Biological Forum – An International Journal **7**(1): 1704-1709(2015)

ISSN No. (Print): 0975-1130
ISSN No. (Online): 2249-3239

Humic acid as an Ecological Pathway to Protect Corn Plants against Oxidative Stress

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(Received 27 April, 2015, Accepted 07 June, 2015)
(Published by Research Trend, Website: www.researchtrend.net hamid_tohidi2008@yahoo.com*)*

ABSTRACT: In order to study effect of humic acid (HA) foliar application and limited irrigation, on growth and quantitative characteristics of corn an experiment was conducted in research field of Varamin in Iran during 2012 growing season. The experimental design was laid out in a randomized complete block with a split plots arrangement of treatments in three replications. Main plots included three different levels of irrigation (complete irrigation, irrigation withholding at 8-leaf stage and irrigation withholding at staminate inflorescence) and four different concentration of HA foliar application (0, 150, 300 and 450 ppm) was allocated to subplots were. The results showed that irrigation withholding conditions in different growth stages significantly decreased seed yield and biological yield but by contrast increased antioxidant enzymes activity and lipid, protein and nucleoside peroxidation. It appears that HA act in plants via a specific form of stress that is detected by anti-stress defense systems in plants. These HA applied to plants can protect against water stress in degraded soils.

Keywords: Humic acid, corn, irrigation withholding, seed yield, antioxidant enzymes.

INTRODUCTION

Across the globe today, maize is a direct staple food for millions of individuals and, through indirect consumption as a feed crop, is an essential component of global food security (Campos *et al.,* 2004). Water stress induces oxidative stress in plants (Hajiboland and Joudmand, 2009). Under conditions of water stress and other types of environmental stress, reactive oxygen species (ROS), such as superoxide anion radicals, hydrogen peroxide and hydroxyl radicals, are generated (Zhu 2000). These free radicals can damage essential membrane lipids as well as proteins (PROT) and nucleic acids (Noctor and Foyer 1998). Plant cells contain an array of protection mechanisms and repair systems that can minimize the occurrence of oxidative damage caused by reactive oxygen species (ROS) (Abdel Latef, 2010). Mechanisms of active oxygen species detoxification exist in all the plants and include activation of enzymatic (superoxide dismuatase, catalase, ascorbat peroxidase, peroxidase, glutathione reductase (Meloni *et al.,* 2003). Humic acids (HA) can protect plants in water deficient-soils. Despite diverging views, great progress has been made toward understanding the action of humic substances (HS) in plants (Singh and Agrawal, 2010). Recently, a new mode of action for HA suggests that HA can cluster in roots to affect transpiration and, therefore, the hydraulic conductivity of the roots via colloidal stress (Asli and Neumann, 2010). Other authors have observed effects on antioxidative defense mechanisms, reporting the stimulation of catalases (CAT) and the generation of reactive oxygen species (ROS) that act as intermediaries in plant growth (Cordeiro *et al.,* 2011). Hence in this field experiment, an attempt was made to investigate the effect of humic acid foliar application on yield and antioxidant enzymes activity of corn plants under compete irrigation and irrigation withholding at different growth stages.

MATERIAL AND METHOD

In order to study effect of humic acid foliar application and limited irrigation, on quantitative and qualitative characteristics of corn an experiment was conducted in research field of Varamin in Iran during 2012 growing season. Site of study was situated at 31º 519 E and 20º 359 N and 1050 m above sea level. Latitude and longitude of research place were 35º, 19' N and 51º, 39' E, respectively and site of study was located 900 m above sea level. Before beginning of experiment, soil samples were taken in order to determine the physical and chemical properties. A composite soil sample was collected at a depth of 0-30 cm. It was air dried, crushed, and tested for physical and chemical properties. The research field had a clay loam soil. Details of soil properties are shown in Table 1. After plow and disk, plots were prepared. The experimental design was carried out in a randomized complete block with a split plot arrangement of treatments in three replications. Main plots included three different levels of irrigation (complete irrigation, irrigation with holding at 8-leaf stage and irrigation withholding at staminate inflorescence appearance stage) and four different concentration of humic acid foliar application (0, 150, 300 and 450 ppm) was allocated to subplots were.

The 18.75 m^2 plots were prepared with 5 m long and consisted of five rows, 0.75 m apart. Between all main plots, 2 m alley was kept to eliminate all influence of lateral water movement. Polyethylene pipeline was performed for control of irrigation as dropping irrigation. Treflan and gallant super were applied to control weeds. According to soil analysis, phosphorus (150 $kg.ha^{-1}$ P) and potassium (200 $kg.ha^{-1}$ k) fertilizers were applied into the soil. Nitrogen was supplied from ammonium nitrate source (300 $kg.ha^{-1}$) at three stages; seed sowing, 8-leaf stage and before flowering stage. The plots were sown with corn seeds (N.S 640) with 75 cm row to row distance and 20 cm between plants. Corn was planted manually in May 2012. Seeds were sown 3-4 cm deep. Two seeds were sown in each position and the plots thinned to the desired plant population (67000 plant per ha). After seed sowing, irrigation was applied as required during the growing season. The humic acid foliar application was applied with a pressurized backpack sprayer (121 capacity) calibrated to deliver 1000 l ha^{-1} of spray solution. Sprayer was equipped with a spiral solid cone spray nozzle. At the end of growing season crop were harvested and seed yield and biological yield were assayed.

A. Antioxidant enzyme activity assay

Superoxide dismutase activity was determined by measuring the ability of the enzyme extract to inhibit the photochemical reduction of nitroblue tetrazolium according to the method of Giannopolitis and Ries (1977). The reaction mixture contained 100 µl l µm riboflavin, 100 µl 12 mM L-methionine, 100 µl 0.1 mM EDTA (pH 7.8), 100 µl 50 mM Na_2CO_3 (pH 10.2), 100 µl 75 µM nitroblue tetrazolium in 2300 nitroblue tetrazolium 25mM sodium phosphate buffer (pH 6.8) and 200 µl crude enzyme extract, in a final volume of 3 ml. Glass test tubes that contained the reaction mixture were illuminated with a fluorescent lamp (120 W), and identical tubes that were not illuminated served as blanks. After illumination for 15 min, absorbance was measured at 560 nm. One unit of Superoxide dismutase activity was defined as the amount of enzyme which caused 50 % inhibition of photochemical reduction of nitroblue tetrazolium.

Catalase activity was estimated by the method of Cakmak and Horst (1991). The reaction mixture contained 100 µl crude extract, 500 µl 10mm H2O2 and 1400 µl 25mm sodium phosphate buffer. The decrease in the absorbance recorded at 240 nm for 1 min by a spectrophotometer.

Glutathione peroxidase activity was measured according to method of Paglia and Valentine (1997) in which 0.56 M (Ph = 7) phosphate buffer, 0.5 M EDTA, 1mM NaN_3, 0.2mM NADPH were added to the extracted solution. Glutathion peroxidase catalyses the oxidation of glutathion by cumene hydroperoxide in the presence of glutathion reductase and NADPH, the oxidized glutathion is immediately converted to the reduced form with the concomitant oxidation of NADPH to NADP. The decrease in absorbance at 340 nm was measured with a spectrophotometer.

B. Destructive biomarkers assay

The level of membrane damage was determined by measuring MDA as the end product of peroxidation of membrane lipids (De Vos *et al.,* 1991). In brief, samples were homogenized in an aqueous solution of trichloroacetic acid (10% w/v), and aliquots of filtrates were heated in 0.25% trichloroacetic acid. The amount of MDA was determined from the absorbance at 532 nm, followed by correction for the non-specific absorbance at 600 nm. The content of MDA was determined using the extinction coefficient of MDA ($\varepsilon = 155\ \mu M^{-1}\ cm^{-1}$).

The level of protein damage was determined by measuring dityrosine assay. 1.2 grams of fresh tissue material were homogenized with 5 ml of ice-cold 50 mM HEPES-KOH, pH 7.2, containing 10 mM EDTA, 2 mM PMSF, 0.1 mM p-chloromercuribenzoic acid, 0.1 mM DL-norleucine and 100 mg polyclar AT. The plan tissue homogenate was centrifuged at 5000 g for 60 min to remove debris. Purification of o,o'-dityrosine in the clear tissue homogenized supernatant fluid was accomplished by preparative HPLC. o,o'-Dityrosine was recovered by gradient elution from the C-18 column (Econosil C18, 250 mm · 10 mm) (Orhanl et al., 2004). The composition of fluent varied linearly from acetonitrile-water-TFA (1:99:0.02) to acetonitrile-water-TFA (20:80:0.02) over 25 min. The gradient was started 5 min after the injection. A flow rate of 4 ml/min was used. o,o0-Dityrosine was analyzed by reversed-phase HPLC with simultaneous UV-detection (280 nm) and fluorescence-detection (ex. 280 nm, em. 410 nm). A phenomenex inertsil ODS 2 (150 mm · 4.6 mm, 5 lm) HPLC column (Bester, Amsterdam, The Netherlands) equipped with a guard column was used for these analyses. A gradient was formed from 10 mM ammonium acetate, adjusted to pH 4.5 with acetic acid, and methanol, starting with 1% methanol and increasing to 10% over 30 min. The flow rate was 0.8 ml/min. A standard dityrosine sample was prepared according to Amado *et al.* (1984). Dityrosine was quantified by assuming that its generation from the reaction of tyrosine with horseradish peroxidase in the presence of H_2O_2 was quantitative (using the extinction coefficient e315 = 4.5 $mM^{-1}\ cm^{-1}$ at pH 7.5). Hydroxyguanosine (8OH-2'dG) was measured in the leaves essentially as described previously (Bogdanov *et al.,* 1999). Briefly, an automated column switching method for 8OH-2'dG is based on the unique selectivity of the integral porous carbon column for purines. Samples were injected onto a C8 column and the band containing 8OH-2'dG was then quantitatively trapped on a carbon column. The selectivity of the carbon column for 8OH-2'dG allows elimination of interfering peaks by washing the column with a second mobile phase and then eluting 8OH-2'dG to an analytical C18 column with an identical mobile phase containing adenosine to displace 8OH-2'dG.

Detection with series colorimetric electrodes provides qualitative certainty for 8OH-2'dG peak by response ratios.

C. Statistical analysis

All data were analyzed from analysis of variance (ANOVA) using the GLM procedure in SAS (SAS Institute, 2002). The assumptions of variance analysis were tested by insuring that the residuals were random, homogenous, with a normal distribution about a mean of zero. Duncan's multiple range tests was used to measure statistical differences between treatment methods and controls.

Table 1: Soil properties of the experimental site.

Depth	EC (ds m^{-1})	pH	O.C (%)	T.N.V (%)	K (ppm)	P(ppm)	Total N (%)	Texture
0-30 cm	4.1	7.4	0.71	<10	368	25.9	0.079	Clay loam

RESULT AND DISCUSSION

Analysis of variance showed that the effect of irrigation withholding in different growth stages was significant on all traits experiment. Also the effect of humic acid foliar application was significant on all measured traits experiment (Table 2). Interaction of experimental factors (irrigation withholding in different growth stages × humic acid foliar application) was not significant on all measured traits experiment. As can be seen from Table 4, seed yield decreased as result of irrigation withholding at 8-leaf and irrigation withholding at staminate inflorescence stage at by 12.91% and 25.95%, respectively with compared complete irrigation treatment conditions. Similar findings have been reported in faba bean (*Vicia faba* L.) (Mwanamwenge *et al.,* 1999). Acceleration of flowering and/or maturity probably contributed to reduce the impact of drought stress in corn plants. However humic acid treatment with high concentration (300 & 450 ppm) improved seed yield under complete irrigation and irrigation withholding in different growth stages. It seems that HA maintain soil nutrients supply, help in moisture retention and mitigation of salinity. Our results are supported by Suganya and Sivasamy (2006), Selim *et al.,* (2009), Buyukkeskin and Akinci (2011), Çelik *et al.,* (2011), Tahir *et al.,* (2011), Yoon-Ha Kim *et al.,* (2012) who have reported that HA increase crop growth and productivity, and help in moisture retention and mitigation of salinity. Biological yield decreased as result of irrigation withholding at 8-leaf and irrigation withholding at staminate inflorescence stage at by 8.68 % and 14.17%, respectively with compared complete irrigation treatment conditions (Table 4). Anyia and Herzog (2004) indicated that water deficit caused between 11 and more than 40% reduction of biomass across the genotypes of cowpea (*Vigna unguiculata* L.) due to decline in leaf gas exchange and leaf area. However humic acid foliar application with high concentration (300 and 450 ppm) could improve biological yield. Our results are in agreement with findings Yoon-Ha Kim *et al.,* (2012) who have reported that HA increase crop growth and productivity. Also the result showed that the highest superoxide dismutase, catalase and Glutathione peroxidase enzyme activity were obtained from Irrigation withholding at staminate inflorescence appearance (Table 3). It was proved that the drought stress increases the production of reactive oxygen species (ROS) (Mittler 2002). To scavenge these ROS, plants either synthesize different antioxidant compounds or activate antioxidant enzymes. Plants can detoxify ROS by up-regulating antioxidant enzymes, such as SOD, CAT and POX as well as some non-enzymatic antioxidant compounds. It is evident that high levels of antioxidants are related to plant water deficit tolerance (Sankar *et al.,* 2007; Tahi *et al.,* 2008). Similar results were reported under drought stress in wheat (Shao *et al.,* 2005a), *Phaseolus acutifolius* (Turkan *et al.,* 2005) and tomato plants (Sa´nchez-Rodr?´guez *et al.,* 2010). The combined action of SOD and CAT converts the toxic O^{2-}, H_2O_2 to water and molecular oxygen, averting the cellular damage under unfavorable conditions such as drought stress (Reddy et al., 2000; Chaitanya et al., 2002). Humic acid treatment with high concentration (300 & 450 ppm) increased superoxide dismutase, catalase and glutathione peroxidase enzyme activity (Table 3). The hypothesis proposed by Asli and Neumann (2010) can be justified by these results because colloidal stress may be one explanation for these plant responses to the presence of HA via oxidative stress mechanisms. As such, the theories proposed by other authors regarding the rupture of the HA super-molecule into smaller fragments by rhizosphere acidification, as well as the entrance of HA fragments into plants that exert hormone-like effects, could further support our findings (Canellas *et al.,* 2010; Dobbss *et al.,* 2010; Suthar, 2010). Also the result showed that the highest malondialdehyde, dityrosine and Hydroxyguanosine content were observed Irrigation withholding at staminate inflorescence appearance (Table 3). It is well known that peroxidation of lipid membranes of higher plants reflects free radical-induced oxidative damage at the cellular level under abiotic stress (Hernandez *et al.,* 1995; Nouairi *et al.,* 2009). Malondialdehyde is often regarded as the product and a reflection of the degree of membrane lipid peroxidation (Ali *et al.,* 2005). Therefore, malondialdehyde content in the leaves corn plants was measured under water stress. With the water stress, leaf malondialdehyde content increased. Dityrosine content is often regarded as the product and a reflection of the degree of protein cell plants.

Table 2: Analysis of variance on corn attributes affected by irrigation with holding in different growth stages and humic acid foliar application.

S.O.V	df	Seed yield	Biological yield	Superoxide dismutase	Catalase	Glutathione peroxidase	Malondialdehyde	Dityrosine	Hydroxyguanosine
Replication	2	2153296.42**	29945437.59ns	4.70ns	2.35 ns	31.90ns	0.001ns	0.0009 ns	0.0007 ns
Irrigation	2	21749189.37**	24256804.34**	395292.49**	30644.53**	27714.81**	131.62**	42.96**	34.61**
Error (a)	4	22996.70	165861.30	439.21	47.55	187.14	0.041	0.01	0.02
Humic acid foliar application	3	946422.91**	849253.41*	4667.18*	1654.72*	1061.27**	0.53*	0.50**	0.37*
Interaction	6	62466.50 ns	410667.23ns	89.53ns	184.67ns	57.41ns	0.04ns	0.02ns	0.03ns
Error (b)	18	39650.63	195065.3	1321.71	433.79	897.23	0.11	0.07	0.08
C.V		14.49	10.78	4.67	10.55	11.66	2.98	4.19	5.84

*,** and ns significant at 0.05, 0.01 and no significant

Table 3: Comparison of main means corn attributes affected by irrigation withholding in different growth stages and humic acid foliar application

Treatments	Seed yield ($kg.ha^{-1}$)	Biological yield ($kg.ha^{-1}$)	Superoxide dismutase	Catalase	Glutathione peroxidase	Malondialdehyde	Dityrosine	Hydroxyguanosine
Irrigation								
Complete Irrigation	a11880.45	a26276.2	c596.92	c92.44	c99.07	c7.36	c4.65	c3.11
Irrigation withholding at 8-leaf stage	b10668.44	b24689.4	b778.36	b147.41	b158.19	b12.62	b6.34	b5.43
Irrigation withholding at staminate inflorescence appearance	c9192.24	c23439.3	a959.91	a193.38	a194.27	a13.47	a8.43	a6.42
Humic acid foliar application								
Untreated (0 ppm)	b10253.52	ab24739.5	b747.64	b130.37	c130.28	a11.49	a6.80	a5.27
Treated (150 ppm)	b10355.02	b24415.8	ab776.12	b137.36	b148.71	b11.16	b6.47	b4.98
Treated (300 ppm)	a10804.59	a24902.9	a789.50	ab148.54	ab164.22	b11.01	b6.36	b4.90
Treated (450 ppm)	a10908.38	a25148.2	a800.33	a161.37	a173.14	b10.95	b6.26	b4.79

Treatment means followed by the same letter within each common are not significantly different ($P < 0.05$) according to Duncan's Multiple Range test

Table 4: Interaction between irrigation withholding in different growth stages and humic acid foliar application on some attributes of corn.

Treatments	Humic acid foliar application	Seed yield	Biological yield	Superoxide dismutase	Catalase	Glutathione peroxidase	Malondialdehyde	Dityrosine	Hydroxyguanosine
Irrigation	**Humic acid foliar application**								
Complete Irrigation	Untreated (0 ppm)	bc11779.2	a26779.6	c574.76	f84.22	c91.85	e7.51	e4.83	f3.20
	Treated (150 ppm)	c11570.3	b25722.9	c594.38	f90.06	c93.61	e7.40	e4.67	f3.14
	Treated (300 ppm)	ab12011.9	ab26135.5	c605.70	f95.40	c103.87	e7.30	e4.60	f3.10
	Treated (450 ppm)	a12160.4	ab26466.6	c612.84	f100.11	c106.97	e7.23	e4.51	f3.00
Irrigation withholding at 8-leaf stage	Untreated (0 ppm)	e10258.6	cde24454.2	b744.13	e133.17	bc143.29	bc13.11	c6.74	cd5.84
	Treated (150 ppm)	e10548.2	cd24551.6	b776.03	de140.75	ab157.50	cd12.63	cd6.34	de5.40
	Treated (300 ppm)	d10921.3	cd24849.0	b791.28	cde154.29	ab164.21	d12.40	d6.20	e5.29
	Treated (450 ppm)	d10945.7	c24902.8	b801.99	bcde161.43	ab167.75	d12.34	d6.10	e5.18
Irrigation withholding at at staminate inflorescence appearance	Untreated (0 ppm)	g8722.8	f22984.8	a924.02	bcd173.73	ab176.05	a13.87	a8.85	a6.77
	Treated (150 ppm)	g8946.6	f22972.9	a957.96	bc181.27	ab189.37	ab13.44	b8.40	ab6.40
	Treated (300 ppm)	f9480.5	ef23724.3	a971.51	ab195.93	a201.61	ab13.33	b8.29	abc6.32
	Treated (450 ppm)	f9619.0	de24075.2	a986.16	a222.59	a210.03	b13.27	b8.18	bc6.19

Treatment means followed by the same letter within each common are not significantly different ($P < 0.05$) according to Duncan's Multiple Range test

Hydroxyguanosine is a nucleoside which is an oxidative derivative of guanosine. Measurement of the levels of hydroxyguanosine is used as a biomarker of oxidative stress. The highest dityrosine and hydroxyguanosine were observed from stressed plants (Table 3). However, the malondialdehyde, dityrosine and hydroxyguanosine content in humic acid foliar application treatments remained lower than that in untreated humic acid foliar application treatment (Table 3), which shows that the antioxidant enzymes activity could alleviate the peroxidation of membrane lipids, protein and nucleoside in plant cells.

CONCLUSIONS

Humic acid foliar application affected the activity of some enzymes in the anti oxidative defense system, thus controlling the ROS levels and on the occurrence of lipid, protein and nucleoside peroxidation. The mechanisms of HA action also involve the expression of genes encoding aquaporins in the tonoplast. This work indicates that HA could act according to a physiological mechanism equivalent to that functioning in plants under stress conditions. HA be applied to estimulate antioxidative stress system and protect plants in water deficient soils.

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