

Impacts of Humic Acid, Indole Butyric Acid (IBA) and Arbuscular Mycorrhizal Fungi (*Glomus mosseae*) as Growth Promoters on Yield and Phytochemical Characteristics of *Hibiscus Sabdariffa* (Roselle)

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ABSTRACT

Field experiments were carried out during the two successive summer seasons of 2015/2016 and 2016/2017. The experiments were accomplished in an open field located at Bader region, El-Beheira Governorate, Egypt to evaluate the effects of humic acid, indole butyric acid (IBA), and arbuscular mycorrhizal fungi (AMF) (*Glomus mosseae*) individually on vegetative growth, yield, and phytochemical characteristics of *Hibiscus Sabdariffa* (Roselle jamica). Experimental field plots were designed as randomized complete block design (RCBD). The obtained results of the two seasons, generally, showed that all treatments (HA, IBA, and AMF) individually enhanced the vegetative growth, yield of calyx, and phytochemical parameters of Roselle plants. The HA treatment was the most effective in enhancing most of the studied parameters and might be recommended for enhancing the vegetative growth, yield of calyx, and calyx phytochemical components of Roselle plants under the environmental conditions of Bader region, El-Beheira Governorate and other similar regions.

Keywords: *Hibiscus Sabdariffa*, Karkade, Humic acid, Indole butyric acid, Mycorrhizal.

INTRODUCTION

Roselle (*Hibiscus sabdariffa* L.) is an annual shrub and belongs to the family Malvaceae. It is cultivated in tropical and subtropical regions for its popular stem fibers, edible calyxes, leaves, and seeds (Mahadevan and Kamboj, 2009). It is a popular plant in Middle Eastern Countries and known with many names such as Roselle, Sorrel, Mesta, and Karkade (Abu-Tarboush and Ahmed, 1996). It is considered as one of the most popular medicinal plants for its antimicrobial and antioxidant activities due to its rich content of a wide range of phenolic compounds (Anokwuru *et al.*, 2011). Also, it is used in folk therapy for abscesses, bilious conditions, cancer, cough, debility, dyspepsia, and fever (Lazim *et al.*, 2014). The leaves are sedative and emollient. Calyxes are used after being boiled in water in folk remedy for cancer (Duke, 1979) and considered a good source of protein (Mukhtar, 2007). Moreover, calyx used in the food and cosmetic industries as a

natural coloring agent in beverage, jam, and jellies (Khalil and Abdel-Kader, 2011 and Sonar *et al.*, 2013). The flowers of *H. sabdariffa* contain glycosides (hibiscin), anthocyanins, and gossyperin, which might exert diuretic and choleric effects and decrease the viscosity of the blood that reduce the blood pressure (Hassan, 2009).

However, environmental stresses including heat, drought, and salinity limit the plant's productivity. Therefore, several additives were attempted to enhance the performance and productivity of plants. For example, humic acid, which is a mixture of compounds that exist in soils with high levels of organic matter and quinine chemicals and was generated by microbial decomposition of plant tissues (Thygesen *et al.*, 2009). These compounds occur widely in soils, sediments, and water that contain carboxylic, phenolic, and methoxy chemicals (Xu *et al.*, 2005). Humic acids directly affect the plants by affecting the root and shoot processes and increasing nutrients absorption (Lobartini *et al.*, 1997). Many studies reported that humic acids could be used as growth promoters similar to gibberellic acids in improving plant growth and stress tolerance responses (Piccolo *et al.*, 1992).

Additionally, plant growth hormones have been reported to affect root processes, such as auxins which commonly used to stimulate root initiation in plants (Looney and McIntosh, 1968). Indole 3-Butyric Acid (IBA) is one of the most important auxins used to promote the formation of roots by breaking root apical dominance induced by cytokinin in plants (Hartmann *et al.*, 1990 and Cline, 2000). Recent investigations on IBA biosynthesis in plants showed that its concentrations might regulate plant responses to various stresses (Ludwig-Müller *et al.*, 1995 and 2000). Also, numerous reports indicated that *Arbuscular mycorrhizal* fungi (AMF) set symbiosis relationship with most plant families (Smith and Read, 2008). The AMF improves plant growth and vegetative development, as well as increases the fruit yield (Dasgan *et al.*, 2008 and Tüfenkçi *et al.*, 2012). *Arbuscular mycorrhizal* fungi

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control root settlement, which leads to better utilization of nutrients, particularly nitrogen, phosphorus, and microelements, from the rhizosphere (Dasgan *et al.*, 2008 and Salvioli *et al.* 2012). Mwangi *et al.* (2011) reported a positive effect of AMF on plant growth and development is enhanced along with the decrease of rhizosphere abundance. Therefore, the objective of the current study was to investigate the effects of HA, IBA, and AMF on enhancing the vegetative growth, yield, and phytochemical characteristics of *Hibiscus Sabdariffa* (Roselle).

MATERIALS AND METHODS

Field experimental design and growth promoter's treatment

Field experiments were conducted during the two successive summer seasons of 2015/2016 and 2016/2017. The experiments were executed in an open field located at Bader region, El-Beheira Governorate, Egypt. Seeds of Roselle (local variety) were obtained from the Medicinal and Aromatic Plants Dept. Desert Research Center El-Mataria, Cairo, Egypt. Seeds were sown on the 1st of April of each season in the field plots (5×5 m), each plot contained 50 plants where the distance between rows was 100 cm and between plants within the row was 50 cm (Attia and Khater 2015). Soil samples were collected for physical and chemical analyses according to Black *et al.* (1965) and the results are shown in Table A. The analyses were carried out in the Department of Natural Resource and Engineering Soil, Faculty of Agriculture, Damanshour University.

Table A. Some physical and chemical analyses of the experimental soil in 2015/2016 and 2016/2017 growing seasons

Parameter & Unit	First season	Second season
Sand (%)	47.3	46.8
Silt (%)	36.2	36.4
Clay (%)	16.5	16.8
Soil texture	Sandy-loam	Sandy-loam
pH (in water)	7.6	7.56
EC (ds·m ⁻¹)	0.96	0.971
Organic matter (%)	0.97	0.972
Total N (%)	0.17	0.16
Assimilable P (ppm)	10.8	10.72
Exchangeable K (ppm)	105	103

Plants were treated with the following treatments: 1- control (tap water), 2- 100 ppm solution of IBA as a spray treatment, 3- Humic acid was added as a ground application at a rate of 3 Kg/Fadden. Indole butyric acid and humic acid were applied twice after 45 and 60 days

of seed germination. 4- Mycorrhizal fungi (AMF) were applied as seed inoculation treatment (*Glomus mosseae*).

The following data were recorded:

Vegetative growth characters and fruit yield

Three plants from each experimental plot were randomly chosen and tagged for vegetative growth traits, notably: plant height (cm), number of branches per plant, leaf area (cm²) according to Zidan (1962). By the end of the experiment, stem diameter (cm), shoot fresh and dry weights per plant (g) were determined without the inflorescences and roots. At harvest stage (after 220 days of sowing), the number of fruits per plant, fruits fresh weight (g/plant), and dry sepals yield per plant (g) were determined.

Chlorophyll and carotenoids analyses in leaves

Leaf chlorophyll a and b and total carotenoid contents (µg/mL) were measured spectrophotometrically according to (Lichtenthaler and Buschmann, 2001).

Phytochemical analyses of calyces

A- Calyces were collected from each treatment then dried at 70°C to constant weights to determine: moisture contents, ash, and crude fiber which were determined by AOAC (2000) methods. Total lipids were extracted with chloroform: methanol (2:1) and quantified gravimetrically (Christie, 1983). The nitrogen content (N) of the sample was estimated by the method described by Kjeldahl (1983) and crude protein was calculated as $N \times 6.25$. Total carbohydrate content was obtained by the difference between the weight of the sample and total weights of its moisture, ash, total lipid, protein, and fiber contents (Muler and Tobin, 1980).

B- Calyces belonging to each treatment were combined and dried at ambient temperature under shade in the open field. Then samples were ground into fine powders and were stored at -20° C until been extracted for the phytochemical analyses. About 5 g of Roselle calyces were homogenized in 100 ml of 0.1 N HCl in ethanol (Du and Francis, 1973) to determine:

1. Total anthocyanin contents

Total anthocyanin contents of Roselle extract was determined calorimetrically according to Du and Francis (1973). Briefly, a 10 ml volume of the filtered extract was diluted to 100 ml with the extracting solvent. The colour intensity was measured at 535 nm for acidified ethanol using Spectrophotometer (model T80 x UVNIS Spectrometer PG Instruments Ltd). The total anthocyanin contents were calculated as mg cyanidin-3-glucoside equivalent/mL of extract.

2. Total flavonoid contents

Flavonoid contents were determined according to Zilic *et al.* (2012). Briefly, 250 μl of 5% NaNO_2 was mixed with 500 μl of extract. After 6 min, 2.5 ml of a 10% AlCl_3 solution was added. After 7 min, 1.25 ml of 1 M NaOH was added, and the mixture was centrifuged at 5000 g for 10 min. The absorbance of the supernatant was measured at 510 nm against the blank. The total flavonoid contents were expressed as μg of catechin equivalent (CE)/mL of sample.

3. Total phenolic contents

Total phenolic contents were determined using the Folin-Ciocalteu reagent according to Singleton *et al.* (1999). Generally, 500 μl from the extract was transferred into a test tube and oxidized with the addition of 250 μl of Folin-Ciocalteu reagent. After 5 min, the mixture was neutralized with 1.25 ml of 20% aqueous Na_2CO_3 solution. After 40 min, the absorbance was measured at 725 nm against the solvent blank. The total phenolic contents were determined through a calibration curve of Gallic acid and expressed as μg of Gallic acid equivalent (GAE)/mL of sample.

4. Determination of ascorbic acid content

The calyx samples were alkaline-hydrolyzed according to Kim *et al.* (2006). About 1 g of each sample was placed in a quick fit conical flask with 20 ml of NaOH (2M) and the flasks were flushed with N_2 and the stopper was replaced. The samples were shaken at 180 rpm for 4 h at room temperature. The pH was adjusted to 2 with HCl (6M). The samples were centrifuged at 5000 rpm for 10 min and the supernatant was collected. Phenolic compounds were extracted twice with 50 ml ethyl acetate and ethyl ether 1:1. The organic phase was separated and evaporated at 45°C and the samples redissolved in 2 ml methanol. Determination of ascorbic acid was determined using the indophenols method (AOAC, 2000).

5. Analysis of phenolic compounds by HPLC

The calyx samples were prepared for HPLC analysis as follows: 100 mg of dry sample was extracted with 0.9 ml of 90 % MeOH (with 0.5 mM meta-phosphoric acid and 0.02 mM EDTA) in 1.5 ml Eppendorf tubes. The tubes were vortexed for 60 s, sonicated for 30 min in cold water (4°C), and centrifuged for 15 min at 4°C at 5000 rpm. The supernatant was collected into a 1.5 ml glass vial. The samples were re-extracted by adding 0.6 ml of extraction buffer as mentioned before and the supernatants were combined into the same 1.5 ml glass vial. Supernatants in glass vials were vacuum-dried for 6–8 h in a speed-vac. Dried-samples were re-solubilized in 500 μl of extraction buffer. Extracts were filtered into a 1 ml HPLC glass vial using a 1 ml syringe and a 0.2

μm nylon filter (Fisher Scientific, Ottawa, ON). About 50 μl of each sample were injected twice into the HPLC.

The HPLC analysis was carried out using Agilent Technologies 1100 series liquid chromatography equipped with an autosampler and a diode-array detector. The analytical column was an Eclipse XDB-C18 (150 \times 4.6 μm ; 5 μm) with a C18 guard column (Phenomenex, Torrance, CA). The mobile phase consisted of acetonitrile (solvent A) and 2% acetic acid in water (v/v) (solvent B). The flow rate was kept at 0.8 ml/min for a total run time of 70 min and the gradient program was as follows: 100% B to 85% B in 30 min, 85% B to 50% B in 20 min, 50% B to 0% B in 5 min and 0% B to 100% B in 5 min. The injection volume was 50 μl and peaks were monitored simultaneously at 280 and 320 nm for the benzoic acid and cinnamic acid derivatives, respectively. Before injection, all samples were filtered through a 0.45 μm Acrodisc syringe filter (Gelman Laboratory, MI). Peaks were identified by congruent retention times and UV spectra and compared with those of the standards.

6. Determination of radical DPPH scavenging activity

The free radical scavenging capacity of samples was determined using the 1,1-diphenyl-2-picrylhydrazyl (DPPH) according to Hwang and Do-Thi (2014). The final concentration was 50 μM for DPPH and the final reaction volume was 3.0 ml. The absorbance at 517 nm was measured against a blank of pure methanol. Percent inhibition of the DPPH free radical was calculated by the following equation:

$$\text{Inhibition (\%)} = 100 \times (A_{\text{control}} - A_{\text{sample}}) / A_{\text{control}}$$

where:

A_{control} is the absorbance of the control reaction (containing all reagents except the test compound).

A_{sample} is the absorbance of the test compound.

Also, the antioxidant activity was determined by means of a calibration curve prepared with Trolox and expressed as mg of Trolox equivalent (TE) per unit (volume or weight) of the sample.

7. Determination of pH sample and total titratable acidity (TTA)

The pH of Roselle calyx was measured using a pH meter (model Cyber Scan 500) standardized with buffer solutions of 4.0 and 7.0 according to the method of AOAC (2000). Total titratable acidity was expressed as % citric acid was determined by standard AOAC (2000) using NaOH (0.1N) and phenolphthalein as an indicator.

8. Mineral content

Iron (Fe), magnesium (Mg), and zinc (Zn) were determined in aliquots of the solutions of the ash that were established according to the method of AOAC (2000) using an Atomic Absorption Spectrophotometer

(Perkin-Elmer Model 2380 manufacture, USA). Calcium (Ca), sodium (Na), and potassium (K) were determined using the Flame Photometer according to the method described by Pearson (1976).

Experimental Design and statistical analysis

The experiments were applied according to complete randomized blocks design (RCBD) with three replicates (Snedecor and Cochran, 1967). Data were statistically analyzed using SAS software program (2013). Comparisons among the means of different treatments were achieved using the revised least significant difference procedure at P= 0.05 level as illustrated by Al-Rawy and Khalf-Allah (1980).

RESULTS AND DISCUSSION

Vegetative growth and yield

Data for vegetative growth parameters of Roselle plants during the two growing seasons of 2015/2016 and 2016/2017 were given in Table (1). Regarding the effect of individual treatments of humic acid, indole butyric acid (IBA) and *Arbuscular mycorrhizal* fungi (AMF), data showed clearly that the application of these treatments significantly enhanced the vegetative growth parameters in both seasons. The obtained results showed that humic acid was the most effective treatment compared to the other treatments in increasing plant height (203.07 and 193.2 cm), number of branches per plant (9.93 and 11.53), stem diameter (2.099 and 1.96 cm), leaf area (3.34 and 3.35 cm²), shoot fresh weight (1068.97 and 1083.70 g) and shoot dry weight (247.19 and 250.91 g) for the first and second season, respectively. The present results were in parallel with those reported by Khalil and Yousef (2014), Al-Mohammad *et al.* (2016) and Hewidy *et al.* (2018) on

Hibiscus sabdariffa L. and Said-Al Ahl *et al.* (2016) on dill.

The augmentation of vegetative growth parameters of Roselle plants might be attributed to the role of humic acid in improving soil structure, changing the physical properties of soil, promoting the chelation of many elements, and making nutrients available to plants. Subsequently, helping in correcting the plant chlorosis and enhancing of photosynthesis density and plant root respiration, which results in improved plant growth (Chen and Avid, 1990). Also, humic acid stimulates plant growth through absorption of major and minor elements, changes in membrane permeability, protein synthesis, enzyme activation, and the activation of biomass production (Ulukan, 2008). Moreover, humic acid plays an important role in improving nitrogen assimilation and at the same time facilitating the synthesis of chlorophyll, sugars, essential amino acids, vitamins, and oils (Khalil *et al.*, 2012). It increases root growth by increasing cell elongation or root cell membrane permeability, which leads to increasing the water and nutrients uptake, so improving plant growth and development (Vaughan, 1974 and Rauthan and Schnitzer 1981).

The increase in fruit number per plant with humic acid treatment might be due to the superiority of this treatment in the number of branches and leaf area (Table 1), which led to increase photosynthesis (Jain, 2002), which in turn was reflected in increasing fruit number. Also, humic acid as an organic fertilizers improved the physical, chemical and microbiological characteristics of the soil (Atiyeh *et al.*, 2000) and increased the holding capacity of soil (Eghbal *et al.*, 2004), which lead to increase yield (Ahmadian *et al.*, 2011 and Kalvanagh and Heris, 2013).

Table 1. Effect of HA, IBA and AMF treatments on vegetative growth characteristics and yield of Roselle plants during the two summer seasons of 2015/2016 and 2016/2017

Parameters	2016				2017			
	Control	HA	IBA	AMF	Control	HA	IBA	AMF
Plant Height (cm)	183.7 c	203.07 a	199.67b	196.27b	161.3d	193.2 a	185.33 b	181.28 c
No. of Branches	5.73 d	9.93 a	8.63 b	7.13 c	7.2 d	11.53 a	10.03 b	8.99 c
Stem Diameter (cm)	1.52 b	2.099 a	1.57 b	1.74 b	1.54 b	1.96 a	1.69 b	1.68 b
Leaf Area (cm ²)	1.70 d	3.34 a	2.14 c	2.81 b	1.67 d	3.35 a	2.19 c	2.75 b
Shoot Fresh Weight (g)	947.67d	1068.97a	999.00 c	1038.00 b	961.93 d	1083.70a	1002.1 c	1039.00b
Shoot Dry Weight (g)	194.33 d	247.19 a	229.38b	221.33 c	200.23 d	250.91 a	230.85 b	220.78 c
No. of Fruits/Plant	68.37 d	124.87a	112.17c	118.54 b	70.25 d	140.67 a	121.8 c	131.67 b
Fruits Fresh Weight /Plant (g)	359.2 d	758.44 a	486.29c	606.93 b	302.4 c	969 a	337.1 c	421.2 b
Dry Sepals Yield / Plant (g)	25.67 d	52.45 a	43.26 c	47.68 b	25.67 d	52.45 a	43.26 c	47.68 b

Means having the same letter (s) within the same row are not significantly different according to LSD for all-pairwise comparisons test at 5% level of probability. *(HA) Humic acid, (IBA) Indole butyric acid, (AMF) Arbuscular mycorrhizal fungi.

The increase in fruit fresh weight and sepals dry weight might be due to the effects of humic acid treatment in increasing the photosynthetic activity and different metabolic processes. Also, humic acid plays an important role in stimulating and increasing cell division, as well as optimized uptake of nutrients and water (Atiyeh *et al.*, 2002 and Chen *et al.*, 2004), which leads to increase fruit fresh weight then sepals dry weight. The aforementioned results were similar to those of Khalil and Yousef (2014), Al-Mohammad *et al.* (2016) and Hewidy *et al.* (2018) on *Hibiscus sabdariffa* L.

Arbuscular mycorrhizal fungi (AMF), have been shown to promote plant growth by many researchers, such as (a) enhancing nutrient uptake (Evelin *et al.* 2012); (b) improving rhizospheric and soil conditions (Asghari *et al.* 2005); (c) producing plant growth hormones and (d) improvement in photosynthetic activity or water use efficiency (Hajiboland *et al.* 2010). The increment of vegetative growth parameters by using AMF are in agree with those reported by Kasliwal and Srinivasamurthy (2016) on *Hibiscus rosa sinensis*.

The role of IBA in increasing plant growth may be to the fact that IBA produced healthier lengthy roots and vigorous root system which enhanced the absorption of minerals and water from the soil resulting in produce more branches, more number of leaves and increasing leaf area. The increase in leaf area increases the photosynthetic activity resulting in increased carbohydrates which results in high growth (Kaur, 2015). These results were similar to the findings of Shahab *et al.*, (2013) on *Alstonia*.

Leaf chlorophyll a and b and total carotenoid contents of leaves of *H. sabdariffa*

Data in Table (2) showed generally that the application of humic acid treatment significantly increased the highest values of leaf chlorophyll a, b and total carotenoid contents compared to the other treatments, in both seasons. However, the differences between humic acid and indole butyric acid in total carotenoid were the same in both seasons. Humic acid treatment recorded the highest values of chlorophyll a (17.72 and 18.03 µg/mL) and chlorophyll b (8.47 and 8.19 µg/mL) in the first and second seasons, respectively. Also, humic acid and indole butyric acid treatments recorded the highest values of total carotenoid contents (3.95 and 4.09 µg/mL) and (4.24 and 4.39 µg/mL) for the first and second seasons, respectively.

The enhancement effect of humic acid in increasing photosynthesis pigments might be due to its ability to increase the activity of soil organisms, which in turn release more nutrients such as iron from unavailable reserves that produce more chlorophyll pigments (Wallace and Khadr, 1996). Moreover, Dekock (1955) reported that humic acid prevents the immobilization of Fe and P and facilitates their translocation from roots to shoots making them available for plants in chlorophyll production. Tahir *et al.* (2011) reported that leaf chlorophyll content was associated with humic acid substances as a result of increased cell membrane permeability, thus increasing the absorption of nutrients, especially nitrogen. Current results of humic acid were in harmony with those reported by Koocheki *et al.* (2016) on *Crocus sativus* L. and Ariaifar and Forouzandeh (2017) on black cumin.

The enhancement effect of indole butyric acid (IBA) in increasing photosynthesis pigments might be due to the stimulatory effect on the amount of metabolites synthesized through enhancement of cell division and chlorophyll accumulation which led to higher rate of photosynthesis (Midan *et al.* 1982 and Ludwig-Müller *et al.* 2000). The obtained results of indole butyric acid were in agreement with those obtained by Shaddad and El-Tayeb (1990) and Abdel-Wahed and Amin (2006) on maize.

Phytochemical analyses of Calyces

Data in Tables (2 and 3) showed the calyx chemical composition of Roselle plants during the two growing seasons of 2015/2016 and 2016/2017. Regarding the effect of individual treatment of HA, IBA and AMF, data in Table 2 showed that there were significant differences between all treatments in increasing calyx chemical composition parameters compared to control, in both seasons. Humic acid was the most effective treatment compared to the rest of treatments in increasing moisture content (13.40 and 13.02%), ash content (13.45 and 13.69 g/ 100g DW), total acidity (19.59 and 19.53 %), total carbohydrate contents (67.63 and 67.2 g/ 100g DW) and total protein content (11.14 and 11.03 g/ 100g DW) for the first and second season, respectively.

The reason behind increasing moisture content as a result of humic acid application might be attributed to the role of humic acid in increasing uptake of both macro and micronutrients availability for plants (Maggioni *et al.*, 1987 and Mackowiak *et al.*, 2001), which lead to exposing water quantities, then increasing moisture availability (Sonnenberg, 2012). The moisture contents might be affected by genetic factors and the long storing period of the Roselle calyces, which cause the dryness (Suliman *et al.*, 2011).

Table 2. The effect of HA, IBA and mycorrhiza (AMF) on leaves pigments content and calyx chemical composition parameters of Roselle plant during the two seasons of 2015/2016 and 2016/2017

Parameters	2016				2017			
	Control	HA	IBA	AMF	Control	HA	IBA	AMF
Leaves pigments content								
Chlorophyll a (µg/mL)	12.17 c	17.72 a	16.12 b	13.94 b	12.71 d	18.03 a	16.50 b	14.65 c
Chlorophyll b (µg/mL)	6.21 c	8.47 a	7.02 b	7.34 b	6.51 b	8.19 a	7.26 ab	7.17 ab
Total Carotenoids (µg/mL)	3.71b	3.95 a	4.42 a	3.22 c	3.75 b	4.09 a	4.39 a	3.43 b
Calyx chemical composition parameters								
Moisture (%)	12.94 c	13.40 a	12.67 d	13.25 b	12.47 b	13.02 a	12.39 b	12.45 b
Ash (g/ 100g DW)	11.71 b	13.45 a	10.18 c	10.21c	10.06 d	13.69a	10.41 c	11.78 b
Acidity (% as a citric acid)	19.18 c	19.59 a	19.06 d	19.37 b	19.14 c	19.53 a	18.98 d	19.26 b
Total Carbohydrates (g/ 100g DW)	66.93 b	67.63 a	65.26 c	66.93 b	66.34 c	67.20 a	65.12 d	66.75 b
Total Protein (g/ 100g DW)	10.44 c	11.14 a	10.37 c	11.06 b	10.56 c	11.03 a	10.15 d	10.89 b
pH	2.73 a	2.60 c	2.72 a	2.65 b	2.74 a	2.61b	2.74 a	2.63 b
Total Lipids (g/ 100g DW)	0.80 b	0.83 a	0.85 a	0.85 a	0.82 b	0.92 a	0.93 a	0.89 a
Crude Fiber (g/ 100g DW)	10.12 c	10.22 b	10.08 c	10.45 a	10.39 c	10.89 b	10.10 d	11.06 a

Means having the same letter (s) within the same row are not significantly different according to LSD for all-pairwise comparisons test at 5% level of probability. *(HA) Humic acid, (IBA) Indole butyric acid, (AMF) Arbuscular mycorrhizal fungi.

Table 3. The effects of HA, IBA and mycorrhiza (AMF) on calyx chemical composition parameters of Roselle plant during two seasons of 2015/2016 and 2016/2017 analyzed using the HPLC

Parameters	2016				2017			
	Control	HA	IBA	AMF	Control	HA	IBA	AMF
Ascorbic Acid (mg/ 100g)	192.26 b	205.06 a	188.30 b	201.65 a	187.19 ab	203.00 a	179.16 b	198.22 ab
Total Phenolics (mg/ 100g)	30.08 c	31.49 a	30.61 b	31.23 a	27.92 c	29.47 a	28.41 b	29.13 a
Total Flavonoids (mg/ 100g)	1.58 d	2.18 a	1.70 c	1.95 b	1.55 c	2.10 a	1.58 c	1.86 b
Total Anthocyanins (mg/100g)	556.09 d	669.71 a	635.79 c	662.75 b	544.92 d	650.38 a	610.26 c	643.73 b
Gallic Acid (µg/ 100g)	138.57 d	178.72 a	145.05 c	160.70 b	139.26 c	178.83 a	143.51 c	162.09 b
Caffeic Acid (µg/ 100g)	3278.51b	3697.07 a	3267.74 b	3104.32 c	2920.00c	3570.00 a	3200.23b	3011.24bc
Syringic Acid (µg/ 100g)	480.23 b	592.51 a	384.33 c	473.86 b	484.11 b	593.66 a	390.30 c	476.25 b
Coumaric acid (µg/ 100g)	43.84 a	43.71 a	37.28 b	36.10 b	44.48 a	43.84 a	37.54 b	36.21 b
Ferulic Acid (µg/ 100g)	64.52 b	83.90 a	64.35 b	64.16 b	63.71 b	83.44 a	63.87 b	63.02 b
Sinapic Acid (µg/ 100g)	53.87 c	74.35 a	49.19 d	61.68 b	53.17 c	73.08 a	48.77 d	60.35 b
Rosmarinic Acid (µg/ 100g)	38.64 d	54.07 a	42.31 c	45.19 b	37.93 c	53.73 a	41.41 b	43.47 b
Rutin (µg/ 100g)	12.61 d	25.14 a	15.83 c	19.58 b	12.98 d	25.41 a	16.25 c	20.51 b
Quercetin (µg/ 100g)	13.72 b	16.38 a	9.77 c	13.58 b	13.65 b	15.74 a	9.52 c	13.25 b
Kaempferol (µg/ 100g)	2.73 c	3.31 a	2.66 c	2.96 b	2.49 c	3.15 a	2.44 c	2.89 b
Chrysin (µg/ 100g)	89.04 c	98.30 a	93.62 b	96.82 ab	88.06 b	97.53 a	91.32 ab	95.32 a
DPPH (µ/ml)	54.58 d	59.64 a	56.74 c	58.15 b	50.77 d	56.93 a	53.24 c	54.73 b

Means having the same letter (s) within the same row are not significantly different according to LSD for all-pairwise comparisons test at 5% level of probability. *(HA) Humic acid, (IBA) Indole butyric acid, (AMF) Arbuscular mycorrhizal fungi.

The increment of ash content might be attributed to the role of humic acid in increasing root growth, nutrient uptake and consequently stimulated plant growth (Fernandez-Escobar *et al.*, 1999), which leads to increasing calyx dry weight then increasing total solids. Our results of moisture and ash content are in harmony

with those of Suliman *et al.* (2011) on *Hibiscus sabdariffa* L.

The high acidity of plants treated with humic acid compared to the non-treated plants could be attributed to the utilization of additional carbon source provided from the organic fertilizers in the production of organic

acids, which are responsible for the fruit acidity (Aminifard *et al.*, 2012). The enhancing effect of humic acid on titratable acidity was in parallel with the findings of Aminifard *et al.* (2012) on hot pepper and Fallahi *et al.* (2017) on Roselle.

The increment of total carbohydrate contents with humic acid application might be due to the role of humic acid in increasing root growth by increasing cell elongation or root cell membrane permeability, which lead to increase water and nutrients uptake by increasing root surface area, so increase the process of photosynthesis and the representation of carbohydrates that reflect positively on the process of storage vehicles (AlTohafi *et al.*, 2015). Moreover, the whole Roselle plant is rich in carbohydrates (FAO, 1998). The obtained results were in agreement with Shehata *et al.* (2011) on strawberry and Gendy *et al.* (2012) and Fallahi *et al.* (2017) on *Hibiscus sabdariffa* L.

The increased total protein by humic acid application might be due to the role of humic acid in the assimilation of major and minor elements, enzyme activation, changes in membrane permeability, protein synthesis and the activation of biomass production (Ulukan, 2008). These results are in harmony with those obtained by Fallahi *et al.* (2017) on *H. sabdariffa* L.

It was noticed that the pH values of sepals ranged from (2.60 to 2.74) in both seasons (Table 2). The highest value of pH was obtained for control (2.73 and 2.74) and IBA (2.72 and 2.74) treatments in both seasons, respectively. But, the lowest value was obtained after humic acid treatment (2.60 and 2.61). The increment of pH might be due to the influence of these treatments in producing nitrogenous compounds that increase the pH value.

Data in Table 2 showed clearly that total lipid contents were less affected by treatments. Although, there were significant differences between all treatments and control in increasing total lipid contents, but there was no significant difference between all treatments of HA, IBA and mycorrhiza (AMF) in increasing total lipid contents. These results were in agreement with that of Ahmed *et al.* (2019) on *H. sabdariffa* L. Also, data indicated that the AMF treatment showed the highest value of crude fiber contents. These results were slightly less than the values reported by Suliman *et al.* (2011) that were ranged between (13.2 and 12 g/100g DW).

It was clear from data in Table 3 that there were significant differences between all treatments in increasing calyx chemical composition parameters i.e., ascorbic acid, total phenolics, total flavonoids, total anthocyanin, gallic acid, caffeic acid, syringic acid, coumaric acid, ferulic acid, sinapic acid, rosmarinic acid, rutin, quercetin, kaempferol and chrysin in both seasons compared to the control. The highest value of

these parameters was observed after the treatment of humic acid compared to other treatments in both seasons. Also, the treatment with AMF led to a significant increment in total phenolics and chrysin in both seasons and ascorbic acid content in the first season only. Moreover, control treatment leads to a significant increment in coumaric acid content in both seasons as compared with IBA and AMF treatments.

Current study reported that gallic, caffeic and syringic acids, were the most abundant phenolics in Roselle. Phenolic compounds partially increased by the application of humic acid in both seasons and these results were in line with earlier findings on tomato, where organic nutrition management increased phenolic compounds in fruit (Toor *et al.*, 2006). Humic substances have been shown to enhance the expression of catalyzes enzyme, which was considered the first step in the biosynthesis of phenolic compounds (Canellas *et al.*, 2015). Moreover, Roselle calyces are one of the excellent sources of anthocyanins. These compounds contribute significantly in enhancing health as a good source of antioxidants as well as a natural food colourant (Chumsri *et al.*, 2008). The increase of anthocyanin in fruits after the humic acid treatment might be attributed to the increase in vegetative growth and leaf area (Table1), which has increased the photosynthesis products that involved in pigment production (Dumas *et al.*, 2003). Also, humates increase the uptake of both macro and micronutrients (Maggioni *et al.*, 1987 and Mackowiak *et al.*, 2001), which play an important role in the production of pigments. Moreover, the role of humic acid (phenolic compound derivatives) as a precursor for the synthesis of anthocyanin (flavonoid structure), which increase the antioxidants activity (Ahmed *et al.*, 2011). Shehata *et al.* (2011) reported that, by using compost, the anthocyanin levels in strawberry fruit have increased. The previous data of calyx anthocyanin content in close conformity with the findings of Gendy *et al.* (2012), Fallahi *et al.* (2017), Hashem *et al.* (2017) and Attiam (2018) on *H. sabdariffa* L.

Antioxidant activity determined by DPPH method (Brand-Williams *et al.*, 1995) showed increased values of DPPH after the humic acid treatment (Table 3). These results were consistent with the values of ascorbic acid, total phenolic, total flavonoids and total anthocyanin content reported by Al-Kahtani and Hassan (1990) and Sukhapat *et al.* (2004). The increment of antioxidants content in Roselle sepals by humic acid application might be due to the role of the humic acid in the provision of nutrients and the increase of the efficiency of absorption by the plant and thus increase the process of photosynthesis and carbohydrates (Hendawy, 2008). Ziadi *et al.* (2001) and Achuo *et al.* (2004) indicated that the foliar application of humic acid

consistently increase the antioxidants such as α -tocopherol, α -carotene, superoxide dismutase, and ascorbic acid concentrations in turf grass species. Finally, these results showed that humic acid was required to provide the soil and the plant with nutrient elements that stimulate all metabolism synthesis processes and then increase the secondary metabolism compounds (Al-Mohammad *et al.*, 2016).

Calyx minerals content

Data in Table (4) indicated that there were significant differences between all treatments in increasing the content of Ca, Mg, K, Na, P, Fe, and Zn in Roselle plants. Arbuscular mycorrhiza treatment was the most effective treatment compared to the rest of treatments in increasing Ca content (936.75 and 945.50 mg/Kg), P (53.01 and 60.50 mg/Kg), Fe content (43.68 and 45.80 mg/Kg) and Zn contents (11.57 and 11.40 mg/Kg) in calyx in the first and second season, respectively. Also, humic acid treatment showed significant difference and higher values of Mg content (326.50 and 304.50 mg/Kg), K content (37.75 and 36.25 mg/Kg) and P contents (63.03 and 60.90 mg/Kg) for the first and second seasons, respectively. Furthermore, Na content was decreased after the applications of HA, IBA and AMF treatments compared to the control in both seasons.

In general, minerals content in plants depends on many factors such as fertilization, and genetic factors. Many studies indicated that negatively charged particles of humates help in chelating most of plant nutrients and water molecules. Humates are beneficial in release

nutrients in the soil so that they became available to plants. Also, humates might promote the uptake of some nutrients and reduce the uptake of toxic elements (Khaled and Fawy, 2011). Humates ameliorate nutrient uptake especially phosphorus, stimulates soil biological activity, and acts as a storehouse of N (Mazhar *et al.*, 2012). Also, the application of humates increased the uptake of both macro and micronutrients availability for plants (Maggioni *et al.*, 1987 and Mackowiak *et al.*, 2001). Humic acid plays a positive effect on cell membrane functions by promoting nutrient uptake, respiration, biosynthesis of nucleic acid, ion absorption, enzyme because it is hormone-like substance (Yang *et al.*, 2004). Mycorrhiza improves the plant growth by capturing relatively immobile nutrients like phosphorus (Souchie *et al.*, 2006), macro-elements (Hodge *et al.*, 2001), and some microelements (Faber *et al.*, 1990 and Anju *et al.*, 2011). Mycorrhizal hyphae simplify the absorption of available forms of nutrients by increasing the effective soil volume and adjustment of soil chemical properties (Sembok *et al.*, 2015). Therefore, uptake of nutrients such as P, N, K, Fe, Zn, Cu, Mg, S, and other ions, is usually improved by mycorrhiza inoculation (Aulia *et al.*, 2009). Earlier studies have also shown that AMF (*Glomus mosseae*) increases the P content, Zn concentration, bio volume index and quality index (Sumana and Bagyaraj, 1999). The aforementioned results are in good accordance with those postulated by Gendy *et al.* (2012) on *H. sabdariffa* L and Kasliwal and Srinivasamurthy (2016) on *H. rosa sinensis*.

Table 4. The effects of HA, IBA and mycorrhiza (AMF) on calyx mineral contents of Roselle plant during the two seasons of 2015/2016 and 2016/2017

Mineral	2016				2017			
	Control	HA	IBA	AMF	Control	HA	IBA	AMF
Ca (mg/Kg)	872.25 d	919.75 b	880.00c	936.75 a	847.50 c	881.00 b	841.50d	945.50a
Mg (mg/Kg)	293.00 d	326.50 a	301.25c	311.25b	284.00 d	304.50 a	288.00c	295.50b
K (mg/Kg)	35.00 c	37.75 a	32.66 d	36.08 b	34.50 c	36.25 a	32.13 d	34.97 b
Na (mg/Kg)	13.38 a	12.43 b	12.63 b	12.62 b	13.80 a	12.35 b	12.10 b	12.90 b
P (mg/Kg)	61.43 b	63.03 a	60.15 c	63.01 a	59.50 b	60.90 a	58.05 c	60.50 a
Fe (mg/Kg)	38.33 d	41.68 b	39.82 c	43.68 a	39.85 d	44.20 b	41.09 c	45.80 a
Zn (mg/Kg)	8.84 d	10.13 c	10.70 b	11.57 a	8.58 d	9.75 c	10.20 b	11.40 a

Means having the same letter (s) within the same column are not significantly different according to LSD for all-pairwise comparisons test at 5% level of probability *(HA) Humic acid, (IBA) Indole butyric acid, (AMF) Arbuscular mycorrhizal fungi.

CONCLUSION

Results of the current study indicated, generally, that application treatments of humic acid, indole butyric acid, and AMF to Roselle plants gave the highest yield and good quality compared to un treated plants. Humic acid might be considered as an optimal treatment for the production of high yield and good quality of Roselle plants under the environmental conditions of EL-Beheira Governorate and other similar regions.

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الملخص العربي

تأثير حامض الهيوميك و حمض الإندول بيوتيريك و فطر الميكروهيذا كمحفّزات للنمو على المحصول والخصائص الكيميائية لنبات الكركديه

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وحمض الإندول بيوتيريك وفطر الميكروهيذا بشكل منفرد قد أدت إلى زياده معنويه فى النمو الخضري ومحصول السبلات ومحتواها الكيماوى لنباتات الكركديه. وقد سجلت معاملة حامض الهيوميك أعلى القيم وذلك لأغلب الصفات موضع الدراسة وبالتالي يمكن اعتبارأنها المعاملة المثلى لزياده النمو الخضري ومحصول السبلات والمحتوى الكيماوى للسبلات لنباتات الكركديه وذلك تحت الظروف البيئية لمنطقه بدر ومحافظه البحيره والمناطق الأخرى المماثله لها.

أجريت تجربتان حقليتان علي مدي موسمين متعاقبين خلال صيف ٢٠١٥/٢٠١٦ وصيف ٢٠١٦/٢٠١٧ في حقل مفتوح يقع في منطقة بدر - محافظة البحيرة - جمهورية مصر العربية. وتم تقييم تأثير المعاملة بواسطه حامض الهيوميك وحمض الإندول بيوتيريك وفطر الميكروهيذا وذلك بشكل فردي على النمو الخضري ومحصول السبلات ومحتواها الكيماوى لنباتات الكركديه. وقد صممت التجربه فى صوره قطاعات كاملة العشوائيه. وأوضحت النتائج خلال الموسمان بشكل عام أن جميع المعاملات: حامض الهيوميك