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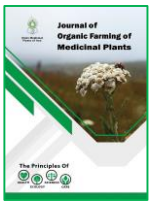

Original Article

Foliar application of humic acid and iron and zinc nano chelates alleviate the salinity damage on chicory (*Cichorium intybus* L.) in hydroponic culture

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ABSTRACT

Chicory has a rich nutritional composition and is a rich source of proteins, vitamins and minerals. Salinity stress can affect its growth and quality to some extent and it is very important to provide the solution to deal with it. For this intention, a factorial experiment was administered in the form of a completely randomized design in three replications on chicory (*Cichorium intybus* L) in Karaj, Iran, in September 2022. The treatments consisted humic acid [0 (control), 500, 1000 mg l^{-1}], micronutrient [0 (control), iron nano chelate and zinc nano chelate], and salinity stress [without stress (control), 30 mM NaCl]. The results confirmed that salinity stress decreased the yield of chicory. Increasing the concentration of humic acid and also the usage of micronutrients increased the yield and qualitative yield of the plants. Humic acid and micronutrients diminished the negative effects of salinity stress. The highest plant yield (679.54 g m^{-2}) was related to interaction of humic acid 1000 mg l^{-1} + iron without salinity stress, and also was observed in humic acid 1000 mg l^{-1}+ zinc spraying (681.15 g m^{-2}). The lowest value of this index (335.97 g l^{-1}) was observed in salinity conditions and control. Salinity stress had the greatest proline content (0.328 mg g FW^{-1}), and the lowest proline content (0.088 mg gFW^{-1}) was seen in humic acid 1000 mg l^{-1} + iron. Therefore, for improving plant yield and resistance to salt stress, humic acid and micronutrient spraying were recommended.

1. Introduction

Nowadays, medicinal plants are the base economic plants that are used and exploited in traditional and modern medicine in raw or processed form. The method of management in the production of these plants is very important. Chicory (*Cichorium intybus* L.) is from the Asteraceae family, which has a high amount of selenium, magnesium, potassium, zinc, and calcium. It is also the main source of some vitamins, like vitamin A, vitamin C, and folate. Chicory is very useful for improving digestive system function, liver function and blood purification (Peña-Espinoza et al., 2018; Anju Javed et al., 2020).

Salinity stress is one of the important stresses that can make an impression on plant nutrition in several ways. Saltiness may cause nutrient deficiency or imbalance in the absorption of nutrients. In salinity conditions, plant growth is reduced, because the toxicity of certain ions (sodium and chlorine) and ionic imbalance affects plant metabolism. Salinity stress can disrupt the photosynthesis of plants and cause a decrease in plant performance (Gong et al., 2018; Kumar, 2020). Salinity may have a deduct impresses on plant physiological processes, one of these effects being the increase in oxidative tension, which cause to the shape of reactive oxygen species (ROS) (Sharifi et al., 2020; Nxele et al., 2017). The results of several reports have shown that salt tension diminishes the yield of various plants, including basil (*Ocimum basilicum* L.) (Caliskan et al., 2017), and different ecotypes of mint (*Mentha spp*.) (Hosseini et al., 2021).

Foliar feeding is a method to diminish the consumption of chemical fertilizers and thus alleviate environmental risks. This feeding method provides the elements to the plant in the fastest time and has favorable effects on the quantitative and qualitative indicators of the plant (Alshaal & El-Ramady, 2017). Humic acid is a suitable natural organic fertilizer multiplex that is created from soil organic matter, lignin and peat decomposition. It

attracts multiple ions to form chelates with micronutrients that abandon the continuously and also slowly. Humic acid improves seed germination, plant resistance in stress condition, growth potential of plants, rate and speed of growth, crop quality and finally crop yield (Pang et al., 2021). In this regard, researchers announced that humic acid has increased quality and the yield medicinal herbs like thyme (*Thymus vulgaris* L.) (Sorkhi, 2020), and basil (Boveiri Dehsheikh et al., 2020).

Spraying of micronutrient elements is one of the ways to increase the quality and performance of plants. Micronutrient elements like copper, boron, iron, manganese and zinc are required by plants in small quantities, but they play a prominent function in plant growth. Plant metabolism, nutrient regulation, chlorophyll synthesis, carbohydrate production, fruit and seed growth are among the functions performed by low-use elements (Nayak et al., 2020). Some micronutrients like iron and zinc have been produced in the form of nano fertilizers and these fertilizers can pass through the pores of the leaves much faster and with maximum efficiency due to their very small particle size and be absorbed by the plant tissues (Qureshi et al., 2018). Iron is a micronutrient required by plants for healthy growth because it is a cofactor for vital enzymes in plants. The results of the other research confirmed that the use of micronutrients (iron and zinc) increased the yield of cucumber (*Cucumis sativus*) (Abkar et al., 2022) and chamomile (*Chamaemelum nobile*) (Shahhoseini et al., 2020). Salinity tension is one of the most important reasons for the decline of agricultural products in several regions of the world, especially in Iran, and it is very substantial to find solutions to combat it. Thus, considering the importance of humic acid and micronutrients in improving the yield of chicory and reducing the negative effects caused by salinity stress, this research aims to interrogate the effect of foliar consumption of humic acid, nano-nutrient elements of iron and zinc, and their combined compounds. It was carried out on the growth and yield of chicory medicinal plants under salinity conditions.

2. Materials and Methods

2.1 *Research site, treatments and cultivation of plants*

This experiment was conducted in a factorial base on a completely randomized design in three replications on the chicory medicinal plant (*Cichorium intybus* L.) in a research greenhouse located in Nazar Abad city, Karaj province, Iran, in September 2022. The treatments of this research included humic acid in three concentrations [0 (control), 500 and 1000 mg l^{-1}], application of micronutrient elements in three levels [0 (control), iron nano chelate, zinc nano chelate] and salinity stress in two levels [No stress (control), 30 mM NaCl].

The concentration of nano zinc fertilizer was 12% and the concentration of nano iron fertilizer was 9% from company of Khazra, and with a registration numbers 29936 and 34428, respectively.

Laboratory sodium chloride was consumed to exert salinity stress. Each experimental unit included 5 pots with 60% v/v coco peat and 40% v/v perlite. The chicory genotype was Mazandaran. First, the seedlings were planted in the seedling tray, and after 3 weeks, in the three-leafed seedlings stage were planted in pots (height 12 cm and diameter 15 cm) with the mentioned substrate. Plants are watered with Hoagland's 1/2 solution regularly every day (once a day) (Utmazian et al., 2007). In order to determine the electrical conductivity, the pots were drained and, if necessary, washed with water. Chicory plants were sampled and harvested after 2 months. Salt stress was applied with a Hoagland nutrient solution. The duration of the salt stress was one month. Control plants received only 1/2 Hoagland nutrient solution (without salt). In order to prevent shock caused by salinity stress on the plants, in the first irrigation after the salinity stress started, 20 mM salt was used together with Hoagland's nutrient solution. Once a week, the root environment of the plants was completely washed with distilled water so that the changes in EC and pH resulting from the accumulation of salts in the planting bed due to the washing process were as low as possible. Humic acid and micronutrient treatments were foliar sprayed three times, and the control treatments were also foliar sprayed with distilled water. The first foliar spraying was done 10 days after planting the plants in the pot; the second foliar spraying was done 20 days after planting; and the third foliar spraying was done 40 days after planting.

2.2 Sample collection and measurement

The measured traits included: plant height, plant dry weight, photosynthetic pigments, relative water content, proline content, catalase enzyme activity and ascorbate peroxidase enzyme activity. Plant height was measured with graduated ruler. After harvesting, the plants were dried for 20 days at room temperature (25°C), then the dry weight was measured by using a digital scale.

2.2.1. Photosynthetic pigments and relative water content (RWC)

The method of Arnon et al. (1967) was applied to measure the photosynthetic pigments of leaves, and finally the absorption value was read in the spectrophotometer at a wavelength of 663 nm for chlorophyll a, 647 nm for chlorophyll b, and 470 nm for carotenoid.

The RWC was computed by the equation (1).

$$RWC= \frac{FW-DW}{SW-DW} \times 100 \quad \text{(Eq.1)}$$

In the above equation, FW = leaf fresh weight, DW = leaf dry weight, SW = leaf saturated weight (Ferrat & Loval, 1999).

In the above equation, FW = leaf fresh weight, DW = leaf dry weight, SW = leaf saturated weight (Ferrat & Loval, 1999).

2.2.2 Proline content

Proline content was measured by Bates et al (1973) method. 0.3 g fresh leaves was combined with 5 ml of sulphosalicylic acid 3%, and homogenized. Then centrifuged at 4□C for 10 minutes at 15000 rpm. Finally, read with spectrophotometer (PG Instruments Itd VIS/UV+T model) at 520 nm wavelength and compared with a control sample.

2.2.3 Catalase and ascorbate peroxidase activity

To measure catalase enzyme activity, 1500 µl of sodium phosphate buffer containing 2% PVPP and 1.3 mM EDTA was added to 350 mg of plant tissue and finally, absorbance changes were recorded with a spectrophotometer (PG Instruments Itd VIS/UV+T model) at 240 nm for 3 minutes (Aebi, 1984). Ascorbate peroxidase enzyme was measured from fresh leaves of plants by spectrophotometric (PG Instruments Itd VIS/UV+T model) method (Sairam et al., 1998).

2.3 Date analysis

The data obtained from this research were analyzed using SAS software (Ver. 9.4) and the mean comparison was done using Duncan's multi-range test with a 5 % error probability.

3. Results

3.1. Plant height

ANOVA results for plant height indicated that the effects of salinity stress, humic acid, and micronutrient elements were significant ($p < 0.01$) (Table 1). The results of the mean comparison illustrated that salinity tension decreased the height of chicory plants. Humic acid usage and increasing its concentration raised the height of the plant. Foliar spraying of zinc and iron raised the height of the plant compared to the control (without foliar spraying). In salinity treatments, the highest plant height (42.32 cm) was seen without salinity tension (control). In humic acid treatments, the highest plant height (38.51 cm) was seen in 1000 mg l^{-1} humic acid foliar spray. In micronutrient treatments, the highest plant nutrition (36.82 cm) was seen in iron foliar spray and also (35.17 cm) in zinc spraying (Table 3).

3.2. Dry matter yield

The effect of salinity tension, humic acid and micronutrient elements and their interaction were significant ($p < 0.01$) (Table 1). The results described that salinity stress reduced chicory dry matter yield. The use of humic acid and increasing its concentration raised the yield of chicory dry matter. Foliar spraying of zinc and iron was also associated with an increase in the dry matter yield of the plant compared to the control (without foliar spraying). The highest dry matter yield (679.54 g m^{-2}) was observed in interaction of without salinity stress (control) + foliar application of humic acid 1000 mg l^{-1} + foliar application of iron and also (681.15 g m^{-2}) related to no salinity stress (control) + humic acid spraying 1000 mg l^{-1} + zinc spraying. The lowest one (335.97 g m^{-2}) was related to salinity stress and the control treatments (Fig. 1).

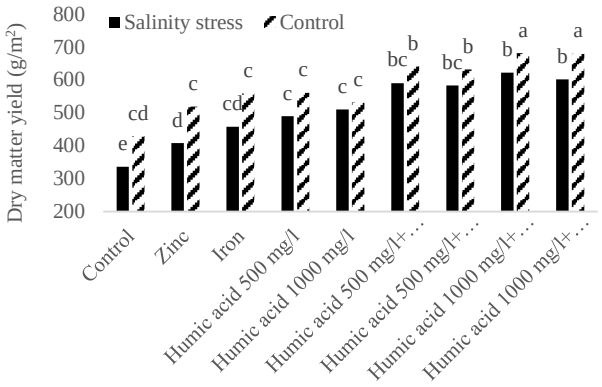

Fig. 1- Interaction effect of humic acid, micronutrient and salinity stress on dry matter yield.

3.3 Photosynthetic pigments

ANOVA results for on chlorophyll (chlorophyll a, b and a+b) showed that the main effect and interaction effect of salinity stress, humic acid, and micronutrient elements were significant ($p < 0.01$) and the main effect of salinity stress, humic acid, and micronutrient elements were significant ($p < 0.01$) on carotenoid (Table 1). The salinity stress reduced the photosynthetic pigments. Fertilization of zinc and iron, as well as the humic acid usage and increasing its concentration constructed an increase in photosynthetic pigments. The highest chlorophyll a (0.99 mg g^{-1} FW) was seen in interaction of without salinity stress (control) + foliar spraying of humic acid 1000 mg g^{-1} FW+ foliar spraying of iron and also was seen in no salinity stress (control) + humic acid 1000 mg g^{-1} FW+ iron (0.98 mg g^{-1} FW). The lowest value of this index (0.25 mg g^{-1} FW) was seen in stress conditions and the control treatments (Fig. 2). The highest chlorophyll b (0.51 mg g^{-1} FW) was observed in without salinity stress (control) + foliar application of humic acid 1000 mg l^{-1} + application of iron, and also was seen in no salinity stress (control) + humic acid 1000 mg.l^{-1} + iron (0.51 mg g^{-1} FW). The lowest chlorophyll b (0.14 mg g^{-1} FW) was observed in salinity stress and the control treatments (Fig. 3). The highest total chlorophyll (1.5 mg g^{-1} FW), was related to without salinity stress

(control) + humic acid 1000 mg l^{-1} + iron and was seen in no salinity stress (control) + humic acid 1000 mg l^{-1} + iron (1.49 mg g^{-1} FW).

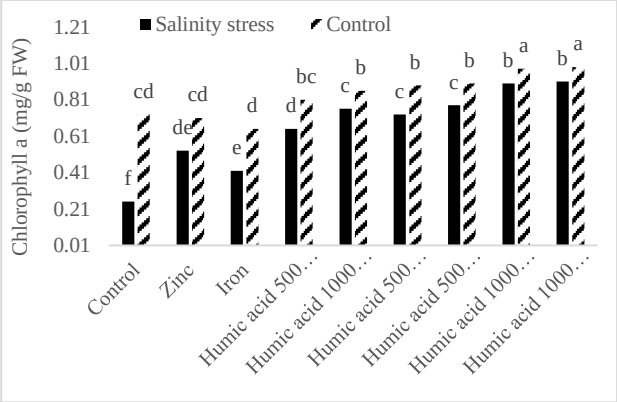

Fig. 2- Interaction effect of humic acid, micronutrient and salinity stress on chlorophyll a.

The lowest one (0.41 mg g^{-1} FW), was seen in the salinity stress and control treatments (Fig. 4). In salinity stress treatments, the highest carotenoid (0.59 mg g^{-1} FW), was observed in non-stress conditions. In humic acid treatments, the highest carotenoid (0.72 mg g^{-1} FW) was seen in humic acid 1000 mg g^{-1}. In micronutrient treatments, the highest carotenoid (0.74 mg g^{-1} FW) was related to iron and also (0.73 mg g^{-1} FW) was seen in zinc spraying (Table 3).

3.4 Relative water content (RWC)

ANOVA results for RWC indicated that the effect of salinity stress, humic acid, and micronutrient elements

Table 1. Results of variance of analysis for plant height, dry matter yield, chlorophylls and carotenoid in chicory under salinity stress, humic acid and micronutrients.

d.f	S.O.V	Mean of squares (MS)					
		Total chlorophyll	Chlorophyll *b*	Chlorophyll *a*	Dry matter yield	Plant height	Carotenoid
Salinity stress (S)	1	52.19**	24.35**	12.21**	719.53**	156.26**	82.75**
Humic acid (H)	2	74.53**	19.72**	18.56**	826.44**	445.97**	64.29**
Micronutrient (M)	2	93.21**	31.56**	23.85**	379.56**	576.52**	101.52*
S×H	2	66.37**	29.31**	16.19**	616.21**	34.09 $^{n.s}$	2.35$^{n.s}$
S×M	2	82.36**	13.53**	31.02**	942.70**	27.65 $^{n.s}$	8.21$^{n.s}$
M×H	4	94.75**	18.95**	28.71**	1256.31**	16.38 $^{n.s}$	2.98$^{n.s}$
S×M×H	4	112.68**	21.39**	35.98**	1729.83**	32.69$^{n.s}$	9.12$^{n.s}$
Error	36	3.43	1.82	2.35	18.46	39.53	13.81
CV (%)			7.95	8.13	7.49	11.21	13.42

ns, * and ** indicate insignificant and the significant differences between traits at P-value<0.05 and P-value<0.01., respectively

Table 2. Results of variance of analysis for ascorbate peroxidase, catalase, proline and relative water content (RWC) in chicory under salinity stress, humic acid and micronutrients.

d.f	S.O.V	Mean of squares (MS)			
		Ascorbate peroxidase	Catalase	Proline	RWC
Salinity stress (S)	1	82.21**	7.83**	69.36**	976.21**
Humic acid (H)	2	65.36**	2.29**	74.21**	1762.85**
Micronutrient (M)	2	81.37*	3.50*	56.73*	2016.21**
S×H	2	12.72$^{n.s}$	0.21$^{n.s}$	63.80**	52.79$^{n.s}$
S×M	2	14.21$^{n.s}$	0.75$^{n.s}$	97.21**	66.43$^{n.s}$
M×H	4	9.86$^{n.s}$	0.52$^{n.s}$	52.75**	71.25$^{n.s}$
S×M×H	4	8.71$^{n.s}$	0.43$^{n.s}$	69.83**	83.76$^{n.s}$
Error	36	21.36	1.59	8.95	97.42
Coefficient of variation (%)		10.28	6.72	9.35	14.21

ns, * and ** indicate insignificant and the significant differences between traits at P-value<0.05 and P-value<0.01., respectively

Table 3. Mean comparison of the effects of salinity stress, humic acid and micronutrients on plant height, carotenoid, relative water content (RWC) and enzymes activity

Treatments	Plant height (cm)	Carotenoid (mg g FW^{-1})	RWC (%)	Catalase (µmole FW min^{-1})	Ascorbate peroxidase (µmol H_2O_2 min^{-1} mg^{-1} protein)
Salinity stress					
Control	32.42 a	0.45 b	72.41 a	0.014 b	0.32 b
30 mM	20.76 b	0.59 a	45.32 b	0.017 a	0.79 a
Humic Acid					
1000 mg l^{-1}	38.51 a	0.72 a	74.21 a	0.018 a	0.38 c
500 mg l^{-1}	30.24 b	0.61 b	69.32 b	0.015 b	0.46 b
Control	25.79 c	0.43 c	51.46 c	0.011 c	0.55 a
Micronutrients					
Iron	36.82 a	0.74 a	71.56 a	0.014 a	0.39 b
Zinc	35.17 a	0.73 a	65.82 b	0.015 a	0.42 b
Control	27.35 b	0.51 b	53.24 c	0.011 b	0.51 a

were significant ($p< 0.01$) (Table 2). Salinity stress decreased RWC. Foliar spraying of zinc and iron, as well as the humic acid usage and increasing its concentration increased RWC. In salinity stress treatments, the highest RWC (72.41%) was observed in non-stress conditions. In humic acid treatments, the highest RWC (74.21%) was seen in humic acid 1000 mg l^{-1} and (71.56%) related to iron foliar spraying (Table 3).

3.5 Proline content

The effect of salinity stress, humic acid, and micronutrient elements were significant ($p< 0.01$) (Table 2). Salinity stress increased proline content and foliar spraying of zinc and iron, as well as the application of humic acid and increasing its concentration decreased proline content. The highest proline content (0.328 mg g^{-1} FW) was seen in salinity tension and the lowest one (0.088 mg g^{-1} FW) was seen in without salinity stress (control) + humic acid foliar spraying 1000 mg l^{-1} + iron foliar spraying (Fig. 5).

3.6 Enzymes (catalase and ascorbate peroxidase) activity

ANOVA results for enzymes indicated that the effects of salinity stress, humic acid, and micronutrient elements were significant ($p<0.05$) (Table 2). Salinity stress increased ascorbate peroxidase. Foliar spraying of zinc and iron, as well as the application of humic acid and increasing its concentration improved catalase activity and decreased ascorbate peroxidase activity. In humic acid treatments, the highest catalase activity (0.018 µmole FW min^{-1}) was observed in humic acid 1000 mg.l^{-1}. In micronutrient treatments, the highest catalase activity (0.014 µmole FW min^{-1}) was seen in iron foliar spraying and (0.015 µmole FW min^{-1}) was related to zinc foliar spraying. In salinity stress treatments, the highest ascorbate peroxidase (0.79 µmol H_2O_2 $min^{-1}mg^{-1}$ protein) was seen in salinity stress. In humic acid treatments, the highest ascorbate peroxidase (0.55 µmol H_2O_2 $min^{-1}mg^{-1}$ protein) was seen in control. In micronutrient treatments, the highest ascorbate peroxidase (0.51 µmol H_2O_2 $min^{-1}mg^{-1}$ protein), was related to control (Table 3).

4. Discussion

Humic acid usage and micronutrients raised the yield of dry matter and plant height, which can be said to be due to the attendance of hormones in humic acid and the creation of suitable growth conditions, as well as the main role of iron in increasing photosynthesis and chlorophyll synthesis. In this study, the dry weight was reduced with the application of salt tension, which can be due to the absorption of water from the pot to the leaves. As a result, the content of water in the leaf cells and then the leaf surface decreases, and these factors lead to a decline in photosynthetic activity and finally a decrease in the dry weight of the plant (Sorkhi, 2020; Boveiri Dehsheikh et al., 2020).

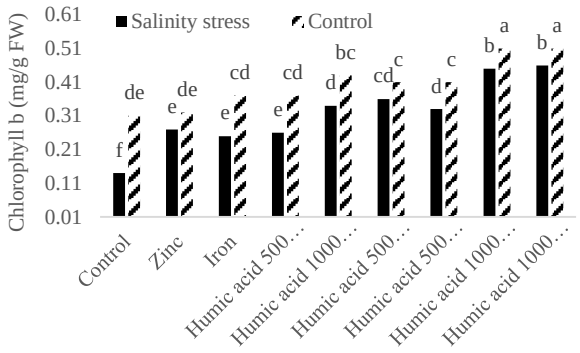

Fig. 3- Interaction effect of humic acid, micronutrient and salinity stress on chlorophyll b

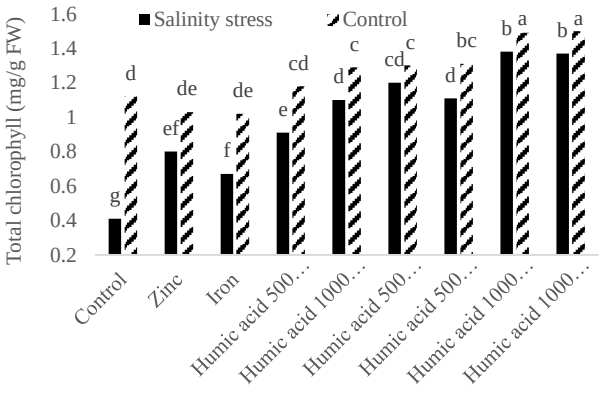

Fig. 4- Interaction effect of humic acid, micronutrient and salinity stress on total chlorophyll

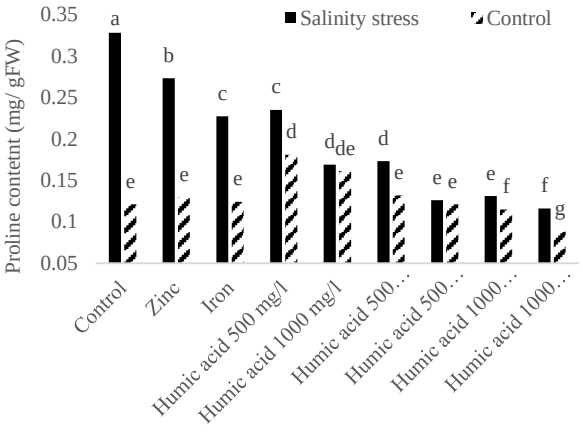

Fig. 5- Interaction effect of humic acid, micronutrient and salinity stress on proline content

The reaction of plants to salinity conditions is different, depending on the grade of ionic toxicity, variations in osmotic potential, terms of stress and the type of plant species (Taïbi et al., 2016; Arora et al., 2020). With the increase in salinity tension in plants, the content of

carotenoids increases. Salinity stress causes oxidative degradation in plant tissues, and the activity of carotenoids in enzymatic and non-enzymatic antioxidant systems reduces the amount of this demotion (Arora et al., 2020). Chlorophyll pigments play an important effect in photosynthesis. Chlorophyll quantity is considered an impressive indicator for monitoring the activity of photosynthesis in plants (Taïbi et al., 2016). Thus, by evaluating the amount of photosynthetic pigments in diverse salinity situations, the salinity tension effect on photosynthetic function can be figured out to some extent (Xu et al., 2017; Taïbi et al., 2016). One of the factors that reduces the amount of chlorophyll during salt stress is the competition and overtaking of glutamine kinase enzyme (the first enzyme of the proline synthesis pathway) during stress by the glutamate ligase enzyme (the first enzyme of the chlorophyll synthesis pathway), which causes glutamate (the common precursor of chlorophyll and proline synthesis) to consume more proline and therefore chlorophyll synthesis will be limited (Mosavi et al., 2018). The results showed that the humic acid usage raised the activity of enzymes (ascorbate peroxidase and catalase). The increase of antioxidant under the influence of humic acid indicates that humic acid causes faster construction of catalase and ascorbate peroxidase from its precursor (superoxide and a peptide called dismutase and porfibrin) (Dadnia, 2017). Raising enzymes activity through foliar application of micronutrients can also be due to the structural role of zinc in enzymes and its direct effect in gene expression and protein production (Pandey et al., 2012). Ascorbate, which is found in high concentrations in chloroplasts and other cell components, is very important in the defense mechanisms of plants during oxidative stress. Ascorbate peroxidase is an important enzyme that helps control ROS in stressed plants. This enzyme converts H_2O_2 to H_2O using ascorbate as a reducing agent (Zelm et al., 2020).

Salinity tension that has an effect on the relative water content (RWC) of plant leaves, so that with the increase in the multiplicity of these stresses, the RWC of leaves decreases. A decrement in the RWC of plant leaves indicates a decrease in turgor, which reduces the amount of water required for morpho physiological processes such as the opening of stomata, cell elongation, and photosynthesis (Khan et al., 2015). Humic acid and iron and zinc micronutrients nano chelates improved the RWC under control and stress condition. Humic acid is believed to have an important role in promotion of plant growth as a biostimulation. It can induce alteration in plant primary and secondary metabolism linked to abiotic stress tolerance, which leads to improved plant growth and increased resistance against abiotic stress (Amer et al., 2021).

The accumulation of proline can be attributed to the maintenance of osmotic balance at the cellular level in many plants grown under salt stress treatments. The cell responds to long-term and short-term salinity stress by synthesizing and accumulating osmotic protective compounds such as proline. These compounds are small and non-toxic molecules that increase the osmotic potential of the cell. The increase in proline caused by the amount of sodium chloride can be explained by the fact that glutamate pathway enzymes are activated under a sodium chloride salinity situation, and proline synthesis increases because sodium chloride stimulates the genes that synthesize these enzymes (Dehghan et al., 2018). Proline declines the cytoplasmic osmotic potential that facilitates water absorption under salt stress condition. In addition to osmotic adjustment, proline has a major role in maintaining the integrity of the plasma membrane, preventing destruction of enzymes and proteins, and scavenging ROS (Dong et al., 2015). The results were consistent with the research of other researchers, they stated that the application of salinity stress and its increase made increase the proline content in coneflower (*Echinacea angustifolia* L.) (Azadbakht et al., 2020).

5. Conclusion

The results of this study illustrated that the NaCl stress reduced the quantitative and qualitative yield of chicory medicinal plants. Humic acid usage and increasing its concentration, as well as the use of iron and zinc micronutrients nano chelates, performed better than control plants under NaCl stress in terms of plant height, dry matter yield, RWC, catalase activity and also the photosynthesis pigments of chicory. In addition, these treatments increased the resistance of chicory to salinity stress, which is believed to be the reason for this increase in the plant's access to nutrients, which made the plant better able to withstand the stress conditions. Integrating humic acid and iron and zinc micronutrients nano chelates can offer several benefits, pointing to the amelioration of NaCl-induced stress development, and physiological adjustment of the chicory. Therefore, humic acid (1000 mg l^{-1}), iron and zinc nano chelates spraying are suggested for improving chicory yield and quality in greenhouse conditions.

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