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Research Article/Araştırma Makalesi

Determination of the physiological and biochemical effects of humic acid application in strawberry plant grown under salt stress

Tuz stresinde yetiştirilen çilek bitkisinde hümik asit uygulamasının fizyolojik ve biyokimyasal etkisinin belirlenmesi

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ABSTRACT

The reduction of salt toxicity in the soil environment significantly contributes to the growth and development of plants. In this study, tolerance and development status of strawberry plant were investigated through irrigation solution including 50 mmol L^{-1} NaCl salinity and the application of 50 mg L^{-1} humic acid (HA). The physiological and biochemical parameters in vegetative and fruit stages and the mineral contents of strawberry plants were assessed if application of HA contributed to salt tolerance mechanisms of strawberry plants. HA improved physiological parameters such as crown and root fresh weight, crown and root dry weight, stomatal conductance as well as improving biochemical parameters and reducing stress metabolites such as (total chlorophyll contents, proline, malondialdehyde, catalase, and peroxidase enzyme activities). HA application (50 mg L^{-1}) under salt stress (50 mmol L^{-1}) also improved the quality parameters such as vitamin C and lycopene contents. We suggest that HA application is beneficial via increasing the tolerance mechanisms of salt-sensitive strawberry plants.

Key Words: NaCl stress, humic acid, strawberry, abiotic stress

ÖZ

Topraktan tuz toksisitesinin azaltılması, bitkilerin büyüme ve gelişimine önemli katkılar sunmaktadır. Bu çalışmada 50 mmol L^{-1} NaCl içeren sulama suyu ile sulanan çilek bitkisinin 50 mg L^{-1} hümik Asit (HA) uygulaması ile tuz stresine karşı toleransı ve gelişim durumu araştırılmıştır. Çilek bitkilerinin tuz toleransına HA uygulamasının katkıda bulunup bulunmadığı, vejetatif ve meyve aşamalarında fizyolojik ve biyokimyasal parametreler ve çilek bitkilerinin mineral içerikleri değerlendirilmiştir. HA, taç ve kök taze ağırlığı, taç ve kök kuru ağırlığı, stomatal iletkenlik gibi fizyolojik parametri ve bunun yanı sıra artan biyokimyasal parametreler ve azalan stres metabolitleri ile (toplam klorofil, prolin, MDA, katalaz ve peroksidaz) bitki gelişimini sağlamıştır. Tuz stresi altında (50 mmol L^{-1}) HA uygulamasının 50 mg L^{-1} vitamin C likopen gibi kalite parametrelerini artırdığı belirlenmiştir. HA uygulaması tuza hasas olan çilek bitkisinin tolerans mekanizmasını artırarak fayda sağlamıştır.

Anahtar Kelimeler: NaCl stres, hümik asit, çilek, abiotik stres

Introduction

Salt stress is a very important abiotic stress affecting plant growth, crop production and quality. Increased salinity in the root media impacts plants during their vegetative growth periods via decreasing plant growth and yield (Wani and Gosal, 2011). Some crop plants have limited capacity to tackle with salt stress. Application of proper fertilization and irrigation or genetic improvement may help to increase the salt tolerance mechanisms in salt-sensitive crop plants. However, additional help is mostly needed to grow them under stressed conditions.

Strawberry is a small fruit crop that is highly valuable all over the world. It belongs to the Rosaceae family with 23 species in the genus Fragaria. (Folta and Davis 2006; Shulaev et al. 2008). Strawberry is an important commercial fruit crop with the increasing production areas and consumption around the world. However, it is considered sensitive to NaCl due to toxic Na^+ or Cl^- ions. NaCl stress not only reduces the crop yield but also deteriorates the quality parameters in strawberry fruits (Jamalian et al., 2013; Garriga et al., 2015).

We could reduce the negative effects of salinity through the application of chemicals. Humic acid (HA) is one of the benefical chemicals to have a key role in the promotion of plant growth as biostimulation. HA can enhance stress tolerance of plants. It has been widely used in water stress, salinity stress and even in biotic stress agent conditions (Aydin et al., 2012). It regulates plant primary and secondary metabolism in stress conditions and increases resistance against abiotic and biotic stresses (Canellas and Olivares, 2014; Canellas et al., 2015). HA could help crop plants enhance a variety of biochemical processes by growing the activity of enzymatic antioxidants including CAT, POD, and SOD, cell membrane stability, regulating water absorption and increasing the uptake of mineral elements and synthesis of proteins and hormones. Humic acid also regulates hormonal homeostasis and positively affects plant growth (Ali et al., 2019). HA also contribute to soil fertility via regulating chemical reactions in the vicinity of plant roots (Trevisan et al., 2009; Geçer, 2020).

In this study, physiological and biochemical responses of Rubygem strawberry cultivar against NaCl salinity were assessed and the effect of HA in terms of growth improvement, mineral contents availability and fruit quality parameters.

Materials and methods

Experimental design and plants growth

This research was conducted in a semi-controlled greenhouse. Fresh strawberry (Rubygem variety) plants were grown in 8-L pots containing peat under natural light conditions. Day and night mean temperatures were 30±2/24±2 °C. A trial was a randomized design with three different factors. The first factor was salinity stress which included two treatments containing 0 (control) and 50 mmol L^{-1} NaCl stress. The second factor was HA treatments used on plant leaf at 50 mg L^{-1} and the third factor was the application of both salt and humic acid (NaCl+HA) treatment. Treatments in each group were replicated five times.

Figure 1. Strawberry plants were grown with or without HA and NaCl applications during the trial

Commercially available strawberry seedlings were transferred to the pots and kept until they formed 4 to 5 fully-developed leaves (5 weeks). Salinity and HA treatments were made once a week. The experimental treatments were made for a period of ten weeks. The whole trial period from the beginning of obtaining strawberry seedlings till the end of harvest took four months (Figure 1).

Plant and fruit analyzes

Plant crown and root fresh weight (FW) were analyzed immediately after the harvest. Plant crown and root dry weight (DW) were determined after drying them at 70° C to reach a constant weight.

Total soluble solids (TSS) were assessed from the fruit juice with a hand refractometer. The results are expressed in percent (%) (Catania et al., 2020).

Stomatal conductivity was determined on the youngest fully expanded leaves on upper branches of the plants with a leaf promoter (SC-1) at midday. Measurements were conducted by clamping the leaves in the leaf chamber. The actual vapour flux from the leaf through the stomata is expressed as mmol $m^{-2}s^{-1}$ (Karlidag et al., 2011).

Strawberry fruits were harvested when 90% of the fruit surface had reached a fully red colour. At the end of the experiment, total fruit weights were determined, and the average fruit yield was calculated and expressed as g $plant^{-1}$.

Lycopene content of strawberry fruits was assessed with the method of Barrett and Anthon (2001) with minor modifications (Karakas, 2013). Strawberry fruit (one gram) was homogenized in 10 mL of ethanol: hexane (4:3) mixture. The homogenate centrifuging the mixture at 10,000 g for 10 min, then 0.1 mL of the supernatant was mixed with 7 mL of ethanol:hexane (4:3) mixture and vortexed. After 1 h of room temperature incubation, 1 mL of H2O was applied to the tubes and vortexed. A sample of the organic (hexane) phase was measured at 503 nm compared with hexane in a UV microplate spectrophotometer (Epoch, SN: 1611187, USA). The results are expressed as mg kg^{-1} FW.

The vitamin C content of fruits was assessed following the method of Oz (2002) with small modifications (Karakas, 2013). Strawberry fruits (five grams) were extracted in 25 mL of oxalic acid. The mixture was centrifuged at 10,000 *g*. Then, 1 mL of the mixture was added to 7 mL of 1% oxalic acid and 8 mL of dye reagent. The ingredients of dye reagent and the experimental protocol was explained in our previous work (Karakas et al., 2016).

Biochemical analyzes

Total chlorophyll contents (Chl-*a* + Chl-*b*) of strawberry plants were assessed following the method of Arnon (1949) with minor modifications (Karakas, 2013). Leaf samples (0.5 g) were extracted with 10 mL acetone: water (80/20, v/v) mixture and filtered through Whatman No.2 filter paper and the supernatants were measured at UV microplate spectrophotometer (Epoch, SN: 1611187, USA) at 663 nm and 645 nm for Chl-*a* and Chl-*b*, respectively. The blank was 80% acetone. The results are calculated as mg L^{-1} and expressed as mg g^{-1} FW.

The proline contents of strawberry leaves were assessed according to the method of Bates et al. (1973) with minor modifications. The detailed protocol was previously described (Karakas et al., 2013). The results are expressed as µmol g^{-1} FW.

Malondialdehyde (MDA) content was assessed following the process of Sairam and Saxena (2000) with minor modifications. The detailed protocol was previously described (Karakas et al., 2013). The results are expressed as nmol g^{-1} FW.

Catalase (CAT, EC. 1.11.1.6) and POX (POX, EC.1.11.1.7) enzyme activities were assessed according to the methods of Milosevic and Slusarenko (1996) and by Cvikrova et al (1994), respectively with minor modifications (Karakas et al., 2013). The detailed protocols for both experiments were made in the previous study (Karakas et al., 2013).

Leaf mineral (K^+, Na^+, Ca^{+2}, Mg^{+2}, Cl^{-1}) contents were assessed following the procedure of Chapman and Pratt (1961) with minor modifications (Karakas, 2013). Dry plant samples (0.5) g were ground in porcelain crucibles. The porcelain crucibles were placed into a muffle furnace, and the temperature was gradually increased up to 500 °C. The cooled ash was then dissolved in 5 mL 2 N hydrochloric acid. After 30 minutes, the volume was made up to 50 mL with distilled water, and the supernatant was filtered through Whatman No. 42 filter paper. Then resulting supernatant containing Na^+, K^+, Ca^{+2}, and Mg^{+2} ions were assessed by Inductively Coupled Plasma (ICP, Perkin Elmer). The concentration of Cl^- in the leaves was read using ion chromatography.

Statistical analyzes

Duncan's Multiple Range Test (DMRT) was used to evaluate the data at the P≤0.05 level using the ANOVA test in SPSS version 22. Data are expressed as a mean value ± the standard error.

Results and Discussion

We determined that crown FW and DW, root FW and DW, stomatal conductance, total soluble solids, fruit average weight and total yield, lycopene, vitamin C, chlorophyll (Chl-*a* and Chl-*b*), leaf ions contents (K^+ and Ca^{2+}) of strawberry plants decreased in NaCl-treated plants. Leaf proline contents, MDA, CAT and POX enzyme activities, leaf mineral contents (Na^+ and Cl^-) of strawberry plants increased with NaCl treatment. Statistical analysis showed that HA application had significant effects on the crown and root FW and DW, SC, TSS, fruit average weight, total yield, lycopene, vitamin C, total chlorophyll, proline content, MDA, POX and CAT antioxidant enzymes, leaf mineral content.

When strawberry plants were exposed to NaCl stress, their crown FW and DW were reduced 53% and 40%, respectively when compared to those of controls. However, application of NaCl+HA increased crown FW and DW contents by 90% and DW 95%, respectively when compared to control plants (Figure 1 A and B). HA application did not contribute to increases in FW and DW of strawberry plants grown in normal conditions. Similarly, root FW and DW of strawberry plants increased upon application of HA to NaCl-treated plants, but the increase of root FW and DW were not as significant as those of increases in crown FW and DW of strawberry plants followed by application of HA (Table 1).

When SC was elucidated, NaCl application reduced SC by 42% however, HA application under NaCl stress led to reduction of SC by only 8%. The application of HA to those NaCl-treated plants significantly improved the stomatal conductance in those plants (Table 1).

Table 1. Strawberry same physiological parameters of strawberry plants HA application and NaCl-treated.

Applications	*Crown FW (g plant^{-1})*	*Crown DW (g plant^{-1})*	*Root FW (g plant^{-1})*	*Root DW (g plant^{-1})*	*SC (mmol m^{-2} s^{-1})*
Control	84.18±3.84^a	9.75±0.26^a	43.06±1.97^a	6.21±0.21^a	234.01±10.28^a
NaCl	39.90±3.32^b	5.90±0.20^b	20.07±1.45^c	2.28±0.14^c	136.40±12.39^c
HA	87.33±3.10^a	10.91±0.42^a	44.86±4.19^a	6.92±0.39^a	246.08±12.81^a
NaCl+HA	75.38±5.63^a	9.24±0.92^a	32.69±2.58^b	4.24±0.22^b	213.78±10.48^b

Significance level at P ≤0.05 was assessed for the HA and NaCl applications via Duncan's Multiple Range Test. Different letters in each column show significant statistical differences.

It was reported that HA promoted soil water holding capacity and reduced irrigation needs for plants (Orzolek, 1993; Hynes and Naidu, 1998). According to some reports, HA regulated hormone level, improved plant growth and enhanced stress tolerance (Piccolo et al., 1992; Moraditochaee. 2012). HA may induce shoot and root growth and improve defence to abiotic stress in plants but the physiological mechanism has not been well determined (Delfine et al., 2005). Masciandaro et al. (2002), Pilanali and Kaplan (2003) stated that HA application mitigated the salinity effect in strawberry, maize and pepper seedlings in saline conditions.

Fruit characteristics

When strawberry plants were exposed to NaCl stress, their crown FW and DW were reduced 53% and 40%, respectively when compared to those of controls. However, application of NaCl+HA increased crown FW and DW contents by 90% and DW 95%, respectively when compared to control plants (Figure 1 A and B).

Average fruit and total fruit weights were significantly dropped 39% and 65%, respectively at NaCl-treated strawberry plants. However, HA application under NaCl stress led to reduction of average fruit and total fruit weights by only 9% and 4%, respectively when compared to controls (Figure 2A and 2B). It was observed that NaCl stress significantly decreased lycopene and vitamin C contents of the fruits. The application of HA improved the quality of fruits that had been exposed to NaCl. Although lycopene and vitamin C contents of the fruits decreased under NaCl stress, this was not that significant as compared to those of other parameters taken in the vegetative stages. However, the application of HA still contributed to increasing of lycopene and vitamin C contents of strawberry fruits (Figure 2C and 2D).

TSS contents did not differ amog the treatments. Although NaCl application reduced the TSS of strawberry plants, the reduction of TSS was not that significant. Application of HA to NaCl-treated plants increased the contents of TSS up to some degree, however, this was not statistically significant either (Figure 2E).

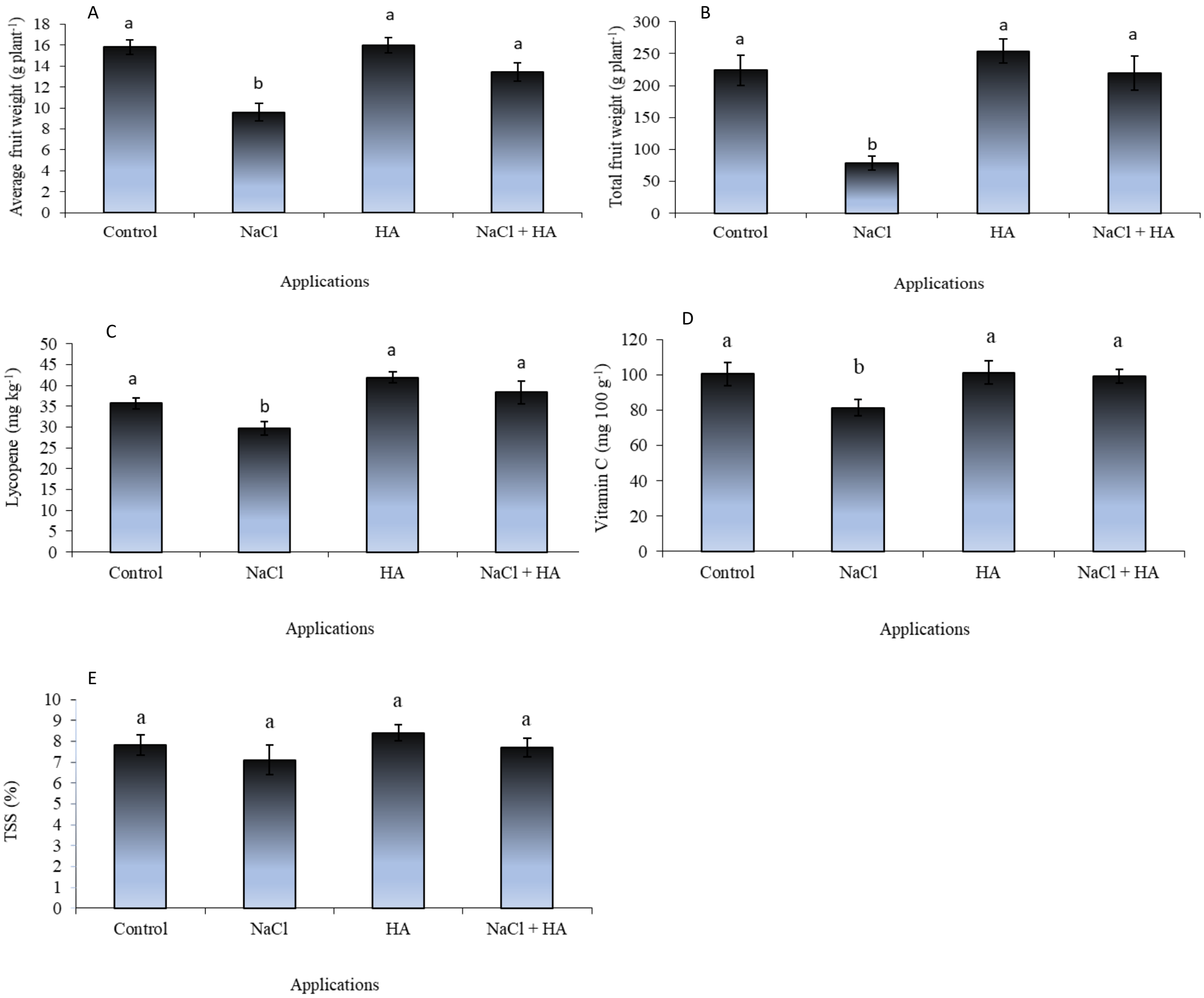

Figure 2. Effect of HA application on average fruit weight, yield lycopene, vitamin C and TSS of NaCl-treated strawberry (Rubygem) plants. Bars indicate the means of the five replicates ± standard error.

Decrease in strawberry yield under salinity stress could be attributed to reduction in photosynthesis and eventually reduction in the flower fruit formation (Saied et al., 2005). Salt stress can decrease fruit quality such as the vitamin C and lycopene content of strawberry (Jamalian et al. 2008; Karakas et al., 2021) Our results are in line with the results of previous studies.

Biochemical characteristics

Total chlorophyll contents of strawberry plants significantly decreased upon exposure to NaCl stress. Although the application of HA increased the total chlorophyll contents of plants, that was not satisfactory to increase the chlorophyll contents (Table 2). On the other hand, HA application on NaCl-treated strawberry plants contributed to increase of chlorophyll contents. The increase of chlorophyll was found statistically satisfactory in which the chlorophyll contents of NaCl-treated plants increased from 1.24 to 1.97 in NaCl+HA-treated plants. One of the main mechanisims behind this could be attributed to increased acctivity of photossnthetic compounds. For example, Fan et al. (2014) stated that chlorophyll contents of *Chrysanthemum* plants were significantly increased upon HA application. It is suggested that the increase of chlorophyll contents were due to increased metabolic activities led by HA application. We state the same pattern in strawberry plants.

Leaf proline content significantly increased as a response to NaCl-treatment. The application of HA significantly decreased the proline contents (Table 2).

Again, leaf MDA contents in strawberry increased with the NaCl-treatment as a response to stress. HA application had also a remarkable effect to decrease MDA level in NaCl-treated strawberry plants. (Table 2).

CAT and POX antioxidant enzyme activities showed a parallel pattern as those of previous parameters. HA application significantly decreased antioxidant enzyme activities NaCl-treated (Table 2).

Table 2. Strawberry biochemical parameters of strawberry plants HA application and NaCl-treated.

Applications	*Total chlorophyll (mg g^{-1})*	*Proline (µmol g^{-1})*	*MDA (nmol g^{-1})*	*CAT (unit mg^{-1} FW)*	*POX (unit mg^{-1} FW)*
Control	2.19±0.10a	2.90±0.42b	1.87±0.13b	1.85±0.20b	4.68±0.40
NaCl	1.24±0.17b	10.56±1.30a	5.31±0.36a	5.49±0.40a	13.69±1.07
HA	2.34±0.14a	2.70±0.24b	1.99±0.21b	1.36±0.18b	4.51±0.50
NaCl+HA	1.97±0.22a	3.68±0.82b	2.57±0.35b	2.22±0.24b	5.20±0.66

Significance level at P ≤0.05 was assessed for the HA and NaCl applications via Duncan’s Multiple Range Test. Different letters in each column show significant statistical differences.

Under saline conditions, compatible solutes like proline and soluble carbohydrates are expected to accumulate (Yaghobi et al., 2016). Increases in proline and soluble carbohydrates can be used as indicators of salinity tolerance ability (Munns and Tester, 2008). It was reported that proline increases positively correlated with HA application under NaCl stress (Jarosova et al., 2016). On the other hand, Jarosova (2016) reported the opposite finding that the amount of proline diminished in plants treated with HA under saline stress. It was suggested that the decrease in proline and soluble carbohydrate content in plants treated with HA may be related to the alleviation mechanism of harmful effects of salinity. In our findings, HA application did not increase proline contents under normal conditions, on the contrary, it decreased proline contents under NaCl stress conditions. NaCl stress was observed to increase proline contents. It was remarkable to see that HA application proline contents in NaCl-treated plants. Our findings were similar to findings observed by Jarosova (2016). We assumed that mostly proline accumulation in salt-sensitive plants caused from the degradation of protein rather than synthesis in cells. It is possible that HA application decreased the damaged proteins and prevented protein oxidation. This was reflected as decreases in proline contents in NaCl stressed plants. These observations were also positively tested when we recorded other parameters such as MDA accumulations. MDA is an important marker to determine if lipid peroxidation or damage to the plasmalemma and membranes of intracellular organelles has occurred as a result of environmental stresses (Halpern et al., 2015). In saline conditions, an increase in the number of free radicals causes membrane fats to decompose and MDA to be production (Bernstein et al., 2010). In this study, HA remarkably diminished MDA content under salinity stress. Since HA regulated the antioxidant enzymes in the host cell and limited the levels of ROS and lipid peroxidation, the application of HA could be benefical for the plants under stress (Lotfi et al., 2015; Saidimoradi et al., 2019). The similar case was proved in our study. The stress marker via MDA measuremet showed that was significantly reduced. This clearly showed that HA was able to reduce the NaCl stress and the metabolites produced for the ease of NaCl stress was spent to increase quality parameters as shown above.

Some leaf mineral contents

The effect of HA and salt stress on leaf mineral contents such as N, P, K^+, Ca^{2+} and Mg^{2+} ions decreased with the increases in salinity levels in strawberry plants. The lowest K^+ and Ca^{2+} ions were determined following NaCl application. HA application enhanced N and P ions, but Mg^{2+} has not been changed significantly in strawberry plants following NaCl treatments. Under saline conditions, increases of Na^+ and Cl^- ions were evident in strawberry plants grown at increasing NaCl salinity, however, employment of HA significantly decreased the Na^+ and Cl^- ions, Table 3.

Table 3. Strawberry leaf mineral contents.

Applications	*N (%)*	*P (%)*	*K^+ (%)*	*Na^- (%)*	*Ca^{++} (%)*	*Mg^{++} (%)*	*Cl^- (%)*
Control	2.21±0.28^a	0.88±0.11^a	2.77±0.10^a	0.21±0.02^c	1.92±0.06^a	0.31±0.02^a	0.30±0.03^c
NaCl	1.81±0.18^b	0.62±0.07^b	1.76±0.20^b	1.13±0.08^a	1.68±0.03^b	0.29±0.01^a	1.40±0.11^a
HA	2.63±0.26^a	1.03±0.14^a	2.60±0.07^a	0.19±0.01^c	2.11±0.12^a	0.34±0.02^a	0.26±0.04^c
NaCl+HA	2.30±0.27^a	0.77±0.05^a	2.54±0.11^a	0.58±0.06^b	1.91±0.11^a	0.33±0.03^a	0.47±0.06^b

Significance level at P ≤0.05 was assessed for the HA and NaCl applications via Duncan's Multiple Range Test. Different letters in each column show significant statistical differences.

Above parameters and mineral nutrient absorption rates are combined to assess the function of HA in reducing the negative effects of salt on plants. The effects of HA on nutrient absorption in plants have been extensively researched in terms of growth conditions and plant varieties. For instance, HA prolonged the uptake of P, K^+, Ca^{2+}, Mg^{2+}, Na^+, Zn^{2+}, Fe^{3+}, Mn^{2+} and Cu^{2+} in *Solanum melongena* L. and tomato plants (Dursun et al.,1999); in maize such as N, P, K^+, Ca^{2+}, Zn^{2+}, Fe^{3+}, Mn^{2+} and Cu^{2+} (Sharif et al., 2002; Eyheraguibel et al., 2008); N, P, K^+ in tomato plants (Abdel-Mawgoud et al., 2007). Effects of HA on mineral uptake in saline-stressed strawberry plants were quite remarkable as shown in this study. NaCl stress significantly increased Na^+ and Cl^- ion accumulation in leaves of strawberry plants while decreasing other beneficial minerals such as N, P, K^+, Mg^{++}, and Ca^{++}. Application of HA not only regulated metabolic homeostasis but also improved beneficial mineral uptake while developing the uptake of toxic ions like Na^+ and Cl^- ions. This is probably due to increased root system and photosynthetic capacity. Positive effects of HA applications were observed on strawberry plants grown in normal conditions. As expressed above, HA application improved the rooting system of those plants grown in normal conditions in which the uptake of mineral elements are increased significantly. Our study showed that saline stress reduced K^+ ion concentration while increasing Na^+

concentration in leaf. It was reported that salinity increased Na^+ deposition ratio in root growing region and decreased the selectivity for K^+ (Saidimoradi et al., 2019). However, HA application improved and regulated mineral homeostasis in strawberry plants. Similar results were reported by Keutgen and Pawelzik (2009) who determined an increase in Na^+ ion content in the leaf and root of strawberry plants when they were exposed to saline stress.

Conclusion

One of the most critical and destructive abiotic stresses affecting agriculture worldwide is salinity stress. Since strawberry is a salt-sensitive plant, it is easily affected by a mild or moderate level of salinity. A very low level of NaCl could significantly reduce the crop yield and quality of fruits.

In this study, strawberry was grown under differing applications (control, 50 mmol L^{-1} NaCl, 50 mg L^{-1} HA, 50 mmol L^{-1} NaCl + 50 mg L^{-1} HA). Strawberry seedlings in NaCl salinity were negatively affected in terms of physiological and biochemical parameters. Defending plants synthesized various stress metabolites such as proline, MDA, POX and CAT antioxidant enzymes to ease the negative effects of NaCl toxicity. However, increases of these metabolites were negatively correlated with the quality-related metabolites such as vitamin C and lycopene contents. HA applications reduced the concentrations of stress metabolites and antioxidant enzyme levels and contributed to increases of vitamin C and lycopene contents indirectly in strawberry plants. This study showed that HA application might be an effective approach to increase the yield production and quality of strawberry fruits. We suggest that use of HA in strawberry plants is a practical approach where salinity is prevalent.

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