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Humic and Oxalic Acid Stimulates Grain Yield and Induces Accumulation of Plastidial Carbohydrate Metabolism Enzymes in Wheat Grown under Sandy Soil Conditions

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Abstract

Humic and oxalic acids have the effects of promoting plant growth. We test whether they are able to positively impact wheat yield under newly reclaimed sandy soil, where water deficiency negatively influences yield. Foliar application of humic acid and oxalic acid on two wheat cultivars, Gemiza-9 and Sakha-93, leads to overall better performance of the plants and increases the yield significantly, irrespective of the cultivar genetic background. However, Gemiza-9 surpassed Sakha-93 in grain yield parameters. The highest values of grain and protein yields/ha were obtained in both cultivars, when the plants were sprayed with a combination of 17 mg/L humic acid and (300 mg/L) oxalic acid. Humic and oxalic acid showed accumulative yield-promoting effect. To understand the mechanism by which humic and oxalic acids promoted grain yield, we performed SDS-PAGE followed by MS-MS-LC analyses. We identified a unique humic acid-induced 52 KDa band in Gemiza-9. The band contained three major proteins, Ribulose bisphosphate carboxylase large chain, ADP-glucose synthase and NADP-dependent glyceraldehyde-3-phosphate dehydrogenase (GAPN). Thus humic acid increased the activity of plastid enzymes involved in photosynthesis, sucrose biosynthesis and starched accumulation to improve the overall performance of the plant.

Keywords

Wheat, Humic and Oxalic Acids, Carbohydrate Metabolism, Calvin Cycle, SDS-PAGE, Mass Spectrum Analysis

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1. Introduction

Wheat (*Triticum aestivum* L.) is one of the most important crops worldwide. It is main crop in winter season for more than 7000 years in Egypt and considered to be a strategic commodity. It provides more than one-third of the daily caloric intake, grains as food for human and straw as fodder for animals. The cultivated fields with wheat supply only 40% of its annual domestic demand of Egyptians. Therefore, the field production should be increased to cover the demanded consumption. The newly reclaimed sandy soil in Sahara Desert of Egypt is exposed to a combination of environmental stress conditions including temperature fluctuations, low water availability, high irradiance and nutrient deprivation. In these areas, the water is the major limiting factor for plant growth. Such stress may lead to reduce plant growth by affecting various physiological and biochemical processes, such as photosynthesis, respiration, translocation, ion uptake, carbohydrates, nutrient metabolism and growth promoters [1]. Under such conditions, the structure and function of the photosynthetic apparatus of plants undergo considerable derangements. Water deficiency adversely affects the pool of photosynthetic pigments (chlorophylls *a* and *b*, carotenes, and xanthophylls) and proteins, electron transport, photophosphorylation, the activity of the photosystems (PSs), particularly PS II, carboxylation reactions, and certain enzymes of photosynthesis. The activity and content of the key enzyme of carbon metabolism, Ribulose bisphosphate carboxylase/oxygenase (Rubisco; EC 4.1.1.39), is a major factor determining the inhibition of photosynthetic carbon dioxide assimilation under water stress [2]-[5]. In addition, water deficiency disturbs the normal coupling of the light and dark phases of photosynthesis and slows down the synthesis, storage and transportation of carbohydrates (starch and sucrose) [2]-[4].

During the initial stages of photosynthetic metabolism, the ratio of carbohydrate and non-carbohydrate pathways of metabolism are determined by the activity of nicotinamide adenine dinucleotide phosphate (NADP), glyceraldehyde-phosphate dehydrogenase (GAPDH) and phosphoenolpyruvate carboxylase (PEPK). GAPDH, responsible for the reduction phase of the Calvin-Benson cycle of carbon and determines the carbohydrate route of photosynthetic metabolism. ADP-glucose pyrophosphorylase (AGP; EC 2.7.7.27), an allosterically regulated heterotetramer consisting of two large and two small subunits, catalyzes the rate-limiting reaction in starch biosynthesis in plants [2]. AGP uses the substrates glucose 1-phosphate and ATP to produce ADP glucose and pyrophosphate [3]. ADP-glucose is used then as the glucose donor for starch biosynthesis. Plastidial ADP-glucose pyrophosphate, starch believed to act as a major integrator of plant metabolic status that accumulates to cope with temporary starvation imposed by the environment [6]. Water stress decreases GAPDH and ADP-glucose activity in the majority of plants studies, including various wheat cultivars [4]. Water deficiency generates ATP and NADPH deficiency in plants, resulting in the uncoupling of the light and dark phases of photosynthesis.

The water stress may be alleviated by using efficient irrigation system, for example certain bio regulators as antioxidants and soil conditioners as humic acid. Humic acid reduces the amount of fertilizer consumption and induces the abiotic stress tolerance like drought stress. The exact mechanism by which humic substances influence alleviate water stress still not completely understood. [7] proposed that humic acid might inhibit pronase activity by either competing with the substrate for the catalytically active sites on the enzyme or by causing conformational changes in the enzyme. [8] showed the inhibitory effect of humic acid on protease activity. Humic substances may also affect the level and the distribution of sugar in maize leaves [9]. The latter effect seems to be mediated by changes in the activities of the enzymes involved in the carbohydrate metabolism. In most plant species like maize and wheat, starch and sucrose are the major products of photosynthesis. Humic acid is also known to promote plant growth and improve the yield [10]. The advantages of bio-stimulants, such as humic acid, lie in their ability to promote hormonal activity in plants as well as promote antioxidant production in plants which, in turn, reduces free radicals. It is involved in increasing root vitality, chlorophyll biosynthesis, and seed germination rate, and improve nutrients uptake [11].

Oxalic acid is a common constituent of plants, and several species accumulate high levels of the simplest dicarboxylic acid. The most striking chemical property of oxalic acid is its strong chelating ability with multivalent cations. Recently, oxalic acid application has received much attention in relation to induced disease systemic resistance and its antioxidant capability [12]-[16]. The objective of this study was to investigate the impact of spraying humic acid, oxalic acid, individually or in combination on yield and yield components of wheat (*Triticum aestivum* L.) cv. Gemiza-9 and Sakha-93 varieties to improve growth, yield and grain quality under drought stress.

2. Material and Methods

2.1. Growth Conditions, Treatments and Experimental Design

Two field experiments were carried out at the Researches and Production Station of National Research Centre, Al-Nubaria district El-Behaira Governorate, Egypt, during two successive winter seasons of 2010/2011 and 2011/2012. The soil of both experimental sites was newly reclaimed sandy soil, where mechanical and chemical analyses are reported in **Table 1** according to [17]. A split plot design was applied with four replications, in which wheat varieties occupy the main plots, while humic acid, oxalic acids and there combinations treatments were allocated at random in sub-plots. Grains of wheat varieties (*Triticum aestivum* L.) were taken from Agricultural Research Centre, Egypt and sown on the 15th November in both season in rows 3.5 meters long, and the distance between rows was 20 cm apart, plot area was 10.5 m^2 (3.0 m in width and 3.5 m in length). The recommended agricultural practices of growing wheat were applied and the seedling rate was 70 kg/fed). Pre-sowing, 357 kg/ha of calcium super-phosphate (15.5% P_2O_5) was applied to the soil. Nitrogen was applied after emergence in the form of ammonium nitrate 33.5% at rate of 357 kg/ha it was applied at five equal doses before irrigation. Potassium sulfate (48.52% K_2O) was added at two equal doses of 119 kg/ha before irrigations. Irrigation was carried out using the new sprinkler irrigation system where water was added every 7 days. Wheat plants were foliar sprayed with humic acid at the rate of (0 and 17 mg/L) and oxalic acid at the rate of (0, 100, 200 and 300 mg/L). In both seasons, foliar application of humic and oxalic acids were carried out twice; where plants were sprayed after 45 and 60 days from sowing the seeds. The two irrigations after spraying humic and oxalic acid were skipped.

At harvest, two central rows from each plot were harvested and sub samples of ten plants were taken randomly to estimate the following yield components: Plant height (cm), spike length (cm), number of spikelets/spike, grain yield (g/plant), straw yield (g/plant), biological yield (g/plant), grain index (100 grains weight in gram). The whole plants of each plot were harvested to determine grain; straw and biological yield/fed. Data were statistically analyzed according to [18]. The combined analysis was conducted for the data of two seasons. The least significant differences (LSD at 5%) used to compare the treatments means.

2.2. SDS-Protein Electrophoresis

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to the method of [19], as modified by [20]. Total proteins of the wheat leaves powder fully were taken from two cultivars Sakha 93 and Gemiza 9 after treatment with oxalic and humic acids under drought conditions. Proteins were separated on 12% polyacrylamide gel using vertical slab (19.8 cm × 26.8 cm × 0.2 cm) of the electrophoresis apparatus manufactured by LABOCONCO. Gels were analyzing using Total Lab TL100.

2.3. Mass Spectrometry Analyses

Differential protein bands were excised from Bis-Tris polyacrylamide gel. The gel was stained with colloidal Coomassie Blue. De-stained with 50% acetonitrile and subsequently digested with sequencing grade trypsin (Roche) overnight. Peptides were extracted with 5% formic acid/50% acetonitrile twice and vacuum dried in a Speed Vac (Thermo Fisher Scientific). Peptides were redissolved in 0.5% formic acid and subjected to LCMS/MS [21] [22].

2.4. Database Search Parameters

Scaffold: version: scaffold-4.0.3 and X!Tandem 2010.01.04 (The Global Proteome Machine Organization), source (opt/Scaffold 4/parameters/unimod.xml) were used for analysing the data. Parameters used in QE XTandem 10 ppm. Fixed modifications consisted of carbamidomethylation of cysteine. Variable modifications considered were oxidation of methionine residues peptide mass tolerance settings/windows were as indicated in the

Table 1. Mechanical and chemical characteristics of the natural soil used in the current study.

Sand %	Silt %	Clay %	PH	Organic matter	$CaCo_3$ %	Ec ds/m	Soluble (N) ppm	Available (P) ppm	Exchangeable (K) ppm
89.5	3.7	5.1	7.3	0.3	1.4	0.3	8.1	3.2	20

individual experiments (between 2 Da and 5 ppm); product mass tolerance = 0.5 Da. Maximum valid E-value for reported peptides was set to 100 (E-values were limited in the data analysis steps) and cyclic permutations to compensate for small search spaces was enabled, with remaining parameters at default. The protein sequence database used was the WheatUni-20130312-QHnGYf database.

3. Results and Discussion

3.1. Gemiza-9 Shows Overall Increased Yield in Sandy Soil in Comparison with Sakha-93

We used two cultivars to investigate the effect of humic and oxalic acid on overall plant performance under growth conditions in sandy soil. First, we tested whether the two cultivars show a basic difference under our experimental conditions. We observed that the grain yield of the two cultivars differed significantly in all the yield parameters that were investigated (**Table 2**). Gemiza-9 cultivar showed lower spike length (cm), grain yield/plant (g) and protein percentage than Sakha-93. In contrast, Sakha-93 significantly showed increased grain yield/plant; spike length and protein percentage in comparison to Gemiza-9. However, Gemiza-9 variety surpassed Sakha-93 variety in grain yield/ha; biological yield/ha and protein yield/ha leading to an overall increase of yield. The superiority of this Gemiza-9 may be due to the increased plant height, no. of spikelet's/spike, straw yield/plant and grain index. Our results are in agreement with those obtained by other investigators [23] [24]. We conclude from these results that high diversity in genetic constituent and the environmental conditions of investigated cultivars are responsible for different tested characters performance consequently yields. .

3.2. Humic and Oxalic Acids Co-Operatively Promote Plant Growth and Yield

To investigate the effect of humic and oxalic on the yield of Gimeza-9 wheat cultivar growing in sandy soil, both acids were sprayed at different concentrations and combinations. Application of humic and oxalic acids individually increased the grain yield/ha. Nevertheless, foliar application of humic acid showed more pronounced effect on the grain yield than oxalic acid. The increase in the grain yield positively correlated with the increased concentrations of oxalic acid (**Table 3**). Similar results showed that humic acid promoted plant growth and

Table 2. Basic yield characteristics of Gemiza-9 and Sakha-93 cultivars under the growth in sandy soil.

Varieties	Plant height (cm)	Spike length (cm)	No. of spikelet's /spike	Biological yield/plant (g)	Grain yield/plant (g)	Straw yield/plant (g)	Seed index (g)	Grain yield (ton/ha)	Biological yield (ton/ha)	Protein %	Protein yield (kg/ha)
Gemiza-9	81.56	11.42	18.52	10.09	3.15	6.94	4.18	3.36	5.59	10.01	341.41
Sakha-93	72.88	11.74	17.33	8.83	3.20	5.63	4.14	2.61	4.36	11.41	299
$LSD_{0.05}$	3.15	0.23	0.66	0.71	0.03	0.87	0.02	0.96	0.25	1.01	5.77

Table 3. Effect of foliar application of humic and oxalic acids on yield characteristics of Gemiza cultivar.

Oxalic and humic acids (mg/L)	Plant height (cm)	Spike length (cm)	No. of spikelet's /spike	Biological yield/plant (g)	Grain yield/ plant (g)	Straw yield/ plant (g)	Seed index (g)	Grain yield (ton/ha)	Biological yield (ton/ha)	Protein %	Protein yield (kg/ha)
Control	65.92	8.17	12.00	5.07	1.64	3.43	3.55	2.08	3.48	9.75	200.25
Oxalic 100	69.50	10.29	15.00	6.90	2.08	4.82	3.89	2.41	4.00	10.37	248.76
Oxalic 200	72.33	11.50	17.00	7.90	2.55	5.35	4.10	2.59	4.31	10.58	273.06
Oxalic 300	75.00	12.50	18.67	9.29	3.27	6.03	4.15	2.74	4.57	10.79	295.43
Humic 17	79.83	11.50	19.08	10.24	3.23	7.01	4.21	3.06	5.12	10.79	327.42
Humic 17 + Oxalic 100	83.17	12.08	19.67	10.81	3.50	7.31	4.30	3.33	5.55	10.79	357.12
Humic 17 + Oxalic 200	84.83	12.67	20.50	12.16	4.07	8.09	4.40	3.59	6.00	11.21	399.75
Humic 17 + Oxalic 300	87.17	13.93	21.50	13.35	5.08	8.27	4.73	4.07	6.78	11.41	459.86
$LSD_{0.05}$	1.05	0.05	0.13	0.21	0.02	0.15	0.06	0.11	0.09	0.13	7.31

improved yield [1] [10]. The beneficial effects of humic acid on plant growth potentiate its role as a regulator of plant growth hormones (gibberellins and auxin). Humic acid might act as an auxin-like compound, as it is apparent from its chemical structure, consequently it affect plant growth and development [25]. In addition, humic acid promotes root development [26]. We further investigated the effect of a combined foliar application of humic and oxalic acids on plant performance. Overall, the combined application of both acids increased the grain yield. Thus, humic and oxalic acids have an accumulative effect. The higher the concentration of oxalic acid, the higher the grain yield was. The highest values of grain, biological and protein yields/ha was obtained at by foliar application of humic 17 mg/L humic acid + 300 mg/L oxalic acid (**Table 3**). The overall increased yield can be explained with the increase in plant height (cm), spike length (cm), no. of spikelet's /spike, biological yield/plant (g), grain yield/plant (g), straw yield/plant (g), grain index (g) and protein percentage. These results are in agreement with those obtained by [11] [25] [27].

3.3. The Yield Promoting Effect of Humic and Oxalic Acids Is Influenced with the Genetic Background

To test whether the positive effect of humic and oxalic acids on plant growth and yield is associated with the plant genetic background; we foliar applied both acids in different concentrations and combinations on Gemiza-9 and Sakha-93 cultivars.

Similar to Gimeza-9, Sakha-93 also showed increased grain yield by the application of humic and oxalic acids. Also there was a positive correlation between the concentration of oxalic acid and the measured parameters for plant growth and yield (**Table 4**). Likewise combined application of humic and oxalic acid resulted in an accumulative effect on plant growth and yield (**Table 4**). Under all tested treatments, Gemiza-9 showed overall higher yield in comparison to Sakha-93. The highest yield was obtained when Gemiza-9 was treated with 17 mg/L humic acid + 300 mg/L oxalic acid (**Table 4**). These results indicate that humic and oxalic acids are able

Table 4. Effect of cultivar genetic background on humic and oxalic acids-enhanced yield.

Varity	Treatment	Plant height (cm)	Spike length (cm)	No. of spikelet's /spike	Biological yield/plant (g)	Grain yield/ plant (g)	Straw yield/ plant (g)	Seed index (g)	Grain yield (ton /ha)	Biological yield (ton/ha)	Protein%	Protein yield (kg/ha)
Gemiza-9	con	66.50	8.00	12.50	5.23	1.57	3.66	3.65	2.26	3.76	8.30	187.26
	OX1	72.67	10.17	14.67	7.67	2.00	5.67	4.07	2.49	4.14	9.54	237.05
	OX2	76.00	11.33	17.33	8.80	2.43	6.37	4.10	2.67	4.44	9.54	254.30
	OX3	79.33	12.67	20.00	9.45	3.53	5.92	4.15	2.94	4.89	10.79	31659
	Humic	86.00	11.33	20.33	10.25	3.17	7.08	4.21	3.43	5.72	9.96	342.01
	h + OX1	89.67	11.83	20.33	11.48	3.70	7.78	4.32	3.93	6.55	10.37	407.34
	h + OX2	91.00	12.33	21.00	13.17	3.83	9.33	4.39	4.25	7.08	10.79	458.34
	h + OX3	91.33	13.67	22.00	14.70	5.00	9.70	4.57	4.90	8.16	10.79	528.41
Sakha-93	con	65.33	8.33	11.50	4.90	1.70	3.20	3.44	1.90	3.17	11.20	213.25
	OX1	66.33	10.41	15.33	6.13	2.17	3.97	3.71	2.32	3.88	11.20	260.49
	OX2	68.67	11.67	16.67	7.00	2.67	4.33	4.09	2.51	4.19	11.62	291.81
	OX3	70.67	12.33	17.33	9.13	3.00	6.13	4.14	2.55	4.24	10.79	274.27
	Humic	73.67	11.67	17.83	10.23	3.30	6.93	4.21	2.69	4.49	11.62	312.85
	h + OX1	76.67	12.33	19.00	10.13	3.30	6.83	4.27	2.74	4.57	11.20	306.90
	h + OX2	78.67	13.00	20.00	11.14	4.30	6.84	4.41	2.94	4.89	11.62	341.15
	h + OX3	83.00	14.20	21.00	12.00	5.17	6.83	4.88	3.25	5.42	12.03	391.34
	$LSD_{0.05}$	1.22	0.13	0.16	0.25	0.17	0.07	0.01	0.11	0.105	0.02	2.41

to enhance plant growth and yield irrespective of the genetic background. However, the better the basic cultivar performance, the more effective humic and oxalic acids can be in directing the overall yield.

3.4. Humic Acid Induces Accumulation of Three Plastedial Proteins in the Gimeza-9 Cultivar

To understand the molecular basis of biochemical changes that occured in response to treatment with humic and oxalic acids in the two wheat cultivars (Sakha-93 and Gemiza-9), SDS-PAGE method was used to identify changes in total protein pattern. Interestingly, we detected a band at molecular weight 52 KDa in Gemiza-9 under treatment with humic acid (**Figure 1**). This band was detectable neither in the control nor in the oxalic acid treatment (**Figure 1**). That could reflect the role of humic acid to enhance the gene responsible for encoding this protein(s) (52 KDa), which could play a role in enhancing plant growth and yield and antioxidant defense system [28]. Moreover, [29] reported that creeping bent grass treated with humic substances has shown positive growth responses. The bent grass and *Acacia saligna* continue growing and producing carbohydrates during times of stress [29] [30].

It has been shown that applying humic acid to creeping bentgrass resulted in increased photosynthesis [11] and [31]. By continuing to produce carbohydrates, bentgrass could be more adept at drought avoidance or act as a signal to induce stress related genes [32]. Resulted that plants sense carbohydrates and transduce a signal which changes gene expression and the activities of many enzymes. This result could explain Gemiza-9 is more tolerant to drought in presence humic compare to Sakha-93 that lake the band 52 KDa. In addition, two bands, 49 and 45 KDa appeared in Gemiza-9 and Sakha-93 after treatments with humic and oxalic acid compared with the control. These changes reflect the role of both humic and oxalic acids on gene expression induction and subsequently on protein accumulation. Similar to our results, [33] found that 13 proteins were up-regulated and 24 were down-regulated upon oxalic acid treatments. These proteins were categorized into several functional groups including protein processing, RNA processing, photosynthesis, signal transduction, stress response, and redox homeostasis. However, in the exact mechanism by which humic and oxalic acids impact on gene expression and alleviate stress is still poorly understood. It seems that oxalic and humic acids regulate gene expression under normal and/or stress conditions in different pathways.

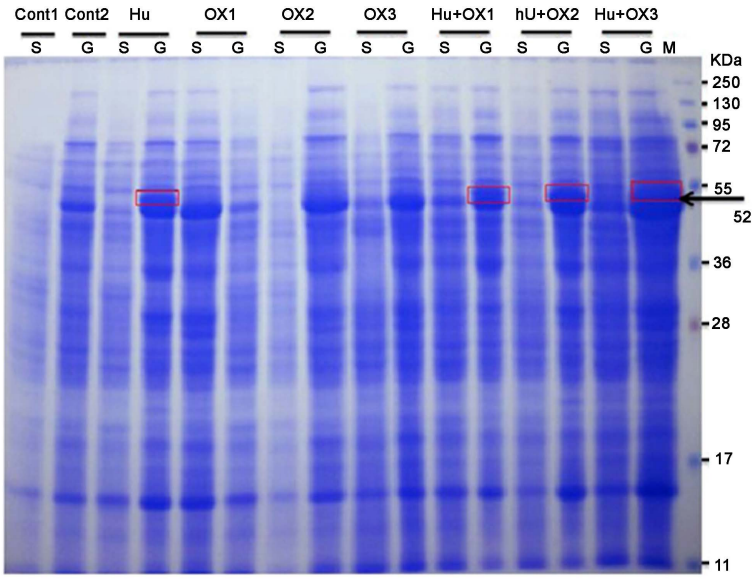

Figure 1. SDS-PAGE profile of proteins extracted from two months leaves of two the Sakha-93 (S) and Gemiza-9 (G) cultivars treated with 0.17 mg/L humic acid (Hu), oxalic acid 100, 200 and 300 mg/L (OX1, OX2, OX3) or 17 mg/L humic acid plus 100, 200 and 300 mg oxalic acid (Hu + OX1, Hu + OX2, Hu + OX3, respectively). The 10% polyacrylamide gel was stained with 0.25% Coomassie Blue R-250.

To identify the protein(s) which were accumulated at 52 KDa by the humic acid treatment in the Gimiza-9 cultivar, the bands excised from the SDS gele and were subjected to mass spectrometry analysis (**Figure 2(A)**). Intriguingly, we detected only 3 proteins (**Figure 2(B)**). To identify these proteins, the Scaffold-4.0.3 and X!Tandem 2010.01.04 were used. The analysis revealed that the detected peptides match the WHEAT Ribulose bisphosphate carboxylase large chain (RuBisCo), WHEAT Glucose-1-phosphate adenylyltransferase (ADPG) and WHEAT NADP-dependent glyceraldehyde-3-phosphate dehydrogenase (GAPN) (**Figure 3**). Percentages of the sequence coverage of the detected proteins were 74, 21, and 17, respectively (**Table 5**). Indeed RuBisCo, ADPG and GAPN had expected molecular weights, 52.85, 53.3 and 53.05, respectively. These proteins play a major role in photosynthesis, sucrose biosynthesis and starch accumulation (**Table 5**). Our results points out the importance of plastidial glycolytic and primary metabolism enzymes in improvement of wheat yield. These results support previous studies concerning the effect of humic acid on biological activity [34] [35], photosynthe-

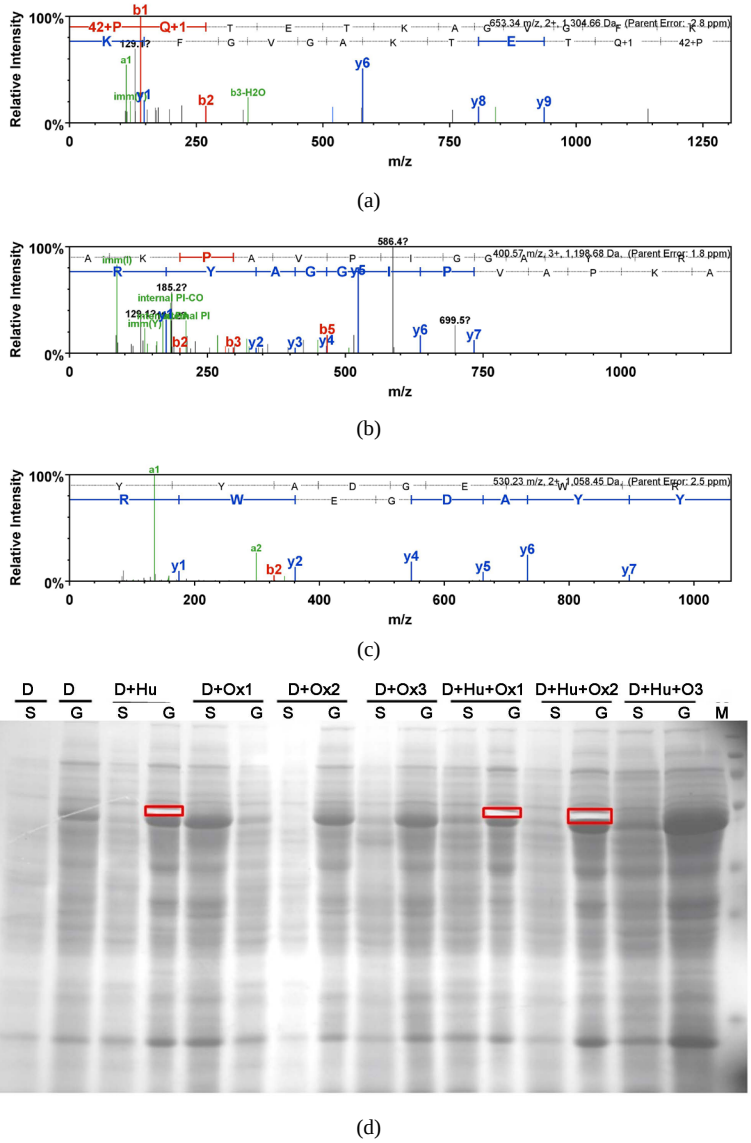

Figure 2. MS spectrum of the differentially accumulated proteins by humic acid treatment in the Gemiza-9 cultivar. Ribulose bisphosphate carboxylase large chain (a), Glucose-1-phosphate adenylyltransferase (b) and NADP-dependent glyceraldehyde-3-phosphate dehydrogenase (c) identified from the specific 52 KDa. (d) The Excised SDS-PAGE gel of 52 KDa protein bands.

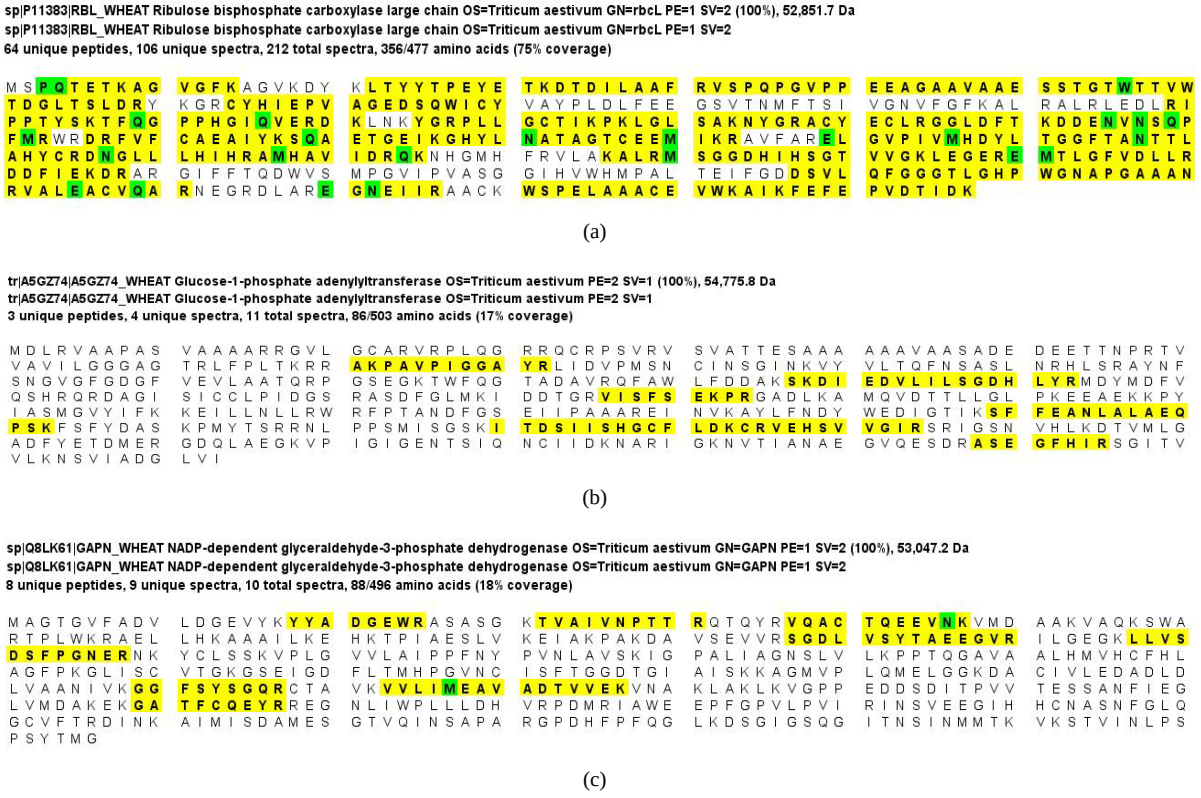

Figure 3. Amino acid sequence of the identified proteins that were accumulated by humic acid application. (a) (b) and (c) correspond to RuBisCo, ADPG and GAPN, respectively. Peptides sequence analysis was done using Scaffold-4.0.3. The peptides matching covering location are indicated in yellow color, 100% protein identification probability indicated in green.

Table 5. List of proteins induced by humic acid application in the leaves of Gemiza-9 cultivar.

Protein name	Accession No.	M.W (KDa)	Seq. Cov.	Function
WHEAT Ribulose bisphosphate carboxylase large chain	P11383	52.85	74%	Calvin cycle carbon dioxide fixation photosynthesis
WHEAT Glucose-1-phosphate adenylyltransferase	A5GZ74	53.322	21%	Starch synthesis
WHEAT NADP-dependent glyceraldehyde-3-phosphate dehydrogenase (GAPN)	Q8LK61	53.05	17%	Photorespiration sucrose metabolism

sis [9] [36] and nitrate assimilation [37] [38]. By identification of the RuBisCo, ADPG and GAPN as a major contributor to the humic acid-promoted plant growth, we started to envisage its biological role.

3.5. Humic Acid Stimulates Carbohydrate Metabolism

Gemiza-9 wheat cultivar responded to the biological effect of humic acid by inducing the genes encoding plastedial enzymes involved in calvin cycle to produce glyceraldhyde-3-phosphate, which could be used to produce an array of different carbohydrate. Three cycles of the calvin cycle would make six glyceraldhyde-3-phosphate of which one could be used by the cell for various purposes. The other five glyceraldhyde-3-phosphates were needed to generate three ribulose-1,5-bisphosphate (RuBP). So, it takes multiple interactions of calvin cycle to allow the production of any useful carbohydrate. Making RuBP from glyceraldhyde-3-phosphate is much more under stress. Another enzyme activated by humic acid treatment is ADP glucose pyrophosphorylase which can couple AMP to the phosphate of glucose-1-phosphate by removing pyrophosphate from ATP. This produces ADP-glucose that is the substrate for starch synthase, which polymerizes it into starch (**Figure 4**). This way we might provide an explanation of how humic acid can enhance the grain filling in the Gimeza-9 cultivar. Taken

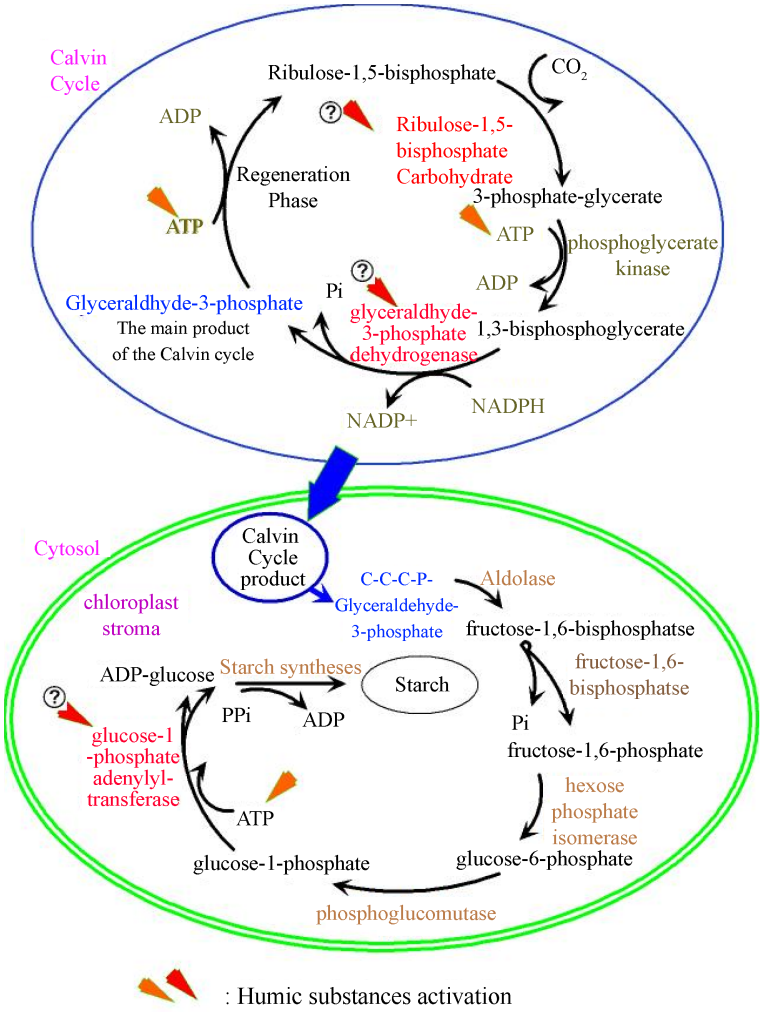

Figure 4. Model of Humic acid stimulated carbohydrate metabolism. The calvin cycle enzymatic pathway in the thylakoid membrane where the carbohydrate molecules are produced, such as glyceraldehyde-3-phosphate (GADP), the major export product from photosynthesis. GADP enters the starch biosynthesis pathway in chloroplast or converted into sucrose in the cytosol. Humic acid activates the two major enzymes in calvin cycle first the Ribulose bisphosphate carboxylase (carboxylation phase), second the NADP-dependent glyceraldehyde-3-phosphate dehydrogenase (reduction phase). In addition to high accumulation of ATP molecules take place. Finally, the humic acid activates the starch biosynthesis pathway through the Glucose-1-phosphate adenylyltransferase enzyme.

together, we conclude that humic acids have a positive effect on carbohydrate metabolism in wheat leaves. This statement is supported also with previous findings that humic acid stimulates the photosynthesis apparatus through increasing the content of chlorophyll and Ribulose bisphosphate carboxylase [39]. However, our findings require more experiments to confirm the direct link between humic acid application and activation of calvin cycle.

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