cortical cell diameter (µm) (10x magnification) and thickness (mm) (4x magnification) of the cortex, epidermis, and xylem parameters were measured (Aras et al., 2021).

The collected data were statistically evaluated using the SPSS 20.0 package program. The Duncan Multiple Range Test determined differences between applications, with a significance level of $p<0.05$. Principal Component Analysis (PCA) categorized all treatments based on the investigated parameters using XLSTAT software.

RESULTS and DISCUSSIONS

In this study, pepper plants were exposed to salt at 150 mM NaCl for 35 days and the effects of exogenous L-tr were evaluated under saline stress. Salinity stress restricted the growth of pepper plants, but the effect of L-tr applications on plant height and stem diameter measurements was statistically significant. (Table 1). Lower plant height and stem diameter values were recorded in all L-tr treatments compared with the control. However, an increase was observed in these parameters compared to the NaCl application. The highest values in plant height and stem diameter measurements were recorded in the control treatment. However, among the applied tryptophan doses, the best positive effect was observed in the NaCl + L-tr 100 µM treatment.

It was observed that the membrane permeability (MP) of the leaves increased in pepper plants under saline conditions. Although the MP was 24.41% in the control group, it increased to 86.28% in the NaCl plants. The membrane permeability, which increased with the effect of salt, decreased with the effect of externally applied L-tr. The most significant reduction, at 67.52%, was recorded with the 100 µM L-tr application (Table 2). As shown in Table 2, the leaf relative water content value (LRWC) decreased with the effect of salt stress. The lowest LRWC was recorded in the NaCl application. In L-tr 100 µM application, 37.1% increase was recorded compared to salt application. The effect of L-tr applications on total phenolic measurements was found to be significant. Total phenolic content was observed to be highest in the NaCl+ L-tr 100 µM application and lowest in the NaCl application (Table 2).

Table 1. The effect of L-tr applications on plant height and stem diameter in pepper

Çizelge 1. Pst Biber bitkilerinde bitki boyu ve gövde çapı ölçümleri üzerinde triptofan uygulamasının etkisi

Treatments	Plant height (cm)	Stem Diameter (mm)
Control	57.33 a	6.85 a
NaCl	32.33 e	3.61 d
NaCl+ L-tr 100 µM	43.33 b	5.49 b
NaCl+ L-tr 50 µM	39.67 c	4.31 c
NaCl+ L-tr 25 µM	37.00 d	4.01 c

Duncan's multiple range test determines the degree of separation within a column. The significance level was 0.05.

Table 2. The effect of L-tr applications on membrane permeability, LRWC and total phenolic measurements in pepper

Çizelge 2. Biber bitkilerinde membran geçirgenliği, LRWC ve toplam fenolik ölçümleri üzerine triptofan uygulamalarının etkileri

Treatments	MP (%)	LRWC (%)	Total Phenolic (µg GAE 100 g $^{-}$1 fw)
Control	24.41 c	84.45 a	3.73 b
NaCl	86.28 a	55.27 d	3.09 d
NaCl+ L-tr 100 µM	67.52 b	75.75 b	4.20 a
NaCl+ L-tr 50 µM	76.72 ab	69.77 bc	3.26 cd
NaCl+ L-tr 25 µM	82.73 a	63.23 c	3.43 c

Duncan's multiple range test determines the degree of separation within a column. The significance level was 0.05.

The effect of tryptophan doses on chlorophyll a content was found to be insignificant. However, significant differences were observed in chlorophyll b content among the application doses, with the highest value recorded in the NaCl + L-tr 100 µM treatment. Salt stress decreased chlorophyll and carotenoid contents in pepper leaves (Table 3). The highest chlorophyll a+b were obtained from the NaCl+ L-tr 100 µM treatment (67.64 µg g–1 fw) while the carotenoid content was increased in L-tr treatment.

Table 3. The effect of L-tr applications on chlorophyll and carotenoid content in pepper under salinity stress

Çizelge 3. Biber bitkilerinde klorofil ve karotenoid içerikleri üzerine triptofan uygulamasının etkisi

Treatments	Chlorophyll a µg g^{-1} fw	Chlorophyll b µg g^{-1} fw	Chlorophyll a+b µg g^{-1} fw	Carotenoid µg g^{-1} fw
Control	35.47 a	31.42 ab	66.89 ab	3.10 a
NaCl	32.34 b	28.72 b	61.06 c	2.53 c
NaCl+ L-tr 100 µM	34.87 a	32.78 a	67.64 a	2.74 b
NaCl+ L-tr 50 µM	34.08 a	29.99 b	66.07 b	2.67 b
NaCl+ L-tr 25 µM	34.19 a	30.44 ab	64.63 ab	2.67 b

Duncan's multiple range test determines the degree of separation within a column. The significance level was 0.05.

Leaf anatomy was investigated at 4x and 10x magnifications and affected L-tr treatment pepper plants." Toluidine Blue O and acid phloroglucinol were used to stain transverse sections of the pepper leaf midrib to define the basic anatomical structure (Figure 1). Salt stress increased the number of cortical cells but decreased their diameter (Table 4). However, L-tr treatment had the opposite effect and increased the cortex cell diameter. Cortex thickness reached the highest value in the control application. Cortex thickness decreased by 25% in salt treatment compared with the control treatment. The values closest to the control were recorded in the NaCl+L-tr 100 µM application.

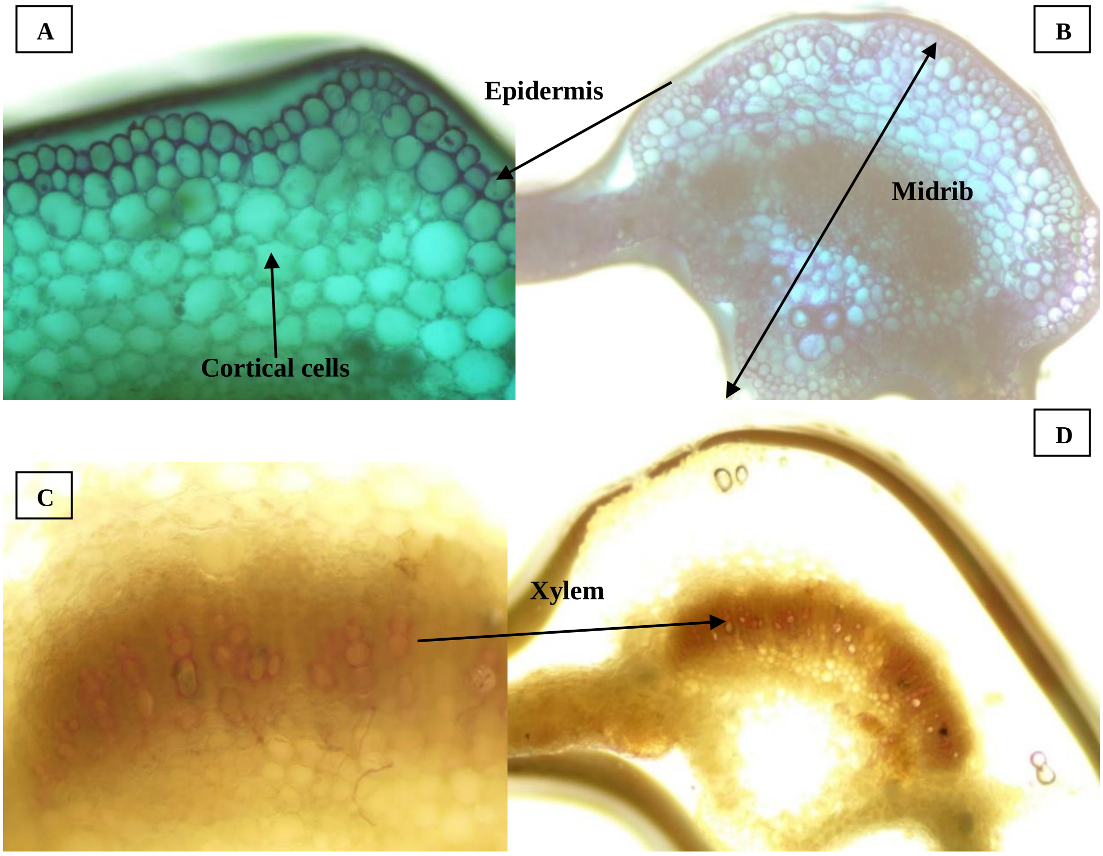

Figure 1. Histological characterization of pepper leaf midrib section images; (A) Toluidine blue dye showing cortical cells and epidermis, (B) Toluidine blue dye showing midrib, (C-D) Phloroglucinol dye showing xylem
Şekil 1. Biber yaprağı orta damar kesiti görüntülerinin histolojik karakterizasyonu; (A) Korteks hücreleri ve epidermisi gösteren Toluidin mavisi boyası, (B) Orta damarı gösteren Toluidin mavisi boyası, (C-D) Ksilemi gösteren Floroglisinol boyası

Table 4. The effect of L-tr applications on cortical cell diameter, cortex thickness and cortical cell number of pepper plant under salinity stress
Çizelge 4. Biber bitkilerinde korteks hücre çapı, korteks kalınlığı ve korteks hücre sayısı ölçümleri üzerine triptofan uygulamasının etkisi

Treatments	**Cortical cell diamater (µm)**	**Cortex thickness (µm)**	**Cortical cell number**
Control	61.89 a	390.50 a	6.31 bc
NaCl	41.79 d	308.25 c	7.38 a
NaCl+ L-tr 100 µM	59.27 a	328.50 b	5.55 d
NaCl+ L-tr 50 µM	53.93 b	319.50 b	5.93 cd
NaCl+ L-tr 25 µM	48.93 c	320.00 b	6.54 b

Duncan's multiple range test determines the degree of separation within a column. The significance level was 0.05.

Table 5 includes midrib, xylem and epidermis measurements. NaCl application decreased the midrib diameter by 21.6% compared to the control application. Moreover, xylem conduits diameter and epidermis thickness values decreased by 25.2% and 28.22%, respectively. The effect of L-tr applications on these parameters was found to be significant.

Table 5. The effect of L-tr applications on midrib diameter, xylem conduits diameter and epidermis thickness of pepper plant

Çizelge 5. *Biber bitkilerinde midrip çapı, ksilem kanal çapı ve epidermis kalınlığı ölçümleri üzerine triptofan uygulamasının etkisi*

Treatments	**Midrib diameter (µm)**	**Xylem conduits diameter (µm)**	**Epidermis thickness (µm)**
Control	1168.00 a	20.98 a	35.12 a
NaCl	916.33 b	15.69 c	25.22 d
NaCl+ L-tr 100 µM	1216.00 a	20.44 a	33.30 ab
NaCl+ L-tr 50 µM	1171.67 a	18.12 b	30.27 bc
NaCl+ L-tr 25 µM	1095.67 a	17.10 bc	27.90 cd

Duncan's multiple range test determines the degree of separation within a column. The significance level was 0.05.

The effect of L-tr on the salinity stress tolerance of pepper plants was evaluated by the Principal Component Analysis (PCA). Fifteen data variables were collected from plants subjected to five treatments (Control, NaCl, NaCl + L-tr 25 µM, NaCl + L-tr 50 µM and NaCl + L-tr 100 µM) were used in PCA analysis. PCA was scattered applications in all four quarters of the biplot. The axes represented 80.25% (F1) and 16.00% (F2), showing 96.25% (F1+F2) of the total variation. Most of the parameters looked at were demonstrated by PC1, which also displayed strong positive relationships. However, there was a negative correlation between MP and cortical cell number parameters with other parameters. Control treatment was generally classified according to plant height, stem diameter, carotenoid, cortex thickness, and epidermis thickness. NaCl + L-tr 100 was generally classified according to total phenolic, chlorophyll a, chlorophyll b, chlorophyll a+b, cortical cell diameter, midrib diameter (Figure 2).

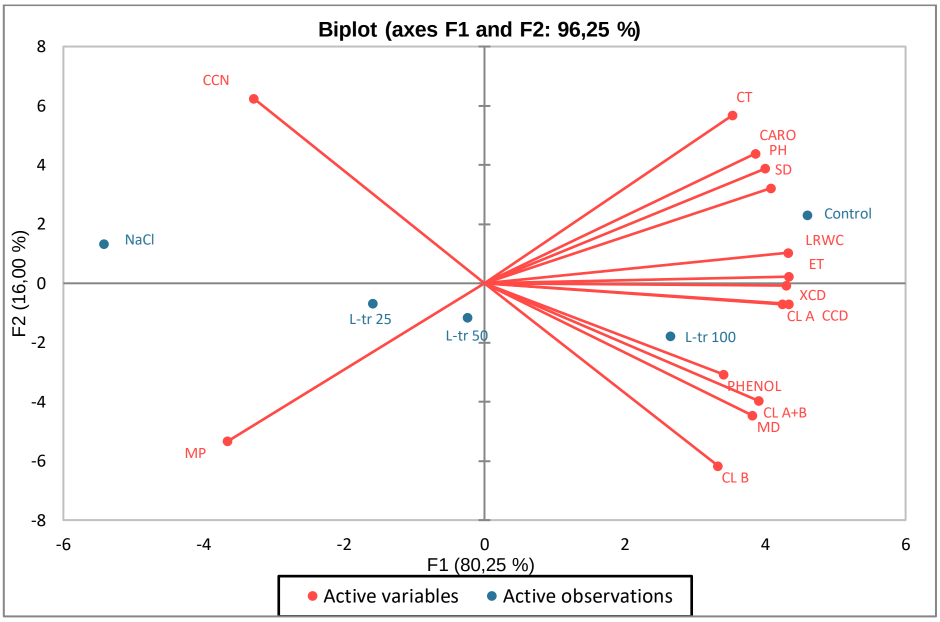

Figure 2. Principle component analysis of physiological and histological traits for different treatments. (PH: Plant height; SD: Stem Diameter; MP: Membrane permeability; LRWC: Leaf Relative Water Content; PHENOL: Total Phenolic; CL A: Chlorophyll a; CL B: Chlorophyll b; CL A+B: Chlorophyll a+b; CARO: Carotenoid; CCD: Cortical cell diameter; CT: Cortex thickness; CCN: Cortical cell number; MD: Midrib diameter; XCD: Xylem conduits diameter; EP: Epidermis thickness)

Şekil 2. Farklı uygulamalar için fizyolojik ve histolojik özelliklerin temel bileşen analizi. (PH: Bitki Boyu; SD: Gövde Çapı; MP: Membran Geçirgenliği; LRWC: Yaprak Oransal Su İçeriği; PHENOL: Toplam Fenolik; CL A: Klorofil a; CL B: Klorofil b; CL A+B: Klorofil a+b; CARO: Karotenoid; CCD: Korteks Hücre Çapı; CT: Korteks Kalınlığı; CCN: Korteksl Hücre Sayısı; MD: Orta Damar Çapı; XCD: Ksilem Kanalları Çapı; EP: Epidermis Kalınlığı)

The salt tolerance can vary among genotypes of the same species. Emirzeoğlu and Basak, (2020) assessed the salinity resistance of different pepper genotypes, noting that the Çetinel variety used in this study demonstrated moderate salinity resistance. Salt stress is known to decrease growth parameters such as plant height (Doğru & Canavar, 2020). In our study, significant decreases in plant height and stem diameter were observed in pepper plants exposed to salt stress, while L-tryptophan (L-tr) applications contributed to the increase in these values. Notably, the NaCl+ L-tr 100 µM application showed a substantial increase of 34% and 52.1% in plant height and stem diameter, respectively, compared to the NaCl application. Similar reductions in plant height and stem diameter under salt stress were reported in the Demre pepper variety by Sönmez and Kaplan, (2004).

The regulation of water content in plant tissues is crucial for salt tolerance, as higher water content indicates increased tolerance to salt stress (Doğru & Canavar, 2020). It has also been reported that exogenous application of Ltryptophan in red pepper plant under Salinity stress led to a significant improvement in LRWC (Jamil et al., 2018). LRWC results in the study are parallel to previous studies.

Membrane permeability (MP) is indicative of ion imbalance resulting from intracellular and extracellular osmotic incompatibility, particularly under salt stress in plants (Ghoulam et al., 2002). It causes soluble substances in the membrane to move out of the tissue and increases tissue electrical conductivity (Altunlu, 2020). In this study, it was determined that the negative effect of salt stress on MP decreased with increasing tryptophan doses.

Changes in phenolic compound levels are observed in pepper plants exposed to salt stress. Phenolic compounds are generally produced through the phenylpropanoid pathway and can be induced by environmental stresses and elicitors (Kusvuran & Dasgan, 2017). These compounds play a crucial role in mitigating oxidative stress caused by high salt concentrations, thus helping the plant cope with adverse conditions (Rice-Evans et al., 1996).

Comparable studies have reported increased phenolic content under moderate salt stress, with a subsequent decrease under severe salt stress (Rezazadeh et al., 2012). The phenolic findings determined in the study support previous studies.

Salt stress typically results in decreased levels of photosynthetic pigments in plants (Doğru & Canavar, 2020). This decrease results in the inhibition of photosynthetic activity (Amuthavalli & Sivasankaramoorthy, 2012). In our study, chlorophyll 'a, b, and a + b' and carotenoid content decreased under salt stress, aligning with general trends reported in the literature. However, L-tr applications effectively increased chlorophyll and carotenoid content compared to the NaCl application. The exogenous application of L-tryptophan significantly improved red pepper's chlorophyll-a, chlorophyll-b, and carotenoid contents (Jamil et al., 2018).

Leaves, being the most crucial organ in a plant's life cycle, are adversely affected by salt stress, impacting cell division and expansion (Taleisnik et al., 2009). Cortical thickness decreased in soybean plants under salt stress (Dolatabadian et al., 2011). The present study's cortical measurements at the end of the investigation suggested that the administration of 100 µM L-tr increased cortical cell diameter while decreasing the number of cortical cells, promoting the development of larger cells. This finding is consistent with Aras et al., (2021) observation that plants with larger cortical cells produced more chlorophyll and chlorophyll precursors in the presence of Ca deficit.

The midrib, a crucial component of leaves, plays a vital role in the movement of water and minerals (Lechthaler et al., 2019; Aras et al., 2021). In our study, salt stress decreased midrib diameter values, while L-tr application increased these values. This increase can be associated with a concurrent increase in the diameter of xylem conduits. Xylem, a vital vascular bundle in higher plants, transports water and minerals. Salt stress can induce changes in the lignification of xylem structures (Dolatabadian et al., 2011). Our findings indicate that L-tr treatments successfully mitigated the adverse effects of salt stress on midrib diameter, epidermis thickness, and xylem conduit diameter.

Principal Component Analysis (PCA) was employed to independently distinguish experimental groups based on the evaluated parameters. The analysis demonstrated that L-tr doses applied to pepper plants exposed to salt stress had varying effects. It was especially noteworthy that the L-tr 100 µM application was in the positive zone, unlike other doses. Similar applications of PCA have been suggested for the selection and distinction of tomato germplasm regarding salt tolerance (Sivakumar et al., 2020).

Increasing environmental pressures due to global warming negatively affect vegetable cultivation. In this study, besides the effects of salt stress on some physiological properties of young pepper plants, histological changes in the cortex, xylem, midrib and epidermis cells were determined. The current study proved that the application of L-tr is one of the appropriate treatments to increase salt tolerance in plants, by regulating the negative effect of stress in plant cells. 100 µM L-tr application improved xylem vessels and cortical cells that may help plants acquire water and minerals more efficiently. The current paper proposes that 100 µM L-tr dose is convenient for application in pepper plants under salinity.

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STATEMENT OF CONFLICT OF INTEREST

The author declare that they have no competing interests.

AUTHOR’S CONTRIBUTIONS

All processes were carried out by the corresponding author.

STATEMENT OF ETHICS CONSENT

Ethical approval is not required as this article does not contain any studies with human or animal subjects.

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