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Exogenously-applied sulphur and ascorbic acid positively altered their endogenous concentrations, and increased growth and yield in *Cucurbita pepo* L. plants grown on a newly-reclaimed saline soil

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Keywords: Squash (*Cucurbita pepo* L.), sulphur, ascorbic acid, salinity, growth, yield.

Abstract

The effects of soil application with sulphur (SAS) at a rate of 500 kg ha^{-1} and/or foliar application with some rates of ascorbic acid (FAA; 0, 1 or 2 mM) on the growth, fruit yield and its components, the concentrations of potassium (K), sodium (Na), sulphur (S) and ascorbic acid (AsA), and the ratio of K/Na in squash (*Cucurbita pepo* L.) plants grown on a newly-reclaimed saline soil were investigated. A 2-season field experiment (2012 and 2013) was performed on a newly-reclaimed saline soil using a design of completely randomized blocks with six treatments, each with four replicates. The results indicated that SAS and/or FAA increased growth traits (i.e., stem length, canopy dry weight $plant^{-1}$, number of leaves $plant^{-1}$, average leaf area and total leaf area $plant^{-1}$), fruit yield and its components (i.e., number of fruits $plant^{-1}$, fruit weight $plant^{-1}$, average fruit weight and fruit yield ha^{-1}), leaf concentrations of K, S and AsA, and K/Na ratio. In contrast, leaf concentration of Na was reduced as a result in SAS and/or FAA. SAS and FAA, therefore, have the potential to be used as a soil and foliar applications, respectively for various crops to overcome the adverse effects of the newly-reclaimed saline soils.

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Introduction

Squash is widely cultivated on newly-reclaimed soils in the Middle Eastern countries, including Egypt. However, most newly-reclaimed soils are affected by salinity and have low fertility and a poor soil structure. The sustainability of crop production is primarily a function of various environmental stress factors, including salinity (Kumar et al., 2009), which is associated with the fertility status of the soil (Sogbedi et al., 2006). Soil fertility is adversely affected by salinity, which has emerged as one of the most serious factors limiting plant growth and productivity, and soil health (Turkan and Demiral, 2009). The loss in plant productivity due to salinity arises as a consequence of an imbalance in ion and nutrient concentrations and osmotic effects (Ashraf, 2009), resulting in the over-production of reactive oxygen species (ROS) compared to their levels in aerobic metabolic processes in chloroplasts, mitochondria, and peroxisomes under normal physiological conditions. The over-production of ROS causes oxidative damages to lipids, proteins and nucleic acids, and affects the properties of cell membranes (Ahmad et al., 2008). The ionic imbalance occurs in the cells due to the excessive accumulation of Na^+ and Cl^-, and the reduction in the uptake of some mineral nutrients such as K^+, Ca^{2+} and Mn^{2+} (Hasegawa et al., 2000; Tester and Davenport, 2003). Salt stress affects plant physiology, both at the whole plant and cellular levels, through osmotic and ionic stress. Salinity generates a 'physiological drought' or osmotic stress by affecting the water relations of the plant (Munns, 2002). Photosynthesis is one of the most severely affected processes under salinity stress (Sudhir and Murthy, 2004), which is mediated by decreased levels of chlorophyll and the inhibition of Rubisco (Soussi et al., 1998). All these and other altered processes lead to poor plant growth and a subsequent loss in productivity. However, plants are well-equipped with antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT), and peroxidase (POX), and non-enzymatic anti-oxidants such as ascorbic acid, glutathione, or carotenoids, to counter any oxidative stress and to protect the plants from oxidative damage (Apel and Hirt, 2004).

Little information is available on the mineral nutrient status of plants and their tolerance to salinity. Among the mineral nutrients, sulphur (S) is increasingly being recognized as the fourth major essential nutrient element after nitrogen (N), phosphorus (P), and potassium (K). S plays an important role not only in the growth and development of higher plants, but is also associated with increased stress tolerance in plants (Nazar et al., 2011; Osman and Rady, 2012). S deficiency negatively affects the chlorophyll and N contents of leaves and photosynthetic enzymes (Lunde et al., 2008), and consequently the reduction in yields and quality parameters of crops (Hawkesford, 2000; Osman and Rady, 2012). Adequate S nutrition improves photosynthesis and the growth of plants, and has regulatory interactions with N assimilation (Scherer, 2008). S is required for protein synthesis, N assimilation, and is a structural constituent of several co-enzymes and prosthetic groups (Marschner, 1995). It is

Received Manuscript 23-November-2013 Accepted Manuscript 30-March-2014

incorporated into organic molecules in plants and is located in thiol (–SH) groups in proteins (cysteine residues) and non-protein thiols (e.g., glutathione). The pool size of some thiol-containing compounds, especially reduced glutathione (GSH) which is sensitive to an oxidizing environment, represents a potential modulator of the stress response (Szalai et al., 2009; Osman and Rady, 2012). Glutathione has been shown to take part in the removal of excess ROS (Noctor and Foyer, 1998), controlling ROS levels (Rausch et al., 2007), and protecting plants from oxidative damage. S was applied to many agricultural areas to ameliorate saline and alkaline soils. In Egyptian soils, which are characterized by a rise in pH, S reduced soil pH values by the oxidation of S to sulphate through various species of soil microorganisms (El-Eweddy et al., 2005; Osman and Rady, 2012). Decreasing soil pH improves the availability of microelements (e.g., Fe, Zn, Mn, and Cu; Hetter, 1985) and improves the chemical properties of alkaline soils as well as increasing yields and related characteristics (Kineber et al., 2004; Osman and Rady, 2012). On the other hand, some trials have been made to alleviate the disturbances in plant metabolism excreted by salinity stress. It has been suggested that some antioxidants, including ascorbic acid (AsA) may help to overcome some of these inhibitory effects. AsA is an important antioxidant defence in plant cells (Foyer and Halliwell, 1976) to protect them by scavenging the ROS. It also stimulates respiration activities, cell division and many enzymes activities (Innocenti et al., 1990). Latterly, there are widespread uses of natural and safety substances such as antioxidants, including AsA for enhancing growth and productivity of many crops. AsA has synergistic effect on plant growth, yield and chemical composition under favourable and unfavourable environmental conditions, i.e. salinity (Rady, 2006; Rady and Migawer, 2010; Osman, 2010).

Since salinity considers a potential threat to agricultural productivity, this work focused on ways to overcome the adverse effects of salinity stress on the growth, yield and its components, the concentrations of potassium (K), sodium (Na), sulphur (S) and ascorbic acid (AsA), and the ratio of K/Na in squash (*Cucurbita pepo* L.) plants grown on a newly-reclaimed saline soil (EC = 7.85 – 7.91 dS m^{-1}) in two seasons (2012 and 2013) using S and/or AsA as soil and foliar applications, respectively.

Methodology

Soil analysis, growth conditions and treatments

Two field experiments were conducted using squash crop during the seasons of 2012 and 2013 in the Experimental Farm at Demo, Faculty of Agriculture, Fayoum University, Egypt. Prior to the initiation of each season, soil samples up to 25cm depth from the experimental site were taken and analyzed according to the published procedures of Wilde et al. (1985) and the results are given in Tables (1 and 2).

Table 1: Some of the physical and chemical properties of the selected soil before planting in 2012 and 2013 seasons

Property	2012	2013
Physical:		
Clay%	28.7	24.2
Silt%	20.4	22
Sand%	50.9	53.8
Soil texture	sandy	sandy
Chemical:		
Organic matter%	1.24	1.14
$CaCO_3$%	11.66	11.82
Soluble cations (meq 100^{-1} g soil):		
Ca^{+2}	22.63	24.52
Mg^{+2}	5.52	5.85
Na^{+}	43.54	45.16
K^{+}	0.66	0.61
Soluble anions (meq 100^{-1} g soil):		
CO_3^{-}	-	-
HCO_3^{-}	3.17	3.13
Cl^{-}	33.23	35.61
SO_4^{2-}	35.45	34.65
Macro-and microelements (ppm):		
N	39.37	34.32
P	5.23	5.15
K	180.16	175.41
Fe	4.17	4.03
Zn	0.63	0.60
Mn	2.02	2.03
Cu	0.47	0.44

Table 2 : Acidity and salinity of the experimental soil before (BSAS) and after 50 days from the soil application with sulphur (ASAS) in 2012 and 2013 seasons

Sample		pH	EC (dS m^{-1})
2012 season	BSAS	8.05	7.85
	ASAS	7.53	6.01
2013 season	BSAS	8.13	7.91
	ASAS	7.62	6.12

Squash seed (cv. Amjed hybrid), imported from Seminis-Petoseed Company, USA, was sown on 14 March 2012 and 17 March 2013. The experimental design was completely randomized blocks with 6 treatments, each with 4 replicates. Each experimental unit measured 14 m^2 and consisted of 5 rows; 4 m long and 0.7 m width, with row spacing averaged 40 cm apart. Each two adjacent experimental unites were separated by 1.2 m alley.

During soil preparation for planting, elemental S (zero or 500 kg ha^{-1}) were broadcasted and incorporated. All experimental areas were also received complete doses of farmyard manure [35 m^3 ha^{-1}; pH 7.16 and 7.11; EC 3.23 and 3.12 dS m^{-1}; organic matter 46.88% (w/w) and 48.46% (w/w); C/N ratios 17.53 and 16.88; total N 1.72% (w/w) and 1.47% (w/w); total P 0.41% (w/w) and 0.45% (w/w); and total K 0.69% (w/w) and 0.72% (w/w) in the 2012 and 2013, respectively] and calcium superphosphate (15.5% P_2O_5) at a rate of 350 kg ha^{-1}. In addition, ammonium nitrate (33.5% N) and potassium sulphate (48% K_2O) at the rates of 450 kg N and 225 kg K_2O ha^{-1}, respectively were applied in two equal applications i.e., at 20 and 35 days after sowing. Other agro-management practices were applied as recommended by the Egyptian Ministry of Agriculture for squash production in newly-reclaimed soils. Squash seedlings were foliar applied with AsA at 0, 1 or 2 mM to run off, three times; 15, 25 and 35 days after sowing. Few drops of Tween-20 were added to the spraying solution as a wetting agent.

Measurements of growth traits

Forty-five days after sowing, five plants were randomly chosen from the two outer rows of each experimental unit and cut off at ground level to measure the stem length, canopy dry weight plant^{-1}, number of leaves plant^{-1}, total leaf area plant^{-1} and leaf area leaf^{-1} was calculated using the following formula:

$$\text{Leaf area leaf}^{-1} = \frac{\text{Leaves area plant}^{-1}}{\text{Number of leaves plant}^{-1}}$$

Measurements of yield and its components

In each experimental unit, plants of the three middle rows were left to grow till the fruits approach the marketable stage. Then, fruits were picked from 20 plants to determine the number of fruits plant^{-1}, fruits weight plant^{-1} and average fruit weight. The total yield of fruit ha^{-1} was calculated from all plants of the three middle rows.

Nutrients and sodium determinations

Leaf samples were collected from the fourth upper leaf of five randomly selected plants from each experimental unit, after 45 days from planting, to determine the concentrations of some nutrients and sodium. The samples were washed with tap water, rinsed three times with distilled water and some of them were dried at 70°C in a forced-air oven till constant weight. Some fresh leaf samples that randomly collected from all experimental units were subjected to the method of Helrich (1990) to determine their concentrations of endogenous ascorbic acid. The dried leaf samples were finely ground and sample weights of the fine powder (0.2 g) were digested using a mixture of sulphuric and perchloric acids to determine the following parameters: Potassium and sodium concentrations were determined using a Perkin-Elmer Flame photometer as outlined by Page *et al.* (1982). Sulphur concentration was measured by atomic absorption spectrophotometer (Analyst 300, Perkin-Elmer, Germany).

Statistical analysis

Data of the two seasons were subjected to the statistical analysis according to the design used (Snedecor and Cochran, 1980). The least significant difference test (LSD) at $p \leq 0.05$ level was utilized to verify the significant difference between treatments.

Received Manuscript 23-November-2013 Accepted Manuscript 30-March-2014

Results

Effect of soil application with sulphur (SAS) and/or foliar application with ascorbic acid (FAA) on vegetative growth traits

Data in Table 3 show that stem length, canopy dry weight plant^{-1}, number of leaves plant^{-1}, average leaf area and total leaf area plant^{-1} increased in squash plants, which FAA at the 2 rates (1 and 2 mM) compared to plants, which had no FAA. Plants received FAA at the rate of 2 mM gave more growth traits than plants received 1 mM. SAS further increased these growth traits. The best growth traits were obtained from the combined treatment of SAS and FAA at the level of 2 mM. The same trend was observed over both the 2012 and 2013 growing seasons.

Table 3: Effect of soil application with sulphur (SAS; kg ha^{-1}) and foliar application with ascorbic acid (FAA; mM) on vegetative growth traits [i.e., stem length (cm), canopy dry weight (g plant^{-1}), number of leaves plant^{-1}, leaf area (dm2 leaf^{-1}) and total leaf area (dm^2 plant^{-1})] of squash plants grown in 2012 and 2013 seasons

Treatment		Stem length	Canopy DW	No. of leaves	Leaf area leaf^{-1}	Leaf area plant^{-1}
SAS	FAA					
				2012		
0	0	3.1c	13.2d	6.6d	1.86c	12.4d
	1	3.5c	15.2d	7.2cd	2.03c	14.5cd
	2	3.8bc	18.2c	8.0bc	2.24bc	17.9bc
500	0	4.3ab	19.2c	8.0bc	2.52ab	20.1b
	1	4.7a	23.0b	8.8ab	2.76a	24.3a
	2	5.0a	25.6a	9.6a	2.76a	26.7a
				2013		
0	0	3.0c	12.7d	6.5d	1.80c	11.6e
	1	3.3c	14.6d	6.9cd	1.97bc	13.4de
	2	3.7bc	17.5c	7.7c	2.17abc	16.3cd
500	0	4.2ab	18.5c	7.7c	2.52a	19.4bc
	1	4.5a	22.1b	8.9ab	2.44ab	21.8ab
	2	4.8a	24.6a	9.7a	2.59a	24.9a

Effect of soil application with sulphur (SAS) and/or foliar application with ascorbic acid (FAA) on yield and its components

Data in Tables 4 show that, except average fruit weight, number of fruits plant^{-1}, fruit weight plant^{-1} and fruit yield ha^{-1} increased in squash plants when received FAA at the 2 rates (1 and 2 mM) compared to plants that had no FAA. Plants received FAA at the rate of 2 mM gave higher fruit yield than plants received FAA at 1 mM. SAS further increased fruit yield of squash plants. The highest fruit yield was obtained when the combined treatment of SAS and FAA at the rate of 2 mM, which was applied. The same trends were seen in both the 2012 and 2013 growing seasons.

Table 4 : Effect of soil application with sulphur (SAS; kg ha^{-1}) and foliar application with ascorbic acid (FAA; mM) on yield and its components [i.e., number of fruits plant^{-1}, fruit weight (g plant^{-1}), average fruit weight (g) and fruit yield (ton ha^{-1})] of squash plants grown in 2012 and 2013 seasons

Treatment		No. of fruits plant^{-1}	Average fruit weight	Fruit weight plant^{-1}	Fruit yield ha^{-1}
SAS	FAA				
			2012		
0	0	5.5e	52.1a	287d	8.00d
	1	5.8de	53.3a	312cd	8.60d
	2	6.1cd	54.7a	335bc	9.55c
500	0	6.5bc	48.9a	312cd	9.38c
	1	6.8b	53.5a	365b	10.76b
	2	7.5a	54.4a	410a	12.33a
			2013		
0	0	5.3d	49.5a	266d	7.81e
	1	5.7cd	53.8a	306c	8.52d
	2	6.0c	55.1a	332bc	9.40c
500	0	6.2bc	51.8a	320c	9.48c
	1	6.6b	53.8a	354b	10.69b
	2	7.2a	54.3a	389a	12.14a

Received Manuscript 23-November-2013 Accepted Manuscript 30-March-2014

Effect of soil application with sulphur (SAS) and/or foliar application with ascorbic acid (FAA) on leaf concentrations of K, S, Na and ascorbic acid (AsA) and K/Na ratio

Data in Table 5 reveal that, except the reduction in the concentration of Na, the concentrations of K, S and AsA, and the ratio of K/Na increased in squash plants, which received FAA compared to those in plants had no FAA. Plants received FAA at the rate of 2 mM had higher K, S and AsA concentrations, and K/Na ratio compared to plants received only 1 mM. Further increased concentrations of K, S and AsA and increased ratio of K/Na were observed with SAS. The concentration of Na showed the reverse trend to other measurements. The best results were obtained from the combined treatment of SAS and FAA at the level of 2 mM. Similar trends were observed in both the 2012 and 2013 growing seasons.

Table 5: Effect of soil application with sulphur (SAS; kg ha^{-1}) and foliar application with ascorbic acid (FAA; mM) on leaf concentrations of potassium (K), sodium (Na), sulphur (S) and ascorbic acid (AsA), and the ratio of K/Na in squash plants grown in 2012 and 2013 seasons

Treatment		K (%)	Na (%)	K/Na ratio	S (%)	AsA (%)
SAS	FAA					
			2012			
	0	2.65d	0.60a	4.42e	0.66c	0.021c
0	1	2.72cd	0.58a	4.69de	0.68c	0.029b
	2	2.82bc	0.56a	5.04d	0.68c	0.041a
	0	2.91b	0.51b	5.71c	0.75b	0.020c
500	1	2.98b	0.48bc	6.21b	0.77ab	0.030b
	2	3.22a	0.46c	7.00a	0.81a	0.042a
			2013			
	0	2.75d	0.63a	4.37d	0.69c	0.019c
0	1	2.83cd	0.61ab	4.64d	0.68c	0.030b
	2	2.94bc	0.58b	5.07c	0.70c	0.039a
	0	3.04b	0.53c	5.74b	0.76b	0.019c
500	1	3.10b	0.52cd	5.96b	0.78b	0.032b
	2	3.34a	0.49d	6.82a	0.85a	0.041a

Discussion

The enhanced results in this study may be attributed to the improvement in K, K/Na ratio, endogenous S and endogenous AsA (Table 5) due to the exogenous application of S and AsA. Soil application of S resulted in the reduction in pH and EC of the tested soil (Table 2), which improved the solubilization of nutrients in the tested soil therefore provided plants with more bio-available nutrients for uptake by plant roots (Osman and Rady, 2012). Khan et al. (2005) have reported that sufficient S application improved photosynthesis and growth through regulating N assimilation. Osman and Rady (2012) also reported that higher N concentrations following S application may result in increased sulphate accumulation by plants which is responsible for increased photosynthesis and plant DW. Increased endogenous concentration of AsA leads to protect plant cells and, consequently, protect the photosynthetic apparatus by scavenging reactive oxygen species (ROS; Zhang and Schmidt, 2000). AsA considers a most powerful ROS scavenger because of its ability to donate electrons in a number of enzymatic and non-enzymatic reactions. It can provide protection to membranes by directly scavenge the O_2^- and OH^- and by regenerate α-tocopherol from tocopheroxyl radical. In chloroplast, AsA acts as a cofactor of violaxanthin de-epoxidase thus sustaining dissipation of excess excitation energy (Smirnoff, 2000). In addition to the importance of AsA in the ascorbate-glutathione cycle, it also plays an important role in preserving the activities of enzymes that contain prosthetic transition metal ions (Noctor and Foyer, 1998). The AsA redox system consists of L-ascorbic acid, monodehydroascorbate and dehydroascorbate. Both oxidized forms of AsA are relatively unstable in aqueous environments while dehydroascorbate can be chemically reduced by glutathione to ascorbate (Foyer and Halliwell, 1976), thus vigorous plant growth will be obtained under stress conditions. In this regard, Elade (1992) stated a positive action for antioxidants, including AsA, on growth and attributed this finding to their effects on counteracting drought, salinity and other abiotic stresses and protecting plant cells against free radicals that are responsible for plant senescence, as well as to their auxinic action. In addition, AsA might regulate cell wall expansion, division and elongation through its action on cell vacuolarization (Gonzalez-Reyes et al., 1994; Cordoba-Pedregosa et al., 1996), improve the nutritional status and absorbing phenolic compounds, which lead to protect the growing tissues from toxic effects of the oxidized phenols (Gupta et al., 1980) and/or enhance the biosynthesis of carbohydrates (Ahmed, 2001) and translocation of sugars (Farag, 1996), which could be explain our results.

Received Manuscript 23-November-2013 Accepted Manuscript 30-March-2014

The improvement occurred in yield might be attributed to the enhanced effect of S on plant growth and activating the bio-chemical processes in plants, *i.e.*, respiration, photosynthesis and chlorophyll content, which increased yield and its components (Hegazi, 2004; Osman and Rady, 2012). On the other hand, data in the Table 5 show that the relative decrease in Na uptake by plants may be due to the positive plant response to the applied S, which combated the negative effects of salt stress in the root zone as well as reduced soil pH and EC. These results are in agreement with those obtained by Osman and Rady (2012), who stated that the pronounced bacterial activity, due to the applied elemental S, is capable to produce some hormones, which induce the proliferation of roots and root hairs that increase nutrient absorbing surfaces, as well as produce organic acids, which solublize inorganic and organic forms of mineral elements, and consequently increase stems and leaves dry wieghts then the yield and its components. Al-Qubaie (2002) stated that AsA as an antioxidant has an auxinic action, a synergistic effect on the biosynthesis of carbohydrates and controlling the incidence of most fungi on plants, which favours a vigorous state and reflects on yields. In addition, Osman (2010) reported an increase in yield bulbs of leek plant as a result in the foliar application with ascorbic acid. Results regarding the beneficial effect of AsA on yield are coherent with those reported by Rady (2006) and El-Yazal (2007). S in combination with AsA reduced soil pH and EC (Table 2) and increased the availability of nutrients (Table 5), resulting in a positive effect on plant growth (Table 3), and also protected soil productivity against excess salt effects. These results indicated that the combined application of S plus AsA was beneficial for newly-reclaimed soils to alleviate the adverse effects of salinity stress and to improve sustainable crop productivity. On the other hand, the relative decrease in Na uptake by plants may be due to the positive plant response to the applied S, which ameliorated the negative effect of salt stress in the root zone (Osman and Rady, 2012). Foyer et al. (1990) stated that, the antioxidant; AsA prevented enzyme inactivation and the generation of more dangerous radicals and allowed flexibility in the production of photosynthetic assimilatory power. Moreover, electron transfer to O_2 prevented over reduction of the electron transported chain, which reduced the risk of harmful back reactions within the photosystem. In addition, Elade (1992) and Farag (1996) proved that most antioxidants were responsible for accelerating the biosynthesis of various pigments. Results of this study are supported by the results of Ahmed and Abd El-Hameed (2004) who reported that the effect of antioxidants on producing healthy plants leads to support the plants to have a greater ability for uptake of elements. Moreover, Gonzalez-Reyes et al. (1994) concluded that ascorbate free radical caused hyperpolarization of plasma membranes, and this energization could then facilitate transport processes across such membranes.

Conclusions

The soil and foliar applications of sulphur (S) and/or ascorbic acid (AsA), respectively have been shown to enhance plant stress-defence responses, to act indirectly by improving general plant performance under stress, and to increase endogenous concentrations of S and AsA, leading to an increase in plant growth and crop yield. Thus, the application of S and/or AsA may provide a novel strategy to reduce the adverse effects of salinity through increased the synthesis of endogenous antioxidant compounds, including AsA. The uptake and assimilation of S (as sulphate) were assisted by AsA and assumed to be a crucial determinant for plant survival under a wide range of adverse environmental conditions since various antioxidants, particularly AsA and S-containing compounds are involved in plant responses to salinity stress. Increased endogenous S and AsA concentrations, in addition to providing a potential efficient antioxidant enzyme system, resulted in less damage to photosynthesis and greater protection from salinity stress. Therefore, the application of S and/or AsA may act to ameliorate the severity of the effects of salinity stress on squash plants grown in newly-reclaimed saline soils.

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Received Manuscript 23-November-2013 Accepted Manuscript 30-March-2014
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