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Alleviation of Salt Stress with Chitosan Foliar Application and Its Effects on Growth and Development in Tomato (*Solanum lycopersicum* L.)

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Abstract: Environmental and climatic fluctuations as well as abiotic stress factors affect agricultural production and cause a loss in quality and yield. It is important to find alternative solutions for the sustainability of agricultural production to feed the increasing population. Salt stress is one of the most devastating abiotic stress factors and tomato (*Solanum lycopersicum* L.) production is also affected by salt stress since it needs extensive irrigation for high yield. The exogenous application of some plant inducers showed promising results in the induction and improvement of plant tolerance to stress factors. Chitosan (2-amino-2-deoxy-b-D-glucosamine), one of the organic compounds, is getting significant attention in agriculture with its potential. Here, we evaluated the potential of chitosan application for salt stress tolerance on tomato. 0.03% and 0.05% chitosan solutions were applied as a foliar spray to the plant and salt tolerance improvement were investigated under untreated (0 mM NaCl) and 100 mM NaCl conditions. The growth-related (root and shoot diameters, above and below-ground biomass, number of leaves and branches, and plant height), photosynthetic parameters (chlorophyll a, b, total carotenoid content), and ion leakage were investigated. According to the results, chitosan application improves plant development in both untreated and salt-stress conditions and improved plant growth. Also, photosynthetic parameters showed that the application of chitosan increased chlorophyll contents under untreated conditions. Our result suggests that the application of chitosan may have a promising effect on salt stress tolerance and further research may shed light on its molecular mechanisms.

Keywords: Chitosan, *Solanum lycopersicum* L., salinity, plant growth, osmotic stress tolerance

1. Introduction

Climate change and population increase coupled with food insecurity force farmers, plant breeders, pre-breeders, and policymakers to find alternative solutions to the sustainable use of resources (Borlaug, 2002; Godfray et al., 2010). The development of new and advanced cultivars with better yield, quality, shelf life, etc. are the main drivers of fighting hunger (Borlaug, 1983; Abberton et al., 2015; Acosta-Motos et al., 2020). However, that is not enough to cope with the fast pacing changes in environmental factors, which can be grouped as abiotic and biotic stress factors (Mahajan and Tuteja, 2005; Liang et al., 2018). Over the last several decades, several new approaches including but not limited to plant elicitors (Bektas and Eulgem, 2015), plant growth-promoting rhizobacteria (PGPR) (Babalola, 2010), biotechnological approaches, such as CRISPR applications (Massel et al., 2021) and priming (Bulgari et al., 2019; Mal et al., 2019) have emerged to improve current cultivars or release new ones to meet the global demand.

Several organic or synthetically derived products have been found useful in the improvement of crop growth, development, and/or stress tolerance (Abd El-Daim et al., 2014; Bulgari et al., 2019; Gully, 2019; Gully et al., 2019; Farooq et al., 2022). As we understand the mechanism and mode of action of these products, new and combined versions are emerging. There is a significant potential for the use of these products in agriculture, since, it is almost impossible to replace all cultivars with new ones. Priming or foliar spraying with c-amino butyric acid (GABA) and b-amino butyric acid (BABA) (Bektas et al., 2016;

Vijayakumari et al., 2016), chitosan (Rashidi et al., 2020), salicylic acid (Hayat et al., 2010) and many other known and newly identified compounds (Valluru et al., 2016; Ashour et al., 2021; Sheikhalipour et al., 2021; Ayed et al., 2022) are reported to enhance growth, development and stress tolerance levels in crops. Therefore, priming, foliar spraying, or other external field applications of these compounds are of great importance for agricultural sustainability (Singh et al., 2015; Pawar and Laware, 2018; Zulfiqar, 2021).

Chitosan (2-amino-2-deoxy-b-D-glucosamine), one of the organic compounds (Balusamy et al., 2022), is getting significant attention in agriculture (Al-Tawaha et al., 2018; Hernandez-Hernandez et al., 2018a, 2018b; Attia et al., 2021; Balusamy et al., 2022; Hidangmayum and Dwivedi, 2022). It is one of the most commonly found natural exoskeleton compounds of insects, shrimp, lobsters, crabs, and cell walls of fungi, nematode eggs, and gut linings (Turk, 2019). It is identified as a plant elicitor in the 80s (Turk, 2019) and found diverse application areas for itself in agricultural production (Balusamy et al., 2022). Since then, chitosan or chitosan-related compounds have been evaluated against salinity in maize (Oliveira et al., 2016; Al-Tawaha et al., 2018) and safflower (*Carthamus tinctorius* L.) and sunflower (*Helianthus annuus* L.) seedlings (Jabeen and Ahmad, 2013), tomato (Hernandez-Hernandez et al., 2018a, 2018b), and bentgrass (Geng et al., 2020). There were also reports of enhanced abiotic stress tolerance with the application of chitosan or related products (Al-Tawaha et al., 2020; Elansary et al., 2020; Hafez et al., 2020). There is a wide range of chitosan applications in agriculture and it is still a relatively unknown compound with great potential in crop production (Hidangmayum and Dwivedi, 2022).

Tomato (*Solanum lycopersicum* L.), one of the most consumed vegetables in the world (Anonymous, 2021), is a staple food and therefore, receives a lot of attention (Foolad, 2004; Shahbaz et al., 2012; Salehi et al., 2019). Abiotic stress factors are the most significant limitation in tomato production (Shahbaz et al., 2012; Behera et al., 2022), similar to other crops (Mahajan and Tuteja, 2005; Munns, 2011; Liang et al., 2018). Since tomato needs extensive irrigation for high yield, salinity becomes an issue for tomato production (Cuartero and Fernandez-Munoz, 1999; Flowers, 2004; Foolad, 2004). There have been several studies and applications to enhance salt stress tolerance in tomato (Cuartero and Fernandez-Munoz, 1999; Ashraf and Harris, 2004; Flowers, 2004; Foolad, 2004; Mayak et al., 2004; Munns, 2011; Shahbaz et al., 2012; Machado and Serralheiro, 2017), however, there is still a significant need for practical applications to reduce or eliminate the destructive effects of salt stress on tomato production. Foliar application of biostimulants would be an easy and cheap strategy to mitigate stress levels on crops (Ayed et al., 2022). Therefore, this study aimed to 1) evaluate the effect of chitosan foliar spraying on growth and development, and 2) the physiological parameters of tomato (*S. lycopersicum* L.) under salt-stressed conditions.

2. Materials and Methods

2.1. Plant material and growth conditions

The study was conducted under semi-controlled conditions in the Department of Agricultural Biotechnology, Siirt University, Siirt, Türkiye (37°58'13.20"N-41°50'43.80"E). Mean temperature and relative humidity ranged between 25-27 °C and 60-70%, respectively. The experiment was conducted under artificial light (6500K) conditions with a 16:8h day/night regime in the growth room. Seeds were surface sterilized with 70% ethyl alcohol (C_2H_5OH) and 5% sodium hypochlorite (NaOCI) for 5 minutes and rinsed with sterile distilled water 3-4 times. Plants were grown in pots (9x8 cm) filled with peat moss and irrigated as needed (approximately 5 days intervals).

2.2. Chitosan and salt stress application

Chitosan (2-amino-2-deoxy-b-D-glucosamine) was dissolved in 2% acetic acid and 5ml of 0.05% and 0.03% of chitosan applied to plants as a foliar spray at the three-four leaf growth stage of the plant, based on preliminary studies. 2% acetic acid was sprayed on plants as a control (5 ml). Acetic acid or chitosan untreated plants were assigned as the negative control. 4 days after the foliar spray, the salt application was started and 100 mM NaCl was applied as a root drench. Plants were irrigated every six days either with 50 ml 100 mM NaCl or distilled water. As a control distilled water was applied to plants and entitled as "untreated". The treatment schema, chitosan, and salt applications were grouped and abbreviated as shown in Table 1.

Table 1. Chitosan and salt-stress doses applied to tomato (*S. lycopersicum* L.) plants and abbreviations used in the study

Stress treatment	Chitosan application	Abbreviation
Untreated (0 mM NaCl)+	Negative control	UNC
	Control (2% acetic acid)	UC
	0.03% Chitosan	U0.03
	0.05% Chitosan	U0.05
Salt-Treated (100 mM NaCl)	Negative control	SNC
	Control (2% acetic acid)	SC
	0.03% Chitosan	S0.03
	0.05% Chitosan	S0.05

The salt application was initiated at the three-four leaf growth stage of the plants. The experiments were repeated 3 times with 3 plants per replication.

2.3. Growth parameters

To evaluate the effect of chitosan on growth and stress tolerance in tomato, stem diameter, and root diameter was measured with a digital caliper. The number of branches and number of leaves was recorded by manual scoring. Plant height was measured with a ruler. Plant shoot and root fresh and dry weights were measured using a precision scale (Weightlab instruments).

2.4. Ion leakage measurements

To determine Electrolyte (ion) leakage; Leaf discs (1.2 cm diameter) were collected from uniform leaves from each of the three plants (replicates) per treatment. Then discs were rinsed and three discs were put into the tubes and shaken for 4 h before the first electrical conductivity (EC1) measurement. Then leaf discs were autoclaved to kill tissues and second electrical conductivity (EC2) was measured (Hanna HI2002-02 Edge® pH Metre, Germany). Ion leakage was measured two times; two weeks before harvest time (Ion leakage 1) and harvesting day (Ion leakage 2). Ion leakage was calculated with the Equation 1 (Yasemin et al., 2017).

$$\text{Ion leakage (\%)} = (EC1/EC2)\times 100 \quad (1)$$

2.5. Chlorophyll content measurements

Chlorophyll parameters (470 nm, 645 nm, 652 nm, and 663 nm) were evaluated using a spectrophotometer (Thermo Fisher Scientific). To do that, 150 mg of leaf tissue was collected and chlorophyll was extracted with 80% of acetone, filtered and the absorbances of the extract solution were measured at 470 nm, 645 nm, 652 nm, and 663 nm on a spectrophotometer. Chlorophyll a, b, total chlorophyll, and total carotenoid content were calculated according to Equations 2 to 5 (Lichtenthaler and Wellburn, 1983).

$$\text{Chlorophyll a (mg g}^{-1}) = (11.75\text{x}A663 - 2.53\text{x}A645)\text{ x }15/150 \quad (2)$$

$$\text{Chlorophyll b (mg g}^{-1}) = (18.61\text{x}A645 - 3.96\text{x}A663)\text{ x }15/150 \quad (3)$$

$$\text{Total chlorophyll (mg g}^{-1}) = (20.02\text{x}A645 + 8.02\text{x}A663)\text{ x }15/150 \quad (4)$$

$$\text{Total carotenoid (mg g}^{-1}) = ((1000\text{x}A470 - 2.27\text{ x chl\_a} - 81.4\text{ x chl\_b})227)\text{ x }15/150 \quad (5)$$

SPAD value was measured with a SPAD-502 (Minolta Corp.) device.

2.6. Statistical analysis

The study was conducted according to a randomized complete blocks design with three replications and three plants per replication. The results were evaluated using analysis of variance (ANOVA) with Statistix software v10 (Analytical Software, Tallahassee, FL).

3. Results and Discussion

Enhancing plant abiotic stress tolerance levels with genetic improvement and/or external applications are the major approaches to gaining sustainability in agricultural production and improving yield per area. Feeding billions will only be possible with the collective and continuous efforts of plant breeders and farmers (Borlaug, 2002; Godfray et al., 2010). Here, one of the novel approaches in agriculture, foliar spraying of chitosan is investigated with tomato (*S. lycopersicum* L.) as a model organism under salt-stressed conditions.

3.1. Plant growth and development under chitosan application

Chitosan application and chitosan x salt stress interactions resulted in significant differences ($p<0.05$). The results showed that stem diameter in UC and U0.03 was significantly higher compared to other groups, while S0.05 and SC applications had the smallest values in stem diameter (Figure 1a). Root diameter between the applications did not differ significantly, however, UNC and U0.03 had the highest, and SC and S0.03 had the lowest root diameter values (Figure 1b). The number of branches per plant was the highest in U0.03 and U0.05, while it was the lowest in UNC and SC applications (Figures 1c and 1d). Plant height varied between 12.3 and 18.1 cm, with U0.03 having the tallest, and S0.05 having the shortest statures (Figure 1e). according to our results, chitosan application itself or its control trigger plants for better plant development under untreated conditions. Especially 0.03% chitosan concentration showed remarkable improvements in the number of branches and leaves as well as plant height. Also, in the salt-applied groups, S0.03 demonstrated prominent plant well-being compared to other groups' improvements in the number of branches and leaves as well as plant height. When we compared our results with the previous reports, we found overall similar outputs on plant growth, development, and stress tolerance. A study on lettuce (*Lactuca sativa* L.) reported improved total

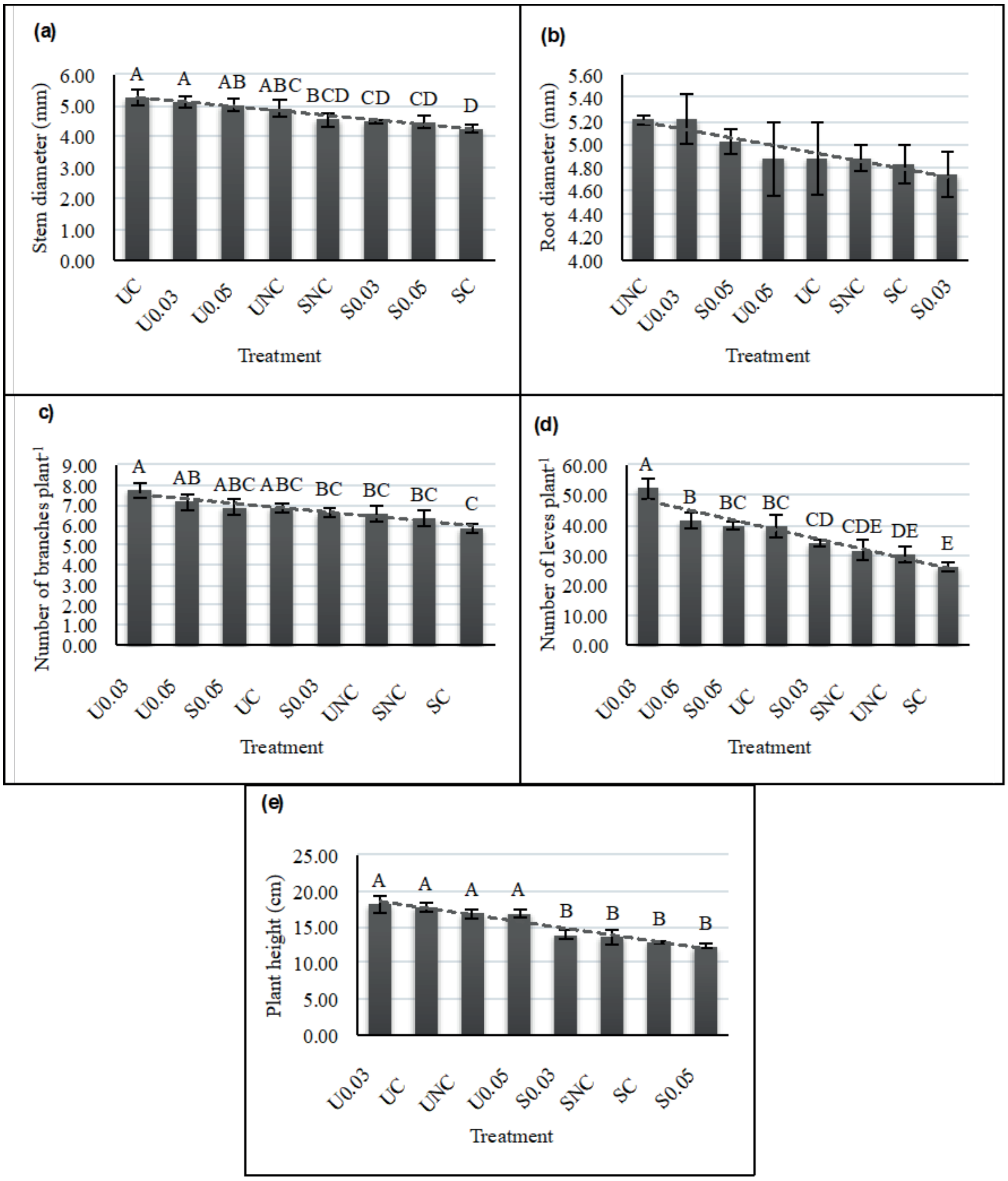

Figure 1. Plant growth parameters under chitosan and salt stress applied conditions. a) Stem diameter, b) root diameter, c) number of branches, d) the number of leaves, e) plant height

Means followed by a different letter in each figure are significantly different at $p<0.05$ level according to the LSD multiple comparison test.

leaf area and growth under chitosan application (Zhang et al., 2021). They suggested that chitosan alleviated the inhibitory effects of salt stress and improved plant growth. Li et al. (2022) evaluated the effect of chitosan oligosaccharide in combination with physcion on maize seedling growth and reported positive outcomes. They suggested the combined application of these two as a seed-coating material to alleviate stress tolerance. In a review by Kociecka and Liberacki (2021), the possible applications and benefits of chitosan are well documented and highlighted. As highlighted by Kociecka and Liberacki (2021), chitosan is the second most common polymer in the world after cellulose. It is available all around the globe, cheap, and easily applicable in all fields of agriculture (Kociecka and Liberacki, 2021). It is reported to enhance growth in almost all cereal crops. We also demonstrated improved growth-related traits (Figure 1) in tomato under chitosan foliar sprayed conditions compared to salt-only or untreated conditions. There are relatively few studies on horticultural crops compared to cereals. So, our study may shed some light on the effect of chitosan on tomato growth.

The effects of chitosan and salt stress applications on tomato growth were evaluated with biomass allocation patterns. The results revealed significant ($p<0.05$) differences between chitosan applied and control groups under untreated and salt stress conditions (Figure 2). Plant fresh and dry weights and root fresh and dry weights were all similar in biomass trends. As expected, the values for the untreated groups were higher than for salt-applied groups. However, chitosan application improved the plant shoot and root fresh/dry weights values (Figure 2). These results were also correlated with plant growth-related traits, and 0.03% chitosan application showed higher values in all applications along with the control group. Also based on our results, the acetic acid application also triggers plant development and demonstrated higher values compared to the UNC. Accordingly, U0.03, U0.05, and UC had the highest values in all biomass indicators. Plant fresh weights varied between 6.22 g and 11.1 g, dry weights between 0.71 and 1.33 g, root fresh weight between 3.36 and 7.84 g, and root dry weight between 0.13 and 0.35 g per plant. While salt application had a negative impact on biomass allocation, chitosan application reversed this effect (Figure 2). Interestingly, SC showed some significant improvement in plant development and biomass. Our results revealed a significant effect of chitosan application on plant biomass along with a positive effect on biomass under non-stressed conditions, which is similar to previously documented results. Zhang et al. (2021) reported enhanced root and shoot biomass under chitosan foliar sprayed and salt-stressed conditions. Their results were similar to our observations. The regulatory role of chitosan in plant growth is also highlighted by Zhang et al. (2021). Another study on maize seedlings (*Zea mays* L.), (Turk, 2019) reported enhanced root length and plant height under chitosan application. They also revealed that chitosan+salt application had higher values in the above parameters compared to salt-only conditions. Sheikhalipour et al. (2021) evaluated the effects of salt stress on bitter melon (*Momordica charantia* L. cv. Palee F1) under chitosan–selenium nanoparticle foliar sprayed conditions. They reported enhanced growth in plant height, shoot and root fresh and dry weigh values with chitosan–selenium compared to

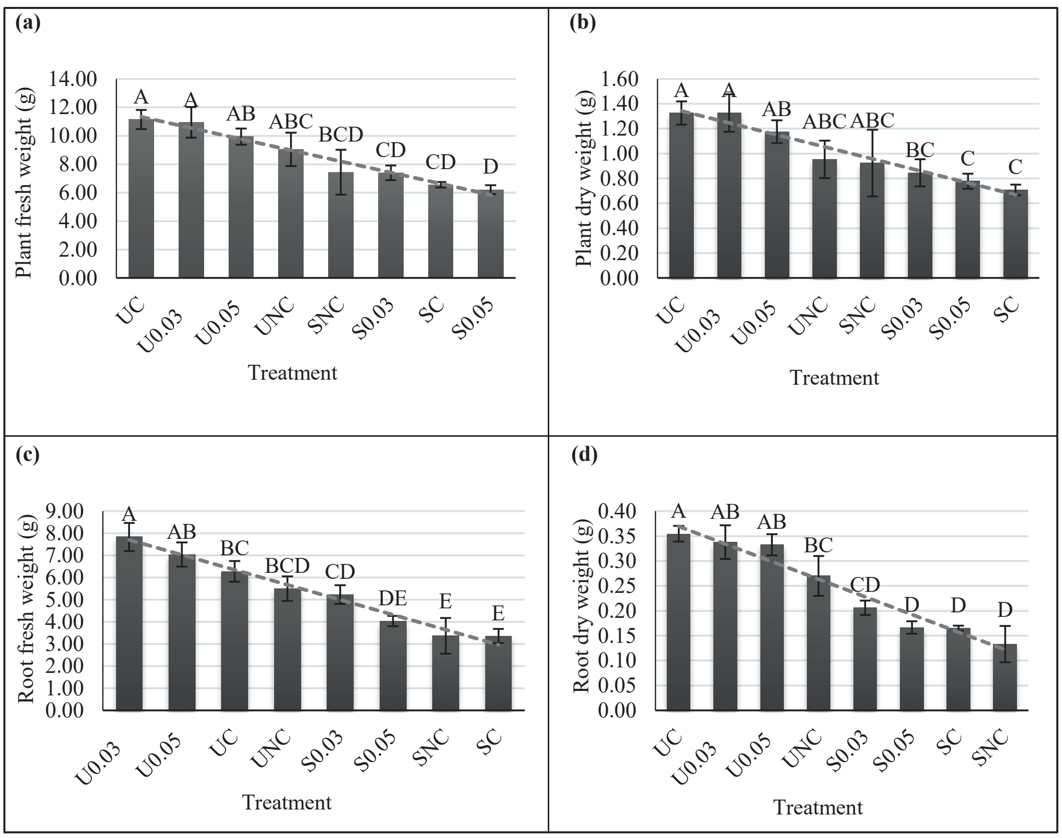

Figure 2. Plant biomass parameters under chitosan and salt stress applied conditions. a) plant fresh weight, b) plant dry weight, c) root fresh weight, and d) root dry weight were evaluated

Means followed by a different letter in each figure are significantly different at $p<0.05$ level according to the LSD multiple comparison test.

control (salt only) conditions. The authors also reported improved values in some key enzymes (peroxidase, ascorbate peroxidase, catalase, and superoxide dismutase) and biochemical parameters. Similar improved plant growth values were also reported by Safikhan et al. (2018), Sen et al. (2020), and Seraj et al. (2021). Our result with support from the literature, suggests that chitosan not only improves plant growth under non-stressed conditions but also alleviates the inhibitory effects of salt stress on plant growth.

3.2. Physiological parameters, stress tolerance, and photosynthesis under chitosan application

To understand the effect of chitosan on plant performance under salt-stressed and non-stressed conditions, we examined the chlorophyll contents and ion leakages. For the leaf chlorophyll concentrations, we used two different methods; SPAD measurements and chlorophyll content extraction with acetone. According to our results, the application of SC, S0.03 or S0.05 seems to trigger an increased chlorophyll-a concentration (Chl_a) under salt-stressed conditions (Figure 3a). Also, similar results were determined with chlorophyll-b (Chl_b), total chlorophyll, and total carotenoid contents (Figure 3b, 3c, 3d). Based on our results, 0.03% and 0.05% chitosan application seems to induce plants and increased the chlorophyll contents of the plants under salt stress conditions. On the other hand, in the untreated groups, these applications showed the lowest chlorophyll contents. Surprisingly, also 2% of acetic acid (SC), the solvent of the chitosan, showed a significant increase in chlorophyll concentration under salt stress conditions compared to the SNC. Correlated with that, also SPAD values showed similar results. Both chitosan application and acetic acid application demonstrated similar effects under salt stress conditions (Figure 3e).

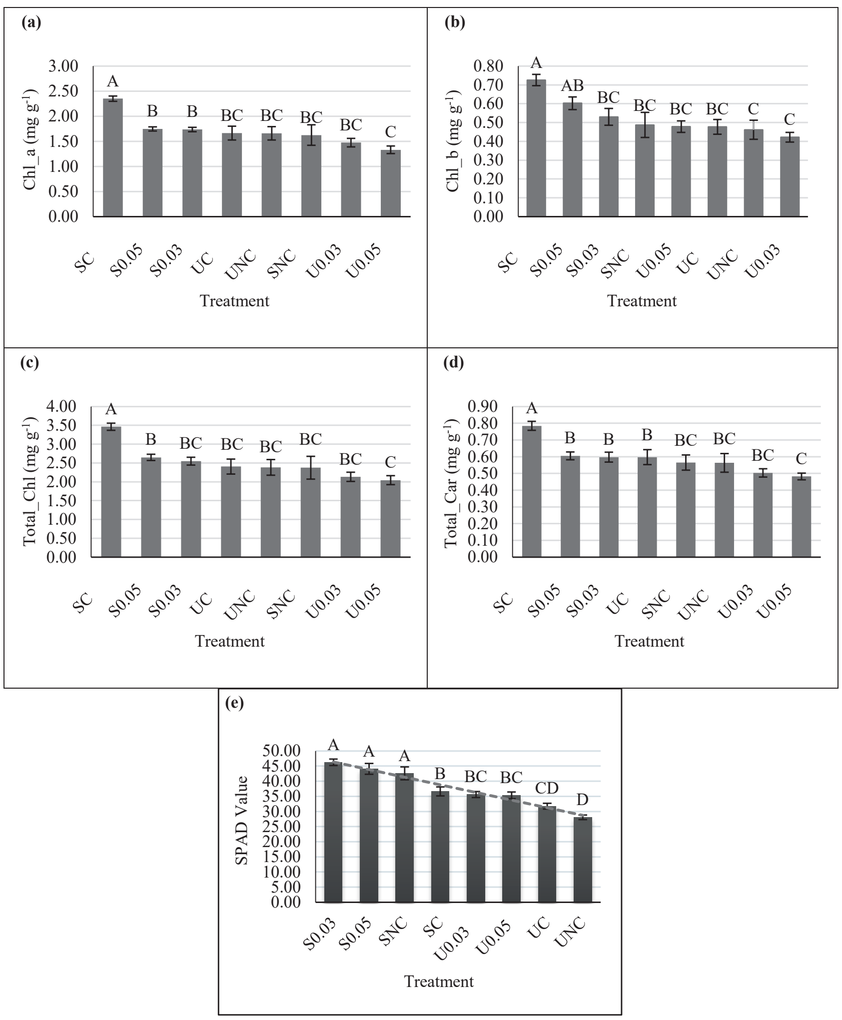

Figure 3. Physiological parameters under chitosan and salt stress applied conditions. a) Chlorophyll a, b) Chlorophyll b, c) Total chlorophyll, d) Total carotenoid, and e) SPAD value were evaluated

Means followed by a different letter in each figure are significantly different at $p<0.05$ level according to the LSD multiple comparison test.

To understand the effect of the foliar application of chitosan on salt stress tolerance, we analyzed the ion leakage of the plants (Figure 4). Ion leakage 1 was the highest in S0.05 and lowest in U0.03 (Figure 4a). Contrarily, ion leakage 2 was the highest in U0.03 and lowest in S0.03 (Figure 3c). Ion leakage 2 was the lowest in all applications with salt (Figure 4b). Several previous studies evaluated the effect mechanism of chitosan on plant physiological processes. Of these, Turk (2019) reported improved mitochondrial respiration and antioxidant enzyme activity under salt stress and chitosan-applied conditions. They suggested that chitosan application could enhance plant growth by improving total respiration rate, cytochrome pathway, and alternative respiration in addition to improved antioxidant enzyme ratios. In a study with water stress and chitosan application, Seraj et al. (2021) reported improved growth and yield in milk thistle (*Silybum marianum* L.). They also reported higher values in proline content and antioxidant enzyme activity under chitosan application and water-stressed conditions. They suggested that chitosan inhibits the stress effects by stimulating osmoregulation with proline and soluble sugar accumulation, as well as reduced malondialdehyde content and higher antioxidant enzyme levels. In a study on mung bean (*Vigna radiata* L. Wilczek) with nano-chitosan priming and its role against salt stress, Sen et al. (2020) reported improved values in protein, antioxidant activity, and phenolic compounds. The authors suggest that priming with normal-sized and nano chitosan, may enhance plant growth and reduce the destructive effects of salt stress in mung beans. Therefore, chitosan application can be suggested to farmers in saline water conditions. Safikhan et al. (2018) also reported similar results in milk thistle (*Silybum marianum* (L.) Gaertn.). Improved abiotic stress tolerance in maize (Oliveira et al., 2016), rice (Moolphuerk and Pattanagul, 2020; Moolphuerk et al., 2021), and soybean (Mehmood et al., 2020) were reported in previous studies. Our results and previous reports clearly demonstrated the positive effect of chitosan on plant growth by improving biochemical and physiological parameters. But, there is still limited knowledge of the mechanism and mode of action as well as the appropriate dose of chitosan or its combinations with other compounds for specific crops. Its interactions with the environmental variables (temperature, humidity, light intensity, etc) are not well documented. There is a need for further studies to shed light on many unknown parts of chitosan applications in agriculture either as priming, foliar spraying, or other methods.

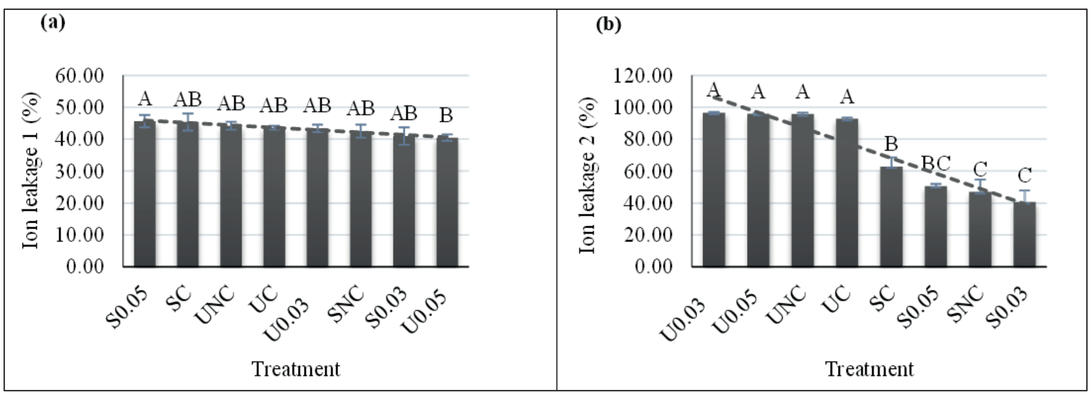

Figure 4. Physiological parameters under chitosan and salt stress applied conditions. a) Ion leakage 1, and b) Ion leakage 2 were evaluated

Means followed by a different letter in each figure are significantly different at p<0.05 level according to the LSD multiple comparison test.

4. Conclusions

This study aimed to investigate the effect of two different chitosan doses on tomato under untreated and salt stress conditions. Based on our results, chitosan application clearly increased some plant growth parameters like stem and root diameter, the number of branches and leaves, plant height, plant shoot, and root fresh and dry weights under untreated conditions. These outcomes provide us with information about the application of chitosan for plant development. Also under salt stress conditions, chitosan applications (0.03% and 0.05%) showed promising improvements in plant development as well. Surprisingly, also acetic acid application as the control showed similar results with chitosan application as well. According to chlorophyll content and ion leakage measurements, also chitosan applications demonstrated promising results. Further studies may reveal the molecular mechanism of these outcomes and may shed light on the possibility of the use of chitosan in agriculture.

Declaration of Author Contributions

The authors declare that they have contributed equally to the article. All authors declare that they have seen/read and approved the final version of the article ready for publication.

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Declaration of Conflicts of Interest

All authors declare that there is no conflict of interest related to this article.

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