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Physiological Responses of Squash (*Cucurbita pepo* L.) to Humic Acid Treatment under NaCl Stress Conditions

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الملخص:

عُومِلَتْ نباتات الكوسة (*Cucurbita pepo* L. cv. Alexandria F1) بمستويات مختلفة من الدبال مصدراً لحمض الهيوميك (HA) وكلوريد الصوديوم (NaCl) مصدراً للملوحة مع مياه الري لدراسة تأثير HA على النمو والعمليات الفسيولوجية تحت ظروف الملوحة. استُخْدِمَتْ ثلاثة مستويات من HA، 0، 200 و400 جزء في المليون مع أربعة مستويات للملوحة: 500 (مياه عذبة)، 1000، 2000 و 3000 جزء في المليون أُعِدّتْ بإضافة 0، 500، 1500 و2500 جزء في المليون من NaCl في الماء العذب. تم قياس الوزن الطازج (FWL) والوزن الجاف (DWL) ووزن الورقة النوعي (SLW) ومحتوى الماء في منتصف النهار (WC) والمحتوى المائي النسبي (RWC) للأوراق لكل معاملة في نهاية التجربة. حُدِّدتْ محتويات الأوراق من النيتروجين (N)، والفوسفور (P)، والبوتاسيوم (K^+)، والصوديوم (Na^+) والكلوريد (Cl^-)، وكذلك نسبة البروتين ومحتوى الكلوروفيل a، b والإجمالي في الأوراق. أدت المعاملة بـ HA بتركيز 400 جزء في المليون إلى الحفاظ على FWL و DWL ، كما منع انخفاض WC للأوراق تحت ظرف الإجهاد بـ NaCl، بينما لم يكن هناك تأثير واضح للمعاملات على قيم SLW و RWC. خفض HA من التأثير السلبي للملوحة على محتوى الأوراق من N و P ، بينما زاد محتوى K^+ ونسبة K:Na. حافظ HA على تركيز أيونات الصوديوم، حيث كانت هناك زيادة ملحوظة في محتوى الأوراق من ايوناته مع زيادة تركيز NaCl المطبق، وبدون أي تأثير لمعاملة HA المشار عنها في هذا الصدد. أدى HA إلى تعزيز شدة تأثير NaCl وتأثيره المخفض على محتوى أوراق الكلوروفيل a، b والإجمالي ونسبة البروتين. رفع HA من قدرة نبات الكوسة على الحفاظ على نسبة WC وتحسين امتصاص العناصر الغذائية وكذلك وقف الزيادة في أيونات الصوديوم في أنسجة النبات. من ناحية أخرى ، لم تنجح معاملة HA في منع زيادة Cl^-، ونتيجة لذلك سُجِّلَ انخفاض في محتوى الكلوروفيل والبروتين في أنسجة الأوراق، وبالتالي سيكون هناك انخفاض عام في النمو والتطور الكلي للنبات.

الكلمات المفتاحية:

الكوسة؛ *Cucurbita pepo* L.؛ حمض الهيوميك؛ NaCl؛ ملوحة.

Abstract

Squash plants (*Cucurbita pepo* L. cv. Alexandria F1) were subjected to different levels of humus as source of humic acid (HA) and NaCl as source of salinity with irrigation to investigate the effect of HA on growth and physiological processes under salinity condition. Three levels of HA: 0, 200 and 400 ppm were used with four levels of salinity: 500 (fresh water), 1000, 2000 and 3000 ppm were prepared by adding 0, 500, 1500 and 2500 ppm of NaCl in fresh water. Fresh weight (FWL), dry weight (DWL), specific leaf weight (SLW), midday water content (WC) and relative water content (RWC) of leaves were measured for each treatment at the end of experimentation. Leaf contents of nitrogen (N), phosphorus (P), potassium (K^+), sodium (Na^+) and chloride (Cl^-), as well as percentage of protein and content of chlorophyll a, b and total in leaves were determined. The treatment with HA at a concentration of 400 ppm led to maintaining the FWL and DWL, also prevented the decrease in WC of the leaves under the condition of NaCl stress. While there was no apparent effect of treatments on the values of the SLW and RWC. HA reduced the negative effect of salinity on the leaf content of N and P, whereas increasing the content of K^+ and K:Na ratio. HA has maintained the concentration of Na^+ ions, and there was a noticeable increase in the leaf content of Cl^- ions with an increase in the applied NaCl concentration, without any effect of HA treatment reported in this regard. HA led to enhance the severity of NaCl reduced effect on leaf content of chlorophyll a, b and the total and percentage of protein. HA has increased the ability of squash to maintain the WC percentage and improving the nutrients uptake as well as stopped the increase in Na^+ ions in the plant tissue. On the other hand, HA treatment was unsuccessful in preventing the increase in Cl^-, consequence a decrease in the content of chlorophyll and protein in leaf tissue has been registered, thus there will be a general reduction in overall growth and development of the plant.

Keywords: Squash; *Cucurbita pepo* L.; Humic acid; NaCl; Salinity.

1. INTRODUCTION

High salinity in the irrigation water is one of the widespread problems that leads to the influence of large areas of agricultural land due to the phenomenon of salt accumulation (salinization of soil), which leads to poor soil construction and low nutrients uptake for the growth and development of plants. Squash (*Cucurbita pepo* L.) is one of the most important vegetable

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crops of the Cucurbitaceae family. It is a summer crop that can be grown most of the year. Most of vegetable crops are usually irrigated with well water, which usually suffer from the salinity problem and thus lead to a negative impact on plants.

Salinity affects plants by decreasing water absorption due to increased salt concentration and high osmotic pressure in the absorption medium[1]. The high salinity of irrigation water resulted in a decrease in the relative water content (RWC) of the squash plant[2]. In addition, effect of saline stress on the cucumber plants was resulted in a decrease in the relative growth rate (RGR) and RWC[3]. The osmotic capacity of cells was significantly affected by increasing salinity from 50-75 to 100 mM on cucumber plants[4]. Also, the pumpkin's leaf content of chlorophyll a, b and total decreased when treated with concentrations of 5 to 10 g/L of NaCl, while the largest values were presented with a concentration of 2.5 g/L[5]. The exposure of squash to salt stress, especially when the salinity concentration increased to 100 mM resulted in a decrease in the leaf content of K^+ and a decrease in Na:K ratio, as well as increased content of Na^+ in the plant[6]. On the other hand, found that salinity has affected the squash plants, and reduced the content of NPK, while its Cl^- and Na^+ ions content increased with increased salt stress[7]. Some studies reported that increasing salt stress concentration from 50-75 to 100 mM on cucumber led to lower protein content[4,8].

The current and expected future water deficit in the world requires the use of means that increase production to bridge the food gap and mitigate the damage caused by irrigation with highly saline water on agricultural soils. Among the methods used is to add humic acid, whether mixed with soil or add it with irrigation water, Humic acid (HA) is an organic acid that is produced naturally as a result of biological and chemical processes from the decomposition of plant and animal wastes by microorganisms[9]. Addition of HA to the soil increases the absorption of nutrients by the plant such as nitrogen, phosphorus, calcium and magnesium[10]. HA also helps to overcome the harmful effect of salt stress by its association with sodium and helping the plant to tolerate high concentrations of it and protect against the impact of toxicity and the osmotic problems associated with these high concentrations[11]. The addition of HA has increased the water content (WC) and the RWC of radish leaves[12]. Also, the studies found that adding HA has increased the leaf content of chlorophyll for squash[13]. A study suggested that the treatment of squash plants with HA and nitrogen fertilizer under conditions of saline stress has increased the benefit of the plant to nitrogen, and thus increased the percentage of NPK, while the percentage of Cl^- and Na decreased as a result of treatment with HA[14]. In addition, adding HA has increased the percentage of protein in cucumber fruits[15].

The aim of this study was to find out the effects of HA by adding humus solution in saline irrigation water to study whether the effect of HA plays a role in reducing the salinity negative effect by inducing physiological responses in squash plant.

2. MATERIALS AND METHODS

The experiment was carried out by cultivating the commercial variety "Alexandria F1" of the squash plant (*Cucurbita pepo* L.) in the research station (Faculty of Agriculture - University of Benghazi - Libya), seeds were sawing in a substrate consisting a 1:1:1 ratio of soil, sand and an organic matter in 30 liters of plastic bags, a fertilization program has been applied by addition the essential nutrients during the growth and development stages of the plant.

Plants were grown under a plastic canopy to avoid the reach of rainfall in case of precipitation, at 14h photoperiod, photosynthetic active radiation reached a daytime peak value of 1200 $\mu mol.m^{-2}.s^{-1}$, the temperature and relative humidity ranged to 30/18°C and 40/75% during day/night periods, respectively.

Treatments were initiated when the plants reached the first leaf stage with different concentrations of salinity and humic acid (HA) in irrigation water. The irrigation was daily carried out with; 4 levels of salt water, fresh water (the concentration of salts is about 500 ppm), 1000, 2000 and 3000 ppm, prepared by adding 0, 500, 1500 and 2500 ppm of NaCl in fresh water, and; 3 levels of humus solution 0, 200 and 400 ppm of HA. Amount of irrigation was adding according to the need of the plant and the change in the daily temperature with respecting the increase in the size of the plant in terms of the gradual increase in the amount of irrigation water needed by the plant to reach the field capacity at each irrigation. Treatments continued until the beginning of the flowering stage.

Fresh weight of leaves (FWL) were measured for each treatment at the end of the experiment. Then the dry weight (DWL) of the leaves was also measured by dried them for the period of three days in an oven adjusted at 65 °C (until there was no decrease in weight). The midday water content (WC) and the relative water content (RWC) were measured by using leaves, that were immediately weighed to obtain a leaf fresh weight. Leaves with the petioles were placed in a beaker submerged with water for overnight at a condition of dark and 4 °C, so leaves could become fully hydrated. Then leaves were reweighed to obtain turgid weight and dried at 70 °C for 3 days to obtain dry weight. The WC was calculated as $[(FW–DW)\times FW^{-1}]\times 100$, while the RWC was calculated as $[(FW–DW)\times(TW–DW)^{-1}]\times 100$ according to Morgan[16]. Where FW is the leaf fresh weight; TW is the turgid weight; and DW is the dry weight. The specific leaf weight (SLW) was determined by dividing values of leaf dry weight by leaf area.

Leaves of four plants were washed with distilled water to remove dust and other residues, and dried in an oven at 65°C for 3 days to determine dry weights. The dried tissues were finely ground and stored in paper bags. After digestion of ground tissue with H_2SO_4 and $HClO_4$, sodium (Na^+) and potassium (K^+) contents in the DW were measured by flame photometer, meanwhile Phosphorus (P) was determined choloremeterically, while those of chloride ($Cl^–$) were determined by titration with $AgNO_3$ in the presence of NaCl, according to AOAC[17]. The total content of nitrogen (N) and percentage of protein in leaves were determined by the micro-Kaeldahl method. Also content of chlorophyll a, b and total were determined choloremeterically according to Moran[18].

The data presented are representative the mean of two independent experiments. The experiment was conducted in four replicates, using factorial experimental 4×3 in completely randomized design, with the treatments of salinity as the first factor and humic acid as the second factor. Data were subjected to analysis of variance using a two-way ANOVA. Differences among means of treatments were compared by Duncan's multiple range test at the 0.05 confidence level.

3. RESULTS AND DISCUSSION

The treatment with the highest concentration of NaCl (3000 ppm) resulted in a decrease in the FWL and DWL, while the treatment with HA at a concentration of 400 ppm resulted in maintaining the weight of the leaves under the condition of saline stress (Table 1.). It was evident that there was no apparent effect of treatment with HA under stress condition with NaCl on the values of the SLW and RWC, when a significant decrease in the value of WC under the effect of stress with NaCl appeared and that adding HA has prevented this decrease and produced comparative values with control treatment (Table 1.).

Table 1. Effect of salinity (NaCl) and humic acid (HA) treatments (ppm) on fresh weight of leaves (FWL), dry weight of leaves (DWL), leaf specific weight (LSW), water content (WC) and relative water content (RWC) of squash plants.

Salinity	HA	FWL	DWL	LSW	WC	RWC
		(g)		(g/cm^2)	(%)	
Fresh water	0	279^{abc}	35^{a}	0.040^{a}	85^{ab}	93^{a}
	200	304^{a}	35^{a}	0.039^{a}	87^{a}	92^{a}
	400	308^{a}	36^{a}	0.040^{a}	85^{ab}	90^{a}
1000	0	275^{abc}	34^{ab}	0.039^{a}	85^{ab}	90^{a}
	200	294^{ab}	34^{ab}	0.040^{a}	85^{ab}	93^{a}
	400	305^{a}	36^{a}	0.039^{a}	86^{a}	91^{a}
2000	0	251^{cd}	33^{abc}	0.038^{a}	83^{b}	90^{a}
	200	271^{abc}	33^{abc}	0.039^{a}	85^{ab}	92^{a}
	400	259^{bc}	33^{abc}	0.037^{a}	86^{a}	90^{a}
3000	0	218^{d}	27^{c}	0.038^{a}	83^{b}	93^{a}
	200	257^{bcd}	30^{bc}	0.042^{a}	86^{a}	92^{a}
	400	268^{abc}	33^{abc}	0.038^{a}	86^{a}	90^{a}

Each value represents mean of four replicates (plants). Means followed by the same letter in each column are not significantly different by Duncan's multiple range test at 5% level

Treatment with HA reduced the negative effect of stress with NaCl on the leaf content of N and P, as the leaves retained a percentage of their nutrient content despite the high level of NaCl applied (Table 2.). Contrary to what was presented, treatment with HA led to a clear increase in the leaf content of K^+ and regardless of the effect of saline stress applied with NaCl treatment(Table 2.). The treatment with HA did not lead to a reduction in the leaves content of the Na^+ ions under normal conditions, but on the other hand it led to maintaining its concentration to levels similar to that of the control treatment under the combined effect of HA and NaCl treatments (Table 2.). In contrast, the ratio of K:Na increased with treatment at a concentration of 400 ppm of HA at all stress levels with NaCl applied in this experiment (Table 2.). On the other hand, there was a noticeable increase in the leaf content of Cl^- ions with an increase in the applied NaCl concentration and without any effect of HA treatment reported in this regard (Table 2.)

Table 2. Effect of salinity (NaCl) and humic acid (HA) treatments (ppm) on content of nitrogen (N), phosphor (P), potassium (K^+), sodium (Na^+), chloride (Cl^-) and K:Na ratio in leaves of squash plants.

Salinity	HA	N	P	K^+	Na^+	Cl^-	K:Na
		(%)		(mg/g)			
Fresh water	0	4.0^{a}	3.5^{a}	73^{d}	0.7^{cde}	24^{g}	102^{b}
	200	4.0^{a}	3.5^{a}	90^{a}	0.8^{b-e}	26^{g}	116^{b}
	400	4.0^{a}	3.7^{a}	88^{ab}	0.7^{cde}	26^{g}	123^{b}
1000	0	3.6^{bc}	3.4^{a}	77^{cd}	0.9^{a-d}	30^{b-g}	88^{bcd}
	200	3.8^{ab}	3.5^{a}	78^{bcd}	0.6^{de}	28^{c-g}	122^{b}
	400	3.9^{a}	3.7^{a}	83^{a-d}	0.5^{e}	32^{b-g}	165^{a}
2000	0	3.5^{bc}	3.3^{a}	59^{e}	1.0^{abc}	40^{abc}	59^{cd}
	200	3.3^{c}	3.3^{a}	83^{a-d}	1.0^{abc}	36^{a-e}	80^{cd}
	400	4.0^{a}	3.6^{a}	83^{a-d}	0.8^{b-e}	36^{a-e}	100^{b}
3000	0	3.4^{c}	2.6^{bc}	56^{e}	1.2^{a}	47^{a}	49^{cd}
	200	3.4^{c}	2.8^{b}	80^{a-d}	0.9^{a-d}	42^{ab}	90^{bc}
	400	4.0^{a}	3.2^{ab}	84^{abc}	0.7^{cde}	39^{a-d}	114^{b}

Each value represents mean of four replicates (plants). Means followed by the same letter in each column are not significantly different by Duncan's multiple range test at 5% level.

The stress of NaCl reduced the leaf content of chlorophyll a, b and the total, however, the addition of HA enhanced the severity of this reduced effect to level of about 50% (Figure 1.). Although an increase in the leaf content of the protein occurred with HA treatment under normal conditions, this increase did not appear when applying the stress with NaCl and consequently led to a reduction in the percentage of protein in the leaves (Figure 1.).

Numerous relevant research indicates physiological changes within plant tissues under the condition of saline stress and the addition of HA, so it is important to focus on the effects of these reactions on plant growth and development. It is also important to know the nature of these events in relation to the nutrients, their movement, and the tissue content of the active substances to enhance the growth and development process in the plant. The effect of saline stress, especially of NaCl, has reduced the growth and development of most plants, which is usually mentioned in many studies[19,20]. On the other hand, treatment with HA has led to positive effects on both the vegetative and reproductive growth of squash and others[21,22,23]. These positive effects of treatment with HA, regardless of how it was added to the plant, had an important role in alleviating the severe effect of saline stress[13]. There are several ideas that have been proven by many previous researches[24], stating that the interaction of the vegetative part of growth with the effect resulting from the addition of any material to the plant, this may have a kind of independence from what is happening in the growth and development of the reproductive part of the plant.

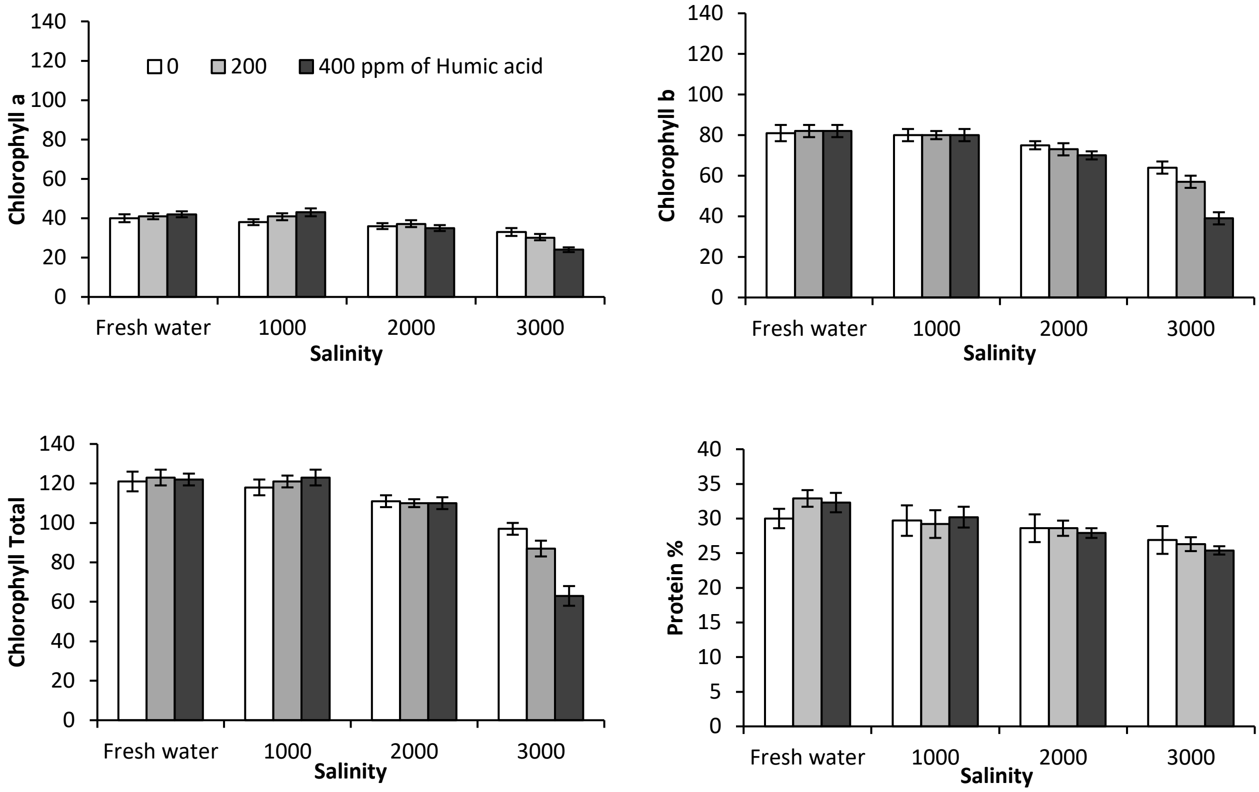

Figure 1. Effect of salinity (NaCl) and humic acid (HA) treatments (ppm) on content of chlorophyll a, chlorophyll b, chlorophyll total (mg.100g^{-1} FW) and protein (%) in leaves of squash.

In this experiment, revealed that the treatment with 400 ppm of HA added to irrigation water led to maintaining both fresh (FWL) and dry (DWL) weights of squash leaves under the stress condition with NaCl (Table 1.). Although the effect of the squash plant treatment with NaCl did not change in some measurements such as LSW and RWC, the values of WC were clearly affected and a decrease in their value occurred. However, when treating with HA, it led undoubtedly to the fact that the percentage value of WC in squash leaves was not affected under the applied stress condition and regardless to the size of the resulting plant (Table 1.). It is recognized that the state of maintaining the WC percentage in the leaves of the plant is reflected in other measurements that support the process of growth and development in the plant, where the studies pointed to an improvement in a group of vital processes in the plant as a result of the treatment with HA, especially nutrients uptake and the occurrence of an osmotic adjustment[25]. Therefore, we found that the leaf content of N and P was preserved from the reduced effect of their content in the leaf tissue under salinity conditions, as well as the emergence of a significant increase in the leaf content of K^+ due to the effect of adding HA (Table 2.).

The appropriate leaf tissue content of macronutrients (NPK) appears to be necessarily sufficient to show improvement in plant growth and development such as leaf weight (Table 1). Other elements provided by exposing the plant to the conditions of saline stress, such as Na^+ and Cl^-, play a role in the viability state of the plant through its physiological and toxic effects. Although there was no significant difference in the leaf content of sodium ions under the condition of interference between saline stress and treatment with HA, this is a positive result as it stopped for increased Na^+ content due to the application of saline stress. HA helps to overcome the harmful effect of saline stress by its association with Na^+, helping the plant to tolerate high concentrations of it and protecting against the toxic effect and the osmotic problems associated with these high concentrations[26]. As a result of these responses in terms of the level of K^+ and Na^+ content, a desired improvement in the K:Na ratio within the tissue of the leaf necessarily occurs (Table 2). The availability of sufficient levels of macronutrients in the leaves and not increasing their Na^+ content may have played the most important role in maintaining the biomass of the leaves as a result of adding HA under the condition of saline stress, as well as neutralizing its toxic role. The positive effects in the vegetative part did not have a clear response in many times in the reproductive part of the plant, perhaps the inability of the HA concentrations applied in this experiment was not sufficient to cause a decrease in the leaf content of chloride ions, but a clear increase occurred due to the effect of the treatment with NaCl on squash plants (Table 2).

Research has stated that treatment with HA has improved the plant leaf content of chlorophyll[27], but in our experience the levels of HA used have enhanced the decrease in leaf content of chlorophyll (a, b and total) under stress condition with NaCl on squash (Fig. 1.). An acceptable explanation for this process can revolve around the cumulative effect of stress with NaCl and treatment with HA by affecting the tissue content of an amino acid (glycine) which is the main source of chlorophyll synthesis. Perhaps a reduced leaf content of protein (Fig. 1.) under stress conditions with NaCl confirms the above hypothesis through the role of Na^+ or Cl^-, or both in this regard.

It is well known from previous researches confirmations related to increasing the tissue content of the protein when treated with HA[28], as we have to consider the concentrations used of HA in this experiment were not at the optimum level required to cause a fundamental change in improving the growth and development of squash under salinity conditions by enhancing protein content, also we cannot ignore the role of the HA addition method in the plant.

4. CONCLUSION

From this study we conclude that the improvement in the biomass of the leaves was due to the addition of HA under salinity conditions with NaCl. More profoundly, this improvement can be attributed to the role of HA in increasing the ability of squash to maintain the WC percentage and improving the nutrients uptake as well as stopping the increase in Na^+ ions and all the consequences of increasing it in the plant tissue. On the other hand, HA was unsuccessful in preventing the increase in Cl^-, which led to a decrease in the content of chlorophyll and protein in leaf tissue, which will necessarily be reflected relatively in the overall growth and development of the plant.

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