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Ameliorative effects of sulphur and humic acid on the growth, anti-oxidant levels, and yields of pea (*Pisum sativum* L.) plants grown in reclaimed saline soil

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SUMMARY
The effects of sulphur (S) and/or humic acid (HA) on the growth, leaf anti-oxidant levels, leaf nutrients, and yields of pea (*Pisum sativum* L.) plants grown on reclaimed saline soil (EC = 8.2 - 8.5 dS m^{-1}) were investigated. Two field experiments were performed in a randomised complete block design with four treatments and four replicates for each treatment. Sulphur and HA were applied at the rates of 500 kg ha^{-1} or 200 kg ha^{-1}, respectively, singly or in combination. Neither S nor HA was included in the controls. Soil application of S and/or HA significantly increased shoot lengths, the number of branches $plant^{-1}$, leaf area $plant^{-1}$, shoot dry weight $plant^{-1}$, the contents of leaf pigments, leaf free proline, leaf macronutrients (N, P, and K), seed protein, and the total yields of pods and seeds ha^{-1}, when compared with non-treated control plants. In contrast, there were significant reductions in leaf Na^+ ion contents under the S and/or HA treatments. The combined S + HA treatment was found to be highly effective at improving the growth and yield of pea plants by alleviating the inhibitory effects of soil salinity stress. The same trends were observed over two growing seasons (2010 and 2011).

Pea (*Pisum sativum* L.) is one of the most popular vegetable crops grown in Egypt. Pea is considered one of the main leguminous crops that are an important component of the agricultural sector in developing countries due to its ability to produce significant quantities of protein, carbohydrates, and nutrient-rich seed. Pea is widely cultivated on newly-reclaimed soils in Egypt. However, most newly-reclaimed soils are affected by salinity, have low fertility, and a poor soil structure. The sustainability of crop production is primarily a function of various environmental stress factors, among them salinity (Kumar *et al*., 2009), which are 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 of plant productivity due to salinity arises as a consequence of an imbalance in ion and nutrient concentrations and osmotic effects (Ashraf, 2009). These result in an 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 damage to lipids, proteins and nucleic acids, and affects the properties of cell membranes (Ahmad *et al*., 2008). 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 during salinity stress (Sudhir and Murthy, 2004), 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 anti-oxidant enzymes such as superoxide dismutase (SOD), catalase (CAT), and peroxidase (POX), and non-enzymatic anti-oxidants such as ascorbic acid, glutathione, and carotenoids, to counteract any oxidative stress and to protect the plants from oxidative damage (Apel and Hirt, 2004).

Over the last few decades, in parallel with breeding and biotechnological strategies to improve plant tolerance to salinity (Maggio *et al*., 2003), several techniques have been proposed to ameliorate the performance of plants in saline environments. These include seed or seedling priming (Azooz, 2009), pre-exposure to moderate salt stress (Friedman *et al*., 2006), applications of stress metabolites that could be recognised and/or integrated by plants as components of stress-induced adaptation responses (Ashraf and Foolad, 2007), and foliar applications of osmo-protective molecules such as anti-oxidants. Most have been shown to have beneficial effects on plants exposed to salt stress (Ali *et al*., 2007; Rady, 2011a). Mineral and organic fertilisers, added singly or in combination, are an important means of plant nutrition, particularly in saline soils. Attention has therefore been focussed on applying combinations of mineral and organic fertilisers, such as sulphur (S) and humic acid (HA), as a technique to overcome the adverse effects of soil salinity on growing plants.

Little information is available on the mineral nutrient status of plants and their tolerance to salinity. Among the

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mineral nutrients, S is increasingly being recognised as the fourth major essential nutrient element after nitrogen (N), phosphorus (P), and potassium (K). Sulphur plays an important role, not only in the growth and development of higher plants, but also as it is associated with increased stress tolerance in plants (Nazar *et al*., 2011). Sulphur deficiency has negative effects on the chlorophyll contents of leaves, N contents, and photosynthetic enzymes (Lunde *et al*., 2008), and consequently reduces the yields and quality parameters of crops (Hawkesford, 2000). Adequate S nutrition improves photosynthesis and the growth of plants, and has regulatory interactions with N assimilation (Scherer, 2008). Sulphur is required for protein synthesis, N assimilation, and is a structural constituent of several co-enzymes and prosthetic groups (Marschner, 1995). Sulphur is incorporated into organic molecules in plants and is located in thiol (-SH) groups in proteins (e.g., 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 oxidising environment, represents a potential modulator of the stress response (Szalai *et al*., 2009). Glutathione has been shown to take part in the removal of excess ROS (Noctor and Foyer, 1998), thereby controlling ROS levels (Rausch *et al*., 2007) and protecting plants from oxidative damage. Sulphur has been applied to many agricultural areas to improve the properties of saline and alkaline soils. In Egyptian soils, which are characterised 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). 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).

Humic substances are commercial products that contain elements which improve soil fertility, increase the availability of nutrient elements and, consequently, have positive effects on plant growth and yield. In addition, humic material reduces the negative effects of chemical fertilisers and removes NO_2^- and NO_3^- ions from the soil (Rady, 2011b). Humic substances can supply growing plants with nutrients, make the soil more fertile and productive, and increase its water-holding capacity. Therefore, humic substances are useful for reclaimed, saline soils because they help plants to resist salinity and drought, help to establish a desirable environment for the development of microorganisms, and stimulate seed germination (Salman *et al*., 2005). These authors also reported significantly improved mineral contents, fruit yields, and fruit quality in watermelon plants due to the application of humic acid (HA), with or without mineral fertiliser. Under different soil conditions, the application of humic substances has been reported to improve plant growth and chemical composition, which are positively reflected in higher crop yields and quality (Selim *et al*., 2009; Mahmoud and Hafez, 2010; Hanafy Ahmed *et al*., 2010).

Since salinity is considered a potential threat to agricultural productivity, this work focussed mainly on ways to overcome the adverse effects of saline stress on the growth, chemical composition of leaves, leaf anti-oxidant levels, yield, and quality of pea (*P. sativum* L.) plants grown on reclaimed saline soil (EC = 8.2 - 8.5 dS m^{-1}) in two experiments (October - December 2010 and October - December 2011) using S and/or HA to elucidate their potential to modulate plant responses to salinity stress.

MATERIALS AND METHODS

Physical and chemical properties of soil, farmyard manure (FYM), and humic acid

The main characteristics of the newly-reclaimed soil (Experimental Farm of the Faculty of Agriculture, Fayoum University, Southeast Fayoum, Egypt; 29º 17'N; 30º 53'E) used in this research were determined according to Wilde *et al*. (1985) and are shown in Table I.

The main characteristics of the two FYMs used in the 2010 and 2011 seasons were: pH 7.32 and 7.23; EC 3.23 and 3.12 dS m^{-1}; organic matter content 66.68% (w/w) and 65.46% (w/w); C/N ratios 32.53 and 30.88; total N 2.05% (w/w) and 2.12% (w/w); total P 0.34% (w/w) and 0.33% (w/w); and total K 0.47% (w/w) and 0.56% (w/w), respectively.

The main characteristics of the HA were: net HA content, 85.01% (w/w) on a dry weight (DW) basis; total N, 0.81% (w/w); total P, 1.15% (w/w); total K, 5.56% (w/w); total Ca, 3.81% (w/w); total Mg, 0.95% (w/w); total S, 0.48% (w/w); Fe, 635 mg kg^{-1} DW; Mn, 355 mg kg^{-1} DW; Zn, 311 mg kg^{-1} DW; Cu, 115 mg kg^{-1} DW; and Na, 212 mg kg^{-1} DW.

Treatments and plant material

Two field experiments were conducted, one in the 2010 season and one in the 2011 season. During soil preparation for sowing, all experimental areas received a complete dose of FYM [60 m^3 ha^{-1}], mineral-P [500 kg ha^{-1} as calcium superphosphate [15.5% (w/w) P_2O_5], mineral-K [60 kg ha^{-1} as potassium sulphate (48% (w/w) K_2O)], and mineral-N [120 kg ha^{-1} as ammonium nitrate (33.5% (w/w) N)]. An additional 240 kg ha^{-1} of ammonium nitrate and 120 kg ha^{-1} of potassium sulphate were added at 3 and at 6 weeks after each sowing. The area was then divided into four 24 m^2 plots. Prior to sowing the pea seed, elemental sulphur (S) and humic acid (HA) were applied individually or in combination to the appropriate plots. The control plots (n=4) did not receive S or HA. Treatment I was S at a rate of 500kgha^{-1}, spread manually on the soil

TABLE I

Physical and chemical properties of the experimental soil before treatment (BT) and on day-50 after treatment (AT) in 2010 and in 2011

	Composition [% (w/w)]				EC	OC#	N	P	K	Ca	Fe	Mn	Zn
Sample	Clay	Loam	Sand	pH	(dS m^{-1})	(g kg^{-1})	(g kg^{-1})	(mg kg^{-1})	(mg kg^{-1})	(mg kg^{-1})	(mg kg^{-1})	(mg kg^{-1})	(mg kg^{-1})
2010 BT	26.4	20.9	52.7	8.3	8.5	11.8	0.81	1.86	84.7	38.1	6.1	4.0	2.3
2010 AT	26.1	21.1	52.8	7.6	5.6	14.2	1.32	2.03	94.0	46.2	8.2	5.4	3.0
2011 BT	27.8	19.2	53.0	8.2	8.2	12.3	0.86	1.77	86.2	43.3	6.7	3.9	2.0
2011 AT	27.6	19.3	53.1	7.4	5.5	16.3	1.46	2.14	98.7	53.1	9.6	5.8	2.7

#OC, organic content.

surface after mixing with an appropriate amount of sand, then mixed into the soil-surface layer. Treatment II was humic acid at a rate of 200 kg ha^{-1}, spread manually after mixing with the same amount of sand, then mixed into the soil-surface layer. The same procedure was carried out for Treatment III in which a mixture of S plus HA (at the above rates) was applied with the same amount of sand. All treatments were applied in a randomised complete block design with four replicates for each of the four treatments. Sowing was conducted on 11 October 2010 and on 1 October 2011 using pea (*P. sativum* L.) seed (cv. Master-B) obtained from the Agricultural Research Center, Cairo, Egypt. Each 24 m^2 plot (8 rows; 5 m long and 0.6 m width) contained 600 plants, spaced at 20 cm (in-row), and three seeds were placed in each hole. All other standard cultural practices were followed, as recommended for commercial pea production.

Plant growth and yield analyses

Fifty-day-old pea plants (n = 3) were removed from each of the four treatment plots and the number of branches $plant^{-1}$ were counted. Shoot lengths were measured using a meter scale, then the shoots were placed in an oven at 80ºC for 24 h. The dried shoots were weighed to record plant DW. Leaf areas were measured manually using a graph sheet, where the squares covered by the leaf were counted to note the leaf area. At the end of each experiment (20 December 2010 and 12 December 2011), all the green pods on each plant in each plot were collected and weighed. The green pea seeds were then extracted from the pods and weighed.

Determination of leaf pigment contents

Total chlorophyll and carotenoids contents (in mg g^{-1} FW) were estimated following the procedure given by Arnon (1949). Leaf discs (0.2 g from each replicate plot of each treatment) were homogenised in 50 ml 80% (v/v) acetone and centrifuged at 10,000 × *g* for 10 min. The absorbance of each acetone extract was measured at 663, 645, and 470 nm using a UV-160A UV-visible recording spectrometer (Shimadzu, Kyoto, Japan).

Determination of leaf free proline contents

Leaf free proline contents (in µg g^{-1} DW) were measured using the rapid colourimetric method, as suggested by Bates *et al*. (1973). Proline was extracted from 0.5 g of each fresh leaf sample (n = 12; i.e., four replicate plots, three plants per replicate of each treatment) by grinding in 10 ml 3% (v/v) sulphosalicylic acid and the mixture was then centrifuged at 10,000 × *g* for 10 min. Two ml of the supernatant was placed in a test-tube, to which 2 ml of a freshly prepared acid-ninhydrin solution was added.The tubes were incubated in a water bath at 90ºC for 30 min and the reaction was terminated in an ice bath. Each reaction mixture was extracted with 5 ml toluene and vortex-mixed for 15 s. The tubes were allowed to stand for at least 20 min in the dark, at room temperature, to allow separation of the toluene and aqueous phases. Each toluene phase was then carefully collected into a clean test-tube and its absorbance was read at 520 nm. The free proline concentration in each sample was determined from a standard curve prepared using analytical grade proline, and expressed on a % DW basis.

Determination of ascorbic acid contents

The extraction and determination of ascorbic acid (AsA) from pea leaf samples were carried out following the method of Kampfenkel et al. (1995). Plant leaf material (1.0 g) was obtained from each replicate plot of each treatment (i.e., 12 leaf samples per treatment), homogenised immediately in liquid N_2 and extracted with 10 ml 5% (w/v) trichloroacetic acid (TCA). The homogenate was centrifuged at 4°C for 5 min at 15,600 × g. The supernatant was transferred to a clean reaction vessel and immediately assayed for AsA content in a 1.0 ml reaction mixture containing 50 µl 10 mM DTT, 100 µl 0.2 M phosphate buffer (pH 7.4), 0.5% (v/v) N-ethylmaleimide, 10% (w/v) TCA, 42% (v/v) H_3PO_4, 4% (v/v) 2,2'-dipyridyl, and 3% (w/v) $FeCl_3$.

Determination of reduced glutathione contents

Determinations of glutathione levels were performed essentially as described by De Kok *et al*. (1986). Briefly, reduced glutathione (GSH) was extracted from 0.2 g fresh weight (FW) of leaf material from each plot in two volumes of extraction buffer [2% (w/v) sulphosalicylic acid, 1 mM Na_2EDTA, and 0.15% (w/v) ascorbate] and homogenised. The homogenate was centrifuged at 12,000 × *g* for 5 min at 4ºC. An aliquot (1.0 ml) of the supernatant was then used to measure GSH content using the glutathione assay kit (Sigma Chemical Co., St. Louis, MO, USA).

Determination of leaf and seed nitrogen (N), leaf phosphorus (P), potassium (K), sodium (Na), and seed protein contents

Leaf and seed N contents (in mg g^{-1} DW) were determined according to Hafez and Mikkelsen (1981). An Orange-G dye solution was prepared by dissolving 1.0 g of 96% (w/w) assay-dye in 1 l of distilled water, with 21.0 g citric acid which acted as a buffer to maintain the correct pH, and 2.5 ml 10% (v/v) thymol in 10% (v/v) ethanol as an inhibitor of microbial growth. Ground plant material (0.2 g leaf tissue or pea seed from each plot) was placed in a centrifuge tube and 20 ml of the dye reagent solution was added. The contents of each tube were shaken for 15 min, then filtered using Whatman No. 1 filter paper. The solution was diluted 100-fold with distilled water and its absorbance was measured at 482 nm. N contents were calculated using the formulae:

$$N\ (\%) = 0.39 + 0.954 \times \textit{Dye absorbed}\ (\text{g}/100\ \text{g}) \text{ and}$$

$$\textit{Dye absorbed}\ (\text{g}/100\ \text{g}) = (a - b / a)\ (cfv / w) \times 100$$

where, *a* was the absorbance of the dye reagent solution at 482 nm without plant material (the blank), *b* was the absorbance of the dye reagent solution at 482 nm with plant material, *c* was the concentration of the dye reagent (1.0 g l^{-1} distilled water), *f* was the purity factor of the dye reagent (96%), *v* was the volume of the dye reagent solution used per sample (20 ml), and w was the weight of ground dry material in g (0.2).

Seed protein contents (in mg g^{-1} DW) were calculated by multiplying the seed N content by 6.25, then converting this to a seed protein percentage (w/w) by dividing by 10 (Jaradat and Rinke, 2010).

TABLE II
Growth parameters in 50-day-old pea plants grown using various soil treatments in 2010 and in 2011

Year	Treatment[#]	Shoot length (cm)	No. of branches plant^{-1}	Leaf area plant^{-1} (cm^2)	Shoot DW plant^{-1} (g)
2010	Control (Sal; EC = 8.5 dS m^{-1})	16.2 ± 3.4d[†]	1.1 ± 0.2d	103.6 ± 5.2d	15.6 ± 2.2d
	Sal + S_{500}	21.7 ± 3.6c	1.4 ± 0.1c	144.8 ± 5.8c	19.5 ± 2.6c
	Sal + HA_{200}	35.5 ± 4.1b	1.7 ± 0.2b	206.9 ± 7.3b	21.7 ± 2.4b
	Sal + S_{500} + HA_{200}	46.9 ± 5.0a	2.2 ± 0.2a	302.5 ± 7.9a	26.8 ± 2.7a
2011	Control (Sal; EC = 8.2 dS m^{-1})	17.0 ± 2.9d	1.2 ± 0.2d	112.4 ± 4.9d	14.8 ± 2.4d
	Sal + S_{500}	22.8 ± 3.4c	1.5 ± 0.2c	161.7 ± 6.2c	19.0 ± 2.3c
	Sal + HA_{200}	37.2 ± 3.2b	1.8 ± 0.4b	221.8 ± 6.9b	21.4 ± 3.1b
	Sal + S_{500} + HA_{200}	49.1 ± 4.7a	2.3 ± 0.3a	323.6 ± 7.7a	25.6 ± 3.4a

[#]Sal, saline soil; S_{500}, sulphur at 500 kg ha^{-1}; HA_{200}, humic acid at 200 kg ha^{-1}.
[†]Mean values (n = 12) ± SD in each column for each year followed by a different lower-case letter are significantly different at $P \leq 0.05$ by Duncan's multiple range test.

The molybdenum-reduced molybdophosphoric blue colour method (Jackson, 1967), in sulphuric acid (with reduction to exclude arsenate), was used to determine P contents (in mg g^{-1} DW). Sulphomolybdic acid (molybdenum blue), diluted sulphomolybdic acid, and 8% (w/v) sodium bisulphite-H_2SO_4 solution were used as reagents.

Leaf K^+ and Na^+ ion contents (in mg g^{-1} DW) were assessed using a Perkin-Elmer Model 52-A Flame Photometer (Glenbrook, Stamford, CT, USA; Page *et al*., 1982).

Statistical analysis

The values for all parameters were subjected to statistical analysis, following the standard procedures described by Gomez and Gomez (1984). The 'F' test was applied to assess the significance of each treatment at the 5% level of probability ($P \leq 0.05$).

RESULTS AND DISCUSSION

Vegetative growth parameters

The application of S and/or HA, individually or in combination, significantly increased shoot lengths, the number of branches plant^{-1}, leaf area plant^{-1}, and shoot DW plant^{-1} when compared to control plants without S or HA added to the soil (Table II). The combined treatment (S + HA) was found to be most effective at enhancing these growth traits. It significantly ($P \leq 0.05$) surpassed all other treatments and exceeded control plant values by 190%, 100%, 192%, and 72% in 2010, and by 189%, 92%, 188%, and 73% in 2011, for shoot length, number of branches plant^{-1}, leaf area plant^{-1}, and shoot DW plant^{-1}, respectively. These positive results may be attributed to the fact that the added S significantly improved leaf contents of chlorophyll, ascorbic acid, and reduced glutathione (Table III). In turn, this may have led to an increase in photosynthetic efficiency, and subsequently to higher plant DWs and crop yields (Anjum *et al*., 2008). Khan *et al*. (2005) reported that application of sufficient S improved photosynthesis and growth through regulating N assimilation. Higher N contents following S application (Table IV) may result in increased sulphate accumulation by plants which is responsible for increased photosynthesis and plant DW. In addition, HA may stimulate plant growth by acting as a plant growth regulator (Rady and Osman, 2011). HA led to higher rates of uptake of K^+ ions (Table IV), and therefore a corresponding increase in chlorophyll fluorescence, which can serve as an indicator of the stress induced by alterations in the balance of endogenous hormones (Marschner, 1995). The increased growth parameters observed following the combined treatment with S and HA may be attributed to their combined positive effects on the soil, which led to an increase in organic matter content and bio-available nutrients, as a result of a reduction in soil pH (Table I). Thus, sulphur plus HA increased the availability of nutrients (Table IV), resulting in a positive effect on plant growth, and also protected soil productivity against excess salt effects. These results indicated that the combined application of S plus HA was beneficial for newly-reclaimed soils to alleviate the adverse effects of salinity stress and to improve sustainable crop productivity.

Leaf pigment, free proline, ascorbic acid, and reduced glutathione contents

Pea plants grown following the combined treatment with S plus HA produced leaves with the highest chlorophyll, carotenoid, free proline, AsA, and reduced

TABLE III
Biochemical composition of 50-day-old pea plants grown using various soil treatments in 2010 and in 2011

Year	Treatment[#]	Chlorophyll content (mg g^{-1} FW)	Carotenoids content (mg g^{-1} FW)	Free proline content (µg g^{-1} DW)	AsA content (µg ascorbate mg protein^{-1})	GSH content (nmol GSH mg protein^{-1})
2010	Control (Sal; EC = 8.5 dS m^{-1})	0.82 ± 0.04c[†]	0.33 ± 0.02d	51.6 ± 1.11d	0.50 ± 0.02c	29.5 ± 1.7d
	Sal + S_{500}	1.19 ± 0.06b	0.42 ± 0.02c	65.9 ± 1.35c	0.58 ± 0.03b	34.3 ± 1.9c
	Sal + HA_{200}	1.33 ± 0.05b	0.50 ± 0.04b	80.3 ± 2.21b	0.59 ± 0.03b	41.0 ± 2.5b
	Sal + S_{500} + HA_{200}	1.67 ± 0.06a	0.62 ± 0.03a	97.5 ± 2.18a	0.65 ± 0.03a	65.2 ± 4.0a
2011	Control (Sal; EC = 8.2 dS m^{-1})	0.85 ± 0.06c	0.35 ± 0.03d	47.5 ± 2.01d	0.48 ± 0.02c	36.2 ± 2.2d
	Sal + S_{500}	1.23 ± 0.05b	0.41 ± 0.05c	62.3 ± 1.78c	0.54 ± 0.02b	42.4 ± 3.1c
	Sal + HA_{200}	1.36 ± 0.06b	0.53 ± 0.03b	75.6 ± 2.32b	0.58 ± 0.03b	51.5 ± 2.8b
	Sal + S_{500} + HA_{200}	1.73 ± 0.08a	0.67 ± 0.04a	94.9 ± 2.84a	0.66 ± 0.03a	62.8 ± 3.6a

[#]Sal, saline soil; S_{500}, sulphur at 500 kg ha^{-1}; HA_{200}, humic acid at 200 kg ha^{-1}.
[†]Mean values (n = 12) ± SD in each column for each year followed by a different lower-case letter are significantly different at $P \leq 0.05$ by Duncan's multiple range test.

TABLE IV
Mineral nutrient contents of 50-day-old pea plants grown using various soil treatments in 2010 and in 2011

Year	Treatment[#]	N (mg g^{-1} DW)	P (mg g^{-1} DW)	K (mg g^{-1} DW)	Na (mg g^{-1} DW)
2010	Control (Sal; EC = 8.5 dS m^{-1})	21.2 ± 1.3c[†]	2.57 ± 0.21d	15.8 ± 1.4c	13.9 ± 1.4a
	Sal + S_{500}	25.8 ± 1.2b	3.29 ± 0.23c	20.2 ± 0.9b	8.9 ± 0.7b
	Sal + HA_{200}	27.0 ± 1.6b	3.67 ± 0.28b	19.8 ± 1.6b	7.1 ± 0.8c
	Sal + S_{500} + HA_{200}	32.2 ± 2.1a	4.54 ± 0.26a	25.0 ± 1.9a	5.8 ± 0.5d
2011	Control (Sal; EC = 8.2 dS m^{-1})	21.7 ± 1.5d	2.65 ± 0.18d	16.2 ± 0.9c	12.6 ± 0.9a
	Sal + S_{500}	24.7 ± 1.7c	3.38 ± 0.22c	20.7 ± 1.3b	8.5 ± 0.8b
	Sal + HA_{200}	28.5 ± 2.2b	3.76 ± 0.19b	20.4 ± 1.1b	7.2 ± 0.8c
	Sal + S_{500} + HA_{200}	33.1 ± 2.5a	4.64 ± 0.29a	25.7 ± 1.8a	5.2 ± 0.6d

[#]Sal, saline soil; S_{500}, sulphur at 500 kg ha^{-1}; HA_{200}, humic acid at 200 kg ha^{-1}.
[†]Mean values (n = 12) ± SD in each column for each year followed by a different lower-case letter are significantly different at $P \leq 0.05$ by Duncan's multiple range test.

GSH contents compared to all other treatments (Table III). Leaves of control plants had the lowest contents of these compounds. The combined treatment of S + HA exceeded control leaf values by 104%, 88%, 89%, 29%, and 121% in 2010, and by 103%, 91%, 100%, 37%, and 73% in 2011, for chlorophyll, carotenoids, free proline, AsA, and GSH, respectively. It was expected that exposure of pea plants to salinity stress could increase levels of anti-oxidants such as AsA and GSH. However, the treatment of saline-stressed plants with S and/or HA further enhanced the levels of AsA and GSH. These compounds react directly, or by enzyme catalysis, with $OH^{\bullet}$, H_2O_2 or $O^{\bullet-2}$, while carotenoids operate directly as quenchers of ROS (Gapper and Dolan, 2006; Bajguz and Hayat, 2009).

The application of S to crops has been shown to enhance plant stress-defence reactions and to act indirectly by generally improving plant performance under stress, as well as by increasing AsA and GSH levels (Rausch and Wachter, 2005). Based on the relationships between pools of AsA and GSH, net photosynthesis, and plant DWs, with or without S, Anjum *et al*. (2008) suggest that the application of S may increase the pools of these compounds in plants to an extent that may lead to an increase in photosynthetic efficiency and, subsequently, to elevated plant DWs and crop yield. Increased contents of GSH and an efficient anti-oxidant system in plants leads to less damage to photosynthesis and greater protection from oxidative stress (Khan *et al*., 2009). In addition, the availability of S regulates the activity of nitrate reductase and the accumulation of N (Pal *et al*., 1976). A larger accumulation of N maintains higher chlorophyll contents, higher activities of enzymes in the Calvin cycle (Lawlor *et al*., 1989), and enhances growth (Khan *et al*., 2005) because of the roles of S and N in cell differentiation, photosynthetic function, and the overall growth of plants (Marschner, 1995). The combined treatment with S plus HA had synergistic effects on increasing nutrient availability to plant roots and increasing leaf nutrient contents (Table IV). This was positively reflected in the extent of plant growth, photosynthesis, and increased levels of proline (Table III). As an osmoticum, proline helps to maintain the turgor of plant cells, favouring those physiological processes that enable plants to overcome the adverse conditions in newly-reclaimed soils. Therefore, the application of S and/or HA may act to reduce the severity of salinity stress.

Leaf nitrogen (N), phosphorus (P), potassium (K), and sodium (Na) contents

The leaves of pea plants grown under saline conditions without added S or HA (control) had increased Na^+ contents, but reduced N, P, and K contents (Table IV). The N, P, and K contents of pea plants treated with S or HA showed significant increases, but Na^+ contents showed significant reductions when compared to untreated control plants. The combined treatment (S + HA) was found to be most effective in mitigating the adverse effects of salinity. In this case, N, P, K, and Na contents exceeded control values by 52%, 77%, 58%, and -58% in 2010, and by 53%, 75%, 59%, and -59% in 2011, respectively. As with S, HA also improved the chemical properties of soils because it increased the number of soil microorganisms which enhance nutrient cycling (Sayed *et al*., 2007) and reduced soil pH (Table I), thus increasing the availability of mineral nutrients to be absorbed by plant roots. Humic acid also promoted plant growth through its effects on ion transfer at the root level by activating the oxidation-reduction state of the medium and increasing the absorption of nutrients by preventing their precipitation in the nutrient solution. The increase in soil nutrients caused by the combined

TABLE V
Total green pod yield, pea seed yield, and seed protein content in pea plants grown after various soil treatments in 2010 and in 2011

Year	Treatment[#]	Total green pod yield (MT ha^{-1})	Total green seed yield (MT ha^{-1})	Seed protein (% DW)
2010	Control (Sal; EC = 8.5 dS m^{-1})	1.52 ± 0.12d[†]	0.35 ± 0.04d	25.9 ± 1.7c
	Sal + S_{500}	2.71 ± 0.18c	0.79 ± 0.06c	28.4 ± 1.5b
	Sal + HA_{200}	3.24 ± 0.25b	1.04 ± 0.06b	28.8 ± 2.2b
	Sal + S_{500} + HA_{200}	3.91 ± 0.40a	1.33 ± 0.11a	31.9 ± 2.5a
2011	Control (Sal; EC = 8.2 dS m^{-1})	1.60 ± 0.21d	0.38 ± 0.03d	24.2 ± 2.1c
	Sal + S_{500}	2.86 ± 0.19c	0.83 ± 0.07c	29.6 ± 2.5b
	Sal + HA_{200}	3.40 ± 0.29b	1.11 ± 0.09b	30.0 ± 2.3b
	Sal + S_{500} + HA_{200}	4.03 ± 0.38a	1.49 ± 0.22a	33.1 ± 2.5a

[#]Sal, saline soil; S_{500}, sulphur at 500 kg ha^{-1}; HA_{200}, humic acid at 200 kg ha^{-1}.
[†]Mean values (n = 12) ± SD in each column for each year followed by a different lower-case letter are significantly different at $P \leq 0.05$ by Duncan's multiple range test.
MT, metric tonnes.

application of S + HA (Table I) was positively reflected in the nutrient composition of the pea plants. Moreover, the optimum leaf nutrient composition obtained following the combined treatment with S + HA could be explained by the improved availability of essential nutrients in the root zone, resulting from their solubilisation caused by the release of organic acids.

Green pea pod and seed yields and seed protein contents

The data in Table V showed that S and/or HA significantly increased green pod and pea seed yields, as well as seed protein contents, when compared with the controls. The combined S + HA treatment was most effective at alleviating the inhibitory effects of salt stress. It improved the green pod yield ha^{-1}, the green seed yield ha^{-1}, and seed protein contents by 157%, 280%, and 23%, respectively in 2010, and by 152%, 292%, and 37%, respectively in 2011, compared to the controls. Thus, the same trends were seen in both growing seasons. The application of S increased N contents in pea plants (Table IV), which was responsible for increased photosynthesis and DW, and, consequently, crop yield and seed protein content.

The positive influence of HA on plant growth and yield could be due to the hormone-like activities present in HA that are involved indirectly in respiration, photosynthesis, oxidative phosphorylation, protein synthesis, anti-oxidant reactions, and various enzyme activities (Muscolo *et al*., 1993; Zhang and Schmidt, 2000; Zhang *et al*., 2003). Although HA is known to increase plant growth, resulting in yield responses similar to those induced by plant hormones, it has not yet been shown conclusively whether HA contain hormone-like components (Muscolo *et al*., 1993).

The application of S in combination with HA increased the uptake of nutrients significantly (Table IV), which ultimately increased chlorophyll levels and photosynthesis, resulting in elevated green pea pod and seed yields, as well as seed protein contents.

CONCLUSIONS

The application of sulphur (S) and/or humic acid (HA) to soils has been shown to enhance plant stress-defence responses, to act indirectly by improving general plant performance under stress, and to increase ascorbic acid (AsA) and reduced glutathione (GSH) contents, leading to an increase in photosynthetic efficiency and, subsequently, to an increase in plant growth and crop yield. Thus, the application of S and/or HA may provide a novel strategy to reduce the adverse effects of salinity through increased N-utilisation and the synthesis of anti-oxidant compounds such as AsA and GSH. The uptake and assimilation of S (as sulphate) were assisted by HA and assumed to be a crucial determinant for plant survival under a wide range of adverse environmental conditions since various anti-oxidants and S-containing compounds are involved in plant responses to salinity stress. Increased AsA and GSH contents, in addition to providing a more efficient anti-oxidant enzyme system, resulted in less damage to photosynthesis and greater protection from salinity stress. Therefore, the application of S and/or HA may act to attenuate the severity of the effects of salinity stress on pea plants grown in reclaimed saline soils.

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