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Photosynthesis, antioxidant status and gas-exchange are altered by glyphosate application in peanut leaves

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Abstract

Glyphosate herbicide caused oxidative stress and exhibited negative effects on photosynthesis and gas exchange of peanut [*Arachis hypogaea* L. cv. Giza (G) 5 and 6] leaves. We demonstrated that glyphosate caused various morphological symptoms, such as chlorosis, yellowing, and appearance of curly edges in leaves treated with high doses of herbicide in both cultivars; however, the G5 cultivar was more sensitive and showed severer symptoms. Glyphosate lowered photosynthesis and reduced contents of pigments and proteins as well as free amino acids in both cultivars. The gas-exchange parameters, such as photosynthetic (P_N) and transpiration rate (E), were highly altered by the glyphosate application. For example, P_N and E were reduced by 65 and 61%, respectively, in G5 treated with high dose of glyphosate compared with control. Antioxidant enzymes, such as peroxidase, catalase, ascorbate peroxidase, and superoxide dismutase were induced by both low and high concentrations in the glyphosate-treated leaves. Moreover, the level of lipid peroxidation, indicated by a malondialdehyde content, as well as the hydrogen peroxide content increased in the glyphosate-treated leaves. However, an increase in total antioxidant activity was detected in leaves and this reflected changes in the antioxidant status and accumulation of antioxidants as a defense mechanism against glyphosate toxicity in peanut.

Additional key words: antioxidant enzymes; gas exchange; glyphosate; lipid peroxidation; peanut; photosynthesis; reactive oxygen species.

Introduction

Glyphosate [N-(phosphonomethyl) glycine] is a wide-spectrum, nonselective, foliar-applied, postemergence herbicide that controls a wide range of species including grasses and broadleaf weeds (Franz *et al.* 1997, Vencill 2002, Gomes *et al.* 2014). However, being a nonselective herbicide, glyphosate does not distinguish between weeds and crops. Glyphosate is considered safe to the environment because of its rapid decomposition by soil microorganisms to phosphate and carbon dioxide (Torstensson 1985).

Gomes *et al.* (2014) reported that mechanisms leading to plant death may be related to secondary or indirect effects of glyphosate on plant physiological processes. Glyphosate kills plants by disturbing the shikimate pathway (Duke *et al.* 2003). It inhibits the enzyme 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS, EC 2.5.1.19), a key enzyme in the shikimate pathway that leads to overproduction and accumulation of shikimate (Steinrücken and Amrhein 1980). It inhibits the biosynthesis of aromatic amino acids (phenylalanine, tyrosine, and tryptophan), which leads to several metabolic disturbances, including the arrest of protein synthesis and prevention of secondary product formation (Steinrücken and Amrhein 1980, Franz *et al.* 1997, Schönbrunn *et al.* 2001). As a consequence, plants die following a slowdown in protein synthesis. In addition, there is a reduction in secondary products of the shikimate pathway and a diversion of carbon into an accumulated pool of shikimate (Tan *et al.* 2006).

Glyphosate has decreased a chlorophyll (Chl) content (Pline *et al.* 1999, Reddy *et al.* 2001), nitrogen fixation, and nitrogen accumulation in some glyphosate-resistant soybean cultivars (King *et al.* 2001, Cerdeira and Duke 2006). A lower amount of leaf Chl is a distinguishing

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Abbreviations: APX – ascorbate peroxidase; C_i – intercellular CO_2 concentration; CAT – catalase; DPPH – 1,1-diphenyl-2-picrylhydrazyl; E – transpiration rate; EPSPS – 5-enolpyruvylshikimate-3-phosphate synthase; g_s – stomatal conductance; MDA – malondialdehyde; P_N – photosynthetic rate; POD – peroxidase; ROS – reactive oxygen species; SOD – superoxide dismutase; TAA – total antioxidant activity.

characteristic in plants exposed to sublethal concentrations of glyphosate (Wong 2000, Tan *et al.* 2006). Glyphosate-tolerant plants are now of great agronomic interest (Racchi 1990). The effects of glyphosate stress on plants in terms of physiological and/or biochemical responses have been well documented (de María *et al.* 2005, Sergiev *et al.* 2006, Ahsan *et al.* 2008).

Sergiev *et al.* (2006) demonstrated that an application of glyphosate to leaves increased significantly the lipid peroxidation and ion flux, which suggests that glyphosate is somehow related to the reactive oxygen species (ROS) production. ROS are produced as byproducts of various metabolic pathways and scavenged by different anti-oxidative defense components that are often confined to particular compartments (Foyer *et al.* 1994, Alscher *et al.* 1997). Under adverse environmental conditions, increase in ROS formation can induce oxidative damage to bio-molecules and cell components (Kellogg and Fridovich 1975, Lai 1977, Wiseman and Halliwell 1996). All plant cells are protected by an antioxidant system, including enzymatic and nonenzymatic components. The photo-synthetic electron transport system is the major source of ROS in plant tissues (Asada 1994) having the potential to generate singlet oxygen and superoxide. The superoxide ion can participate directly in oxidation and reduction reactions with cell components leading to toxic effects.

Peanut (*Arachis hypogaea*, Fabaceae), is an econo-mically important food crop in many countries worldwide. It is native to South Africa, Mexico, South America, and Mediterranean region. Peanut is frequently subjected to drought stresses of different duration and intensities (Oyinlola *et al.* 2004, Aydin 2007, Yu *et al.* 2007). Although there are many reports concerning the change in physiological properties after treatments with herbicides, limited information is available on the indirect effects of glyphosate on transpiration, gas exchange, photosynthesis, and antioxidant enzyme activities in some economically important crops such as peanut. Therefore, this work was aimed to evaluate the effects of glyphosate herbicide on two different peanut cultivars.

Materials and methods

Experiment preparation and treatments: Seeds of two peanut cultivars (*Arachis hypogaea* L., *cv*. Giza 5 and Giza 6, Fabaceae) were sown in a mixture of sand and clay soil in clean plastic pots (ten seeds per pot); these pots were irrigated by tap water. After four weeks, plants of the same growth were chosen, other plants were removed, and the pot contained five similar plants finally. Then the pots with similar plants were selected and divided into groups with four replicates for each treatment [control, low (LD) and high dose (HD) of glyphosate, *i.e.*, 360 and 720 g ha^{-1}, respectively]. The plant groups were treated as presented in the table below. Different plants from different pots were chosen for measurements. In addition to biological replicates, four technical replications were carried out for each measurement.

Group	Abbreviation	Treatment
1	G5 (control)	Giza 5 healthy control plants
2	LD G5	Giza 5 sprayed with 360 g(glyphosate) ha^{-1}
3	HD G5	Giza 5 sprayed with 720 g(glyphosate) ha^{-1}
4	G6 (control)	Giza 6 healthy control plants
5	LD G6	Giza 6 sprayed with 360 g(glyphosate) ha^{-1}
6	HD G6	Giza 6 sprayed with 720 g(glyphosate) ha^{-1}

Photosynthetic pigments content and gas-exchange properties: The method of Lichtenthaler and Buschmann (2001) was used for determination of photosynthetic pigments [chlorophyll (Chl) *a*, Chl *b*, and carotenoids (Car)]. Extraction was carried out at 4°C and under dark conditions. A leaf sample (0.25 g) was mashed in a mortar and pestle with 80% acetone (v/v), the extract was filtered through two layers of nylon and centrifuged in sealed tubes for 5 min at 15,000 × *g*. The supernatant was collected and the absorbance was read using *UV-VIS* spectrophotometer (*T80*, *PG Instruments*, UK) at 663, 647, and 470 nm for Chl *a*, Chl *b*, and Car contents, respectively. The concentrations for Chl *a*, Chl *b*, and the sum of leaf Car (xanthophylls and carotenes) were given in mg ml^{-1} extract solution according to the equations of Lichtenthaler and Buschmann (2001):

$$\text{Chl } a = 12.25\ A_{663} - 2.79\ A_{647}$$
$$\text{Chl } b = 21.50\ A_{647} - 5.10\ A_{663}$$
$$\text{Car} = (1{,}000\ A_{470} - 1.82\ \text{Chl } a - 95.15\text{l Chl } b)/225$$

For gas-exchange properties, control and treated leaves were subjected to analyses of P_N, E, stomatal conductance (g_s), and intercellular CO_2 concentration (C_i) using an infrared gas analyzer (*LI-6400 System, Li-COR*, Lincoln, NE, USA).

Analyses were carried out at midday between 9:00–11:00 h with light intensity of PAR ranging 1,400–1,800 µmol(photon) m^{-2} s^{-1}. Throughout the whole measure-ments, the atmospheric CO_2 concentration (C_{ref}) was 347.2 µmol mol^{-1} and the leaf temperature was 29–32°C. The relative air humidity was 65–70%. The conditions did not differ between treatments over the experimental period.

The values are the means of eight individual measure-ments. Water-use efficiency (WUE) was calculated as follows: $WUE = P_N/E$.

Total protein and total free amino acid contents: Total protein contents were determined according to Lowry *et*

al. (1951). Fresh tissue samples were extracted in 10 ml of 0.1 N NaOH. The extracts were centrifuged for 15 min at 1788.8 ×*g* and the supernatants were collected. One ml of extract was added to 5 ml of alkaline reagent (50 ml of 2% Na_2CO_3 prepared in 0.1 N NaOH +1 ml of 0.5% $CuSO_4 \cdot 5H_2O$ prepared in 1% sodium potassium tartarate) and thoroughly mixed and then allowed to stand for 10 min. A half ml of Folin phenol reagent diluted 1:1 (v/v) was then added and mixed immediately. After 30 min, the absorbance against appropriate blank was measured using *UV-VIS* spectrophotometer (*T80*, *PG Instruments*, UK) at 700 nm. Results were expressed as mg g^{-1}(dry mass, DM).

Total free amino acids were extracted from plant tissues and determined according to the method of Moore and Stein (1948). Dry tissue samples were extracted in distilled water by heating in water bath at 90°C for 2 h. The extracts were then centrifuged and the supernatants were collected. The supernatant was added to 1 mL of ninhydrin solution with stannous chloride. The mixture was heated in boiling water bath for 20 min. A purple color was developed. Diluents (5 mL) were added and mixed. After 15 min, the intensity of the purple color against a reagent blank was measured at 570 nm using *UV-VIS* spectrophotometer (*T80*, *PG Instruments*, United Kingdom). The color is stable for 1 h. The free amino acids concentrations were calculated as mg g^{-1}(dry mass, DM).

Peroxidase (POD) and catalase (CAT) activities: POD (EC 1.11.1.7) activity was determined in extracts prepared by grinding 0.05 g of the fresh leaves in 5 mL of phosphate buffer (pH 7.0) in a mortar at 4°C. The homogenate was centrifuged at 25,155 × *g* at 10°C for 15 min. Supernatants were collected for measurement of POD activity. Fifty microliters of extracted samples in 10 ml of assay mixture were spectrophotometrically measured at 470 nm (extinction coefficient = 26 mM cm^{-1}) using *UV-VIS* spectrophotometer (*T80*, *PG Instruments*, United Kingdom). The assay mixture for POD activity contained 40 mM potassium phosphate (pH 7.2), 0.1 mM EDTA, 5 mM guaiacol, 0.3 mM H_2O_2. The enzyme activity was calculated in terms of nmol (product) g^{-1}(fresh mass, FM) h^{-1} at 25 ± 2°C (MacAdam *et al.* 1992, Zhang 1992).

CAT (EC 1.11.1.6) activity was measured according to Chandlee and Scandalios (1984). CAT activity was measured by monitoring the disappearance of H_2O_2 by measuring the decrease in absorbance at 240 nm (extinction coefficient = 0.036 mM^{-1} cm^{-1}) of a reaction mixture consisting of 25 mM potassium phosphate buffer (pH 7.0), 10 mM H_2O_2 and enzyme extract The enzyme activity was calculated in terms of nmol (product) g^{-1}(fresh mass, FM) h^{-1} at 25 ± 2°C.

Ascorbate peroxidase (APX) and superoxide dismutase (SOD): Half gram of leaves was grinded in 5 ml of phosphate buffer (pH 7.0) and centrifuged at 4,000 rpm for 10 min at 4°C. The extract for APX (EC 1.11.1.11) assay was then mixed with the assay medium containing 50 mM phosphate buffer (pH 7.0), 0.1 mM EDTA, 0.3 mM ascorbate, and 0.06 mM H_2O_2. The decrease in ascorbate concentration was followed by a decline in absorbance at 290 nm and the activity was calculated using the extinction coefficient (2.8 mM^{-1} cm^{-1}) (Nakano and Asada 1981). The enzyme activity was calculated in terms of nmol (product) g^{-1}(FM) h^{-1} at 25 ± 2°C.

According to Beauchamp and Fridovich (1971), SOD (EC 1.15.1.1) activity was analyzed with some modifications. A known FM of leaves was homogenized in the extraction buffer consisting of 50 mM phosphate buffer, pH 7.8, 0.1% (w/v) ascorbate, 0.05% (w/v) ß-mercaptoethanol. The assay mixture in 3 ml contained 50 mM phosphate buffer (pH 7.8), 9.9 mM L-methionine, 0.025% (w/v) nitroblue tetrazolium (NBT), and 0.0044% (w/v) riboflavin. The photoreduction of NBT (formation of purple formazan) was measured using *UV-VIS* spectrophotometer (*T80*, *PG Instruments*, UK) at 560 nm. One unit of SOD activity was defined as extract volume that caused 50% inhibition of the photoreduction of NBT.

Malondialdehyde (MDA) content: Lipid peroxidation was expressed as the MDA content and determined as 2-thiobarbituric acid (TBA) reactive metabolites according to Zhang (1992). One gram of fresh leaves with removed veins was grinded in 5% trichloroacetic acid (TCA) and then centrifuged for 10 min at 1,788.8 × *g*. Two mililitres of the supernatant was mixed with 2 mL of 0.03 mM TBA and incubated for 15 min at 94°C to develop the $(TBA)_2$-MDA adduct. The mixture was cooled using tap water and the absorbance was measured using *UV-VIS* spectrophotometer (*T80*, *PG Instruments*, UK) at wavelength 532 nm. Lipid peroxidation was expressed as nmol g^{-1}(FM) by using an extinction coefficient (155 mM cm^{-1}).

H_2O_2 content: H_2O_2 was extracted by homogenizing 1 g of leaf tissue with 3 ml of phosphate buffer (50 mM, pH 6.5). The homogenate was centrifuged for 25 min at 4024.8 ×*g*. To determine H_2O_2 content, 3 ml of extracted solution was mixed with 1 ml of 0.1% titanium sulfate in 20% H_2SO_4. The mixture was then centrifuged for 15 min at 4024.8 × *g*. The pellet was then dissolved in 5 ml of 2 M H_2SO_4 and the intensity of the yellow color of the supernatant at 410 nm was measured using *UV-VIS* spectrophotometer (*T80*, *PG Instruments*, UK). The H_2O_2 content was calculated using the extinction coefficient (0.28 mol^{-1} cm^{-1}) (Jana and Choudhuri 1981).

Total antioxidant activity: Extracts of leaves in methanol were subjected to the free radical scavenging activity assay using 1,1-diphenyl-2-picrylhydrazyl (DPPH) and the method described by Shimada *et al.* (1992). Extracts (0.2–10 mg ml^{-1}) in methanol (2 ml) were mixed with the same volume of freshly prepared methanolic solution containing 80 ppm of DPPH radicals. Mixtures were shaken vigorously and left to stand for 30 min in the dark. The absorbance was then measured using *UV-VIS*

spectrophotometer (*T80*, *PG Instruments*, UK) at 517 nm. The percentage of DPPH scavenging activity was calculated as follows:

$$\text{DPPH scavenging ability} = [1 - (A_i - A_j)/A_c] \times 100.$$

A_i is absorbance of extract + DPPH, A_j is absorbance of extract + methanol, and A_c is absorbance of DPPH + methanol. The lower absorbance indicates a higher scavenging effect.

Statistical analysis: The obtained data were tested for significance by using analysis of variance (*ANOVA*) test (Duncan 1951). Means were compared by least significant differences (LSD) test at levels $P<0.05$ and $P<0.01$. All statistical tests were carried out using *SPSS* software (*Version 15, SPSS Inc.*, IL, USA).

Results

Growth and morphology of leaves: Leaves of both peanut cultivars (G5 and G6) showed some distinct symptoms after the glyphosate application (Fig. 1). These symptoms included chlorosis, yellowing, and bleaching and were detected in both peanut cultivars (Fig. 1*C*,*D* for G5; Fig. 1*E*,*F* for G6). Moreover, leaf edges showed curly appearance in the G5 leaves as a response to HD (Fig. 1*E*). The glyphosate-treated leaves were smaller compared with controls. Obviously, the G5 cultivar was severely affected; it was more sensitive to glyphosate than the G6 cultivar (Fig. 1*E*,*F*).

Photosynthetic pigments content and gas-exchange properties: The glyphosate treatment caused significant reductions in contents of all pigments. Reductions in the pigment contents were more apparent in the G5 leaves than that of G6. Regarding the G5 cultivar, Chl *a* was reduced to one third of the control with LD, while HD decreased Chl *a* to 26% of the control. Moreover, Chl *b* detected in G5 was lowered to 28% after the glyphosate application. Furthermore, the G5 leaves showed a significantly lowered Car content than that of the corresponding control. On the other hand, the G6 cultivar showed almost 50% reductions in all pigments. The Chl *a/b* ratios for both G5 and G6 cultivars were reduced in response to glyphosate spraying. This reduction was more significant in G5 than that of G6 (Table 1).

The glyphosate-treated leaves showed significant reduction in P_N in both G5 and G6 cultivars. P_N were reduced to 65 and 52% of the controls for G5 and G6, respectively (Fig. 2*A*). Leaves treated with HD showed reductions in both E, g_s, and WUE in comparison with the control plants (Fig. 2*B,C,E*). Furthermore, C_i was enhanced compared with the control. Trends in E, g_s, C_i, and WUE were consistent with the increasing glyphosate concentration (Fig. 2). Treatment with HD altered C_i, which was 13% higher (Fig. 2*D*), and WUE, which was 9% lower than those of controls. Both cultivars responded similarly towards glyphosate in their gas exchange properties (Fig. 2*A–E*).

Total proteins and total free amino acids: The control G5 and G6 leaves showed almost similar protein contents (Table 2). Both cultivars responded oppositely towards the glyphosate application. The glyphosate treatment caused a significant reduction in protein contents of the G5 leaves. Contrary, the G6 leaves showed higher protein contents when treated with HD.

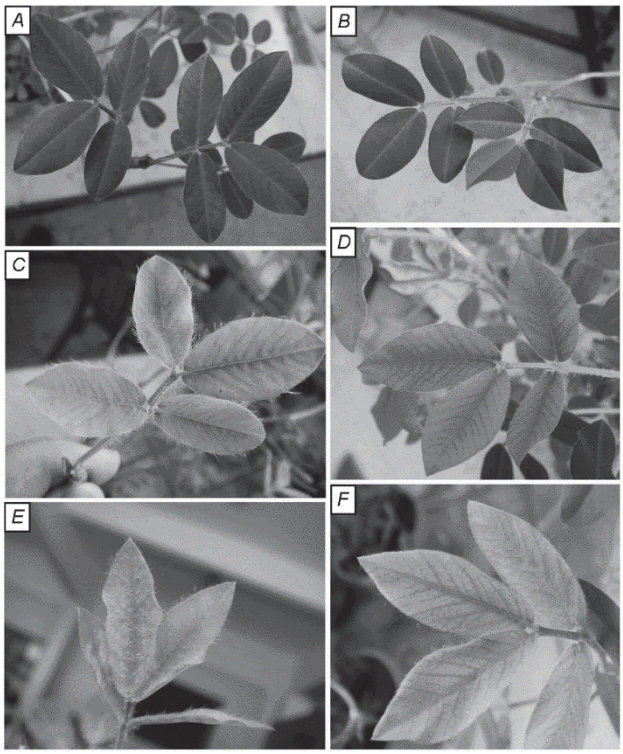

Fig. 1. Effect of glyphosate herbicide on peanut leaves (*Arachis hypogaea* L. cv. Giza 5 and Giza 6). *A*, *C*, and *E* are Giza 5 control, low and high doses, respectively. *B*, *D*, and *F* are Giza 6 control, low and high doses, respectively.

The G5 leaves had higher total free amino acid contents than that of G6 in the control plants. The content of free amino acids declined due to the glyphosate application. The reduction was noticed in both cultivars and it was highly significant. For example, the glyphosate treatment of the G5 leaves reduced the protein content to almost half of the corresponding control value (Table 2).

Table 1. Effect of glyphosate treatments on pigment contents of *Arachis hypogaea* L. (cv. Giza 5 and Giza 6) leaves. The values are means of four replicates ± SD. $^{*-}$ significant at $P<0.05$; **– significant at $P<0.01$. Chl – chlorophyll; Car – carotenoids.

Cultivar	Treatment	Chl *a* [mg g^{-1}(FM)]	[%]	Chl *b* [mg g^{-1}(FM)]	[%]	Car [mg g^{-1}(FM)]	[%]	Chl *a/b*
Giza 5	Control	1.15 ± 0.11	100	0.53 ± 0.08	100	0.12 ± 0.02	100	2.17
	LD	0.39 ± 0.03**	33.6	0.15 ± 0.01**	27.9	0.05 ± 0.01**	41.7	2.61
	HD	0.30 ± 0.05**	26.0	0.15 ± 0.01**	28.5	0.03 ± 0.01**	28.6	1.98
Giza 6	Control	0.99 ± 0.15	100	0.41 ± 0.06	100	0.12 ± 0.08	100	2.39
	LD	0.54 ± 0.15**	54.6	0.24 ± 0.07*	58.5	0.06 ± 0.09**	53.5	2.23
	HD	0.48 ± 0.10**	48.9	0.23 ± 0.04**	55.1	0.06 ± 0.01**	47.5	2.12

Antioxidant enzyme activities: A highly significant increase in POD and CAT activities were detected in the glyphosate-treated leaves. In the G5 cultivar, the increase in POD and CAT activities reached up to 3.5 and 2.4 fold of the control, respectively. POD activity was doubled in the G6 leaves in response to HD. Moreover, the G6 leaves responded to glyphosate by 76.5% increase of the CAT activity compared with the control. It seems that the increase in activities of POD and CAT were dose-dependent. Furthermore, comparing POD and CAT activities after the glyphosate treatments, it was clear that POD responded more than CAT to the same dose of applied glyphosate (Table 3).

Activities of APX and SOD showed enhanced activities after the glyphosate treatment in both G5 and G6 cultivars (Table 4). In details, the increase in APX and SOD activities reached 152 and 175 %, respectively, of the corresponding control in G5. In G6, the increase in APX activity tended to be similar to that of G5, while SOD activity in G6 was lower than that detected in G5. Both enzymes showed glyphosate dose-dependent responses (Table 4).

MDA content and DPPH free radical scavenging assay: Obviously, MDA accumulated in response to the glyphosate treatment (Table 5). The increase in the MDA content was dependent on the concentration. The accumulation of MDA was noticed in both cultivars, G5 and G6. Lipid peroxidation was more obvious in G5. Application of HD to the G5 leaves caused an increase in MDA up to 282.1%, while the MDA content reached 129.7% in the G6 leaves compared with the corresponding controls.

DPPH scavenging assay revealed significant enhancement in TAA in both cultivars (Table 5). Comparing G5 and G6 cultivars, the control G6 leaves showed TAA values higher than that of G5. Application of HD increased TAA in the G5 leaves more than that of G6 in comparison with the control (Table 5).

Discussion

In the present work, glyphosate herbicide severely affected both cultivars of peanut. The negative effects noticed in the glyphosate-treated leaves were related to changes in the physiological and antioxidant status. Some morphological symptoms were noticed in the leaves sprayed with glyphosate. As herbicides affect cell metabolism of plants, specifically their photosynthetic machinery, changes in leaf color or bleaching due to stress is mostly related to alterations in photosynthetic pigment contents (Radwan and Soltan 2012). In this experiment, the contents of pigments were lowered due to glyphosate application in both cultivars. Dalla Vecchia *et al.* (2001) reported that herbicides caused severe photo-oxidative damage to maize chloroplasts. Cobb and Reade (2010) reported that further metabolic consequence of glyphosate treatment was the development of chlorotic areas on leaves. This may be due to an inhibition of δ-aminolaevulinic acid (δ-ALA) synthetase, an early reaction in the biosynthesis of all porphyrin-containing molecules, including Chl and cytochromes. The reduction of the Chl content might be a good indicator for monitoring damage to plant growth and development (Yin *et al.* 2008). This damage might be due to accumulation of ROS that leads to oxidative stress. A significant reduction in P_N was observed and this reduction was concomitant with the glyphosate dose applied. Although glyphosate has its specific target site, there is also evidence that P_N, photosynthetic pigments, or photosynthesis-related proteins have decreased after the glyphosate application (Geiger *et al.* 1986, Servaites *et al.* 1987, de María *et al.* 2005, Sergiev *et al.* 2006). Previous studies have indicated a decreased P_N in plants following glyphosate exposure (Yamauchi *et al.* 2008, Mateos-Naranjo *et al.* 2009, Zobiole *et al.* 2012). Moreover, glyphosate affects photosynthetic electron transport indirectly by inhibiting sink processes. In other words, glyphosate affects photosynthesis indirectly by inhibiting the biosynthesis of Car, Chls, fatty acids, or amino acids (Fedtke and Duke 2005). The changes in gas-exchange parameters are more sensitive biomarkers for glyphosate toxicity (Olesen and Cedergreen 2010). Leaf C_i remained constant despite decreases in g_s following glyphosate treatment, indicating slowing of carbon assimilation rather

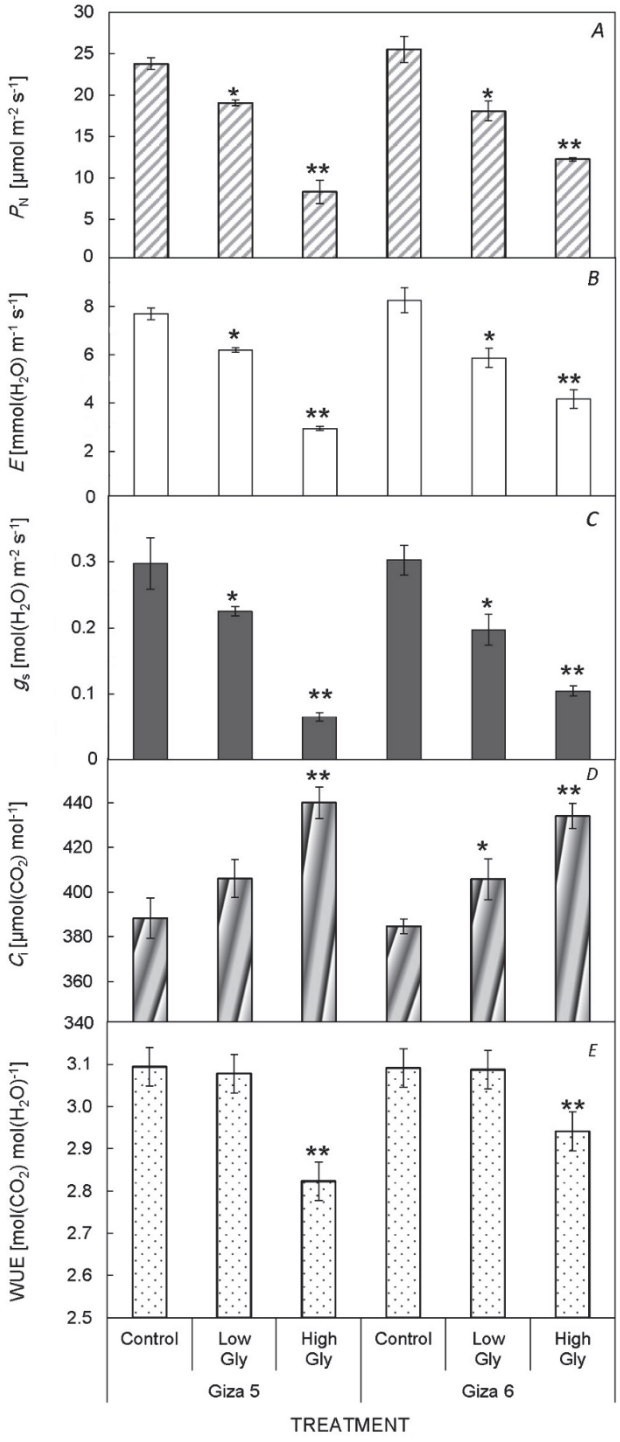

Fig. 2. Effect of glyphosate on gas-exchange properties of peanut leaves (*Arachis hypogaea* L. cv. Giza 5 and Giza 6). (*A*) photosynthetic rate (P_N); (*B*) transpiration rate (*E*); (*C*) stomatal conductance (g_s); (*D*) intercellular CO_2 concentration C_i); (*E*) water-use efficiency (WUE). Values are means of four replicates ± standard deviation. Statistical significance of differences compared to control: * – significant at $P<0.05$; ** – significant at $P<0.01$.

than decreased cause of ribulose-1,5-bisphosphate (RuBP) depletion. A reduction in the rate of regeneration of RuBP could be mediated by a glyphosate-induced lowering of ATP or NADPH supply (Geiger *et al.* 1987).

It is well known that the major function of proteins in metabolism is to serve as enzymes for metabolic activities (Hoppe 1989). In this experiment, reductions in the protein content were noticed in the treated G5 leaves while more proteins were detected in the G6 leaves. Glyphosate blocks the action of the EPSP synthase in the shikimate pathway which is vital in the biosynthesis of three amino acids, such as phenylalanine, tyrosine, and tryptophan. Interestingly, glyphosate competes with one substrate, phosphoenol pyruvate (PEP), but forms a very stable herbicide–enzyme complex with the other, resulting in a 'full stop' in the metabolic pathway. This also subsequently reduces the ability of plants to synthesize a number of metabolites, such as hormones, flavonoids, and lignins (Cobb and Reade 2010). It is unclear how glyphosate leads to plant death; various hypotheses, such as depletion of protein stocks and drainage of carbon from other vital pathways, have been suggested (Duke and Powles 2008).

Srivalli *et al.* (2003) reported that the cellular anti-oxidant system serves as a sensor for accumulation of ROS. Any perturbation of the balance between formation and scavenging of ROS affects cellular redox homeostasis. Recent studies have demonstrated that pollutants, such as excessive herbicides and other toxic organic substances, are able to induce intracellular overproduction of ROS and damage plant cells (Radwan *et al.* 2008, Yin *et al.* 2008). In our experiment, all analyzed antioxidant enzymes (POD, CAT, APX, and SOD) were induced in the glyphosate-treated leaves at both LD and HD applied. The increase was concomitant with the herbicide concentration and the increase was higher in POD and APX than that of CAT and SOD activities. The cooperative function of antioxidants, such as POD, APX, and CAT, plays an important role in scavenging ROS and maintaining the physiological redox status of organisms (Fecht-Christoffers *et al.* 2003, Cho and Seo 2005). Elevated POD activity in plant tissues has been used as a biomarker for various contaminant stresses (Song *et al.* 2007). The increase in the POD activity in wheat leaves as a result of herbicide treatment was probably due to peroxidation of the membrane lipids. Moreover, the increased POD activities in roots and leaves exposed to lower herbicide concentrations may suggest that the plant experienced moderate stress. However, the decreased POD activities in tissues represented the damage of the plant capacity to control the oxidative stress (De Prado *et al.* 1999, Jiang and Yang 2009). Furthermore, POD and CAT may remove H_2O_2 by oxidizing various hydrogen donor molecules and help to improve protection in plant tissues, when exposed to chemicals (Xue *et al.* 2008). Duckweed tissues treated with glyphosate also showed higher CAT and APX

Table 2. Effect of glyphosate treatments on proteins and total free amino acid contents of *Arachis hypogaea* L. (cv. Giza 5 and Giza 6) leaves. The values are means of four replicates ± SD deviation. * – significant at $P<0.05$; **– significant at $P<0.01$.

Cultivar	Treatment	Proteins [mg g^{-1}(DM)]	[%]	Amino acids [mg g^{-1}(DM)]	[%]
Giza 5	Control	320.47 ± 48.38	100	116.30 ± 57.30	100
	LD	282.70 ± 17.46	88.2	67.13 ± 5.95**	57.7
	HD	232.83 ± 12.75**	72.7	68.27 ± 13.17**	58.7
Giza 6	Control	330.00 ± 39.06	100	104.93 ± 18.77	100
	LD	336.97 ± 18.55	102.1	102.33 ± 7.49	97.5
	HD	312.03 ± 12.12*	94.6	66.00 ± 22.26*	62.9

Table 3. Effect of glyphosate treatments on peroxidase (POD) and catalase (CAT) activities of *Arachis hypogaea* (L. *cv.* Giza 5 and Giza 6) leaves. The values are means of four replicates ± SD. * – significant at $P<0.05$; **– significant at $P<0.01$.

Cultivar	Treatment	POD [nmol g^{-1}(FM) h^{-1}]	[%]	CAT [nmol g^{-1}(FM) h^{-1}]	[%]
Giza 5	Control	13.15 ± 0.51	100	57.75 ± 2.42	100
	LD	43.68 ± 1.52**	332.3	84.00 ± 3.81*	145.5
	HD	47.07 ± 2.64**	358.1	138.25 ± 4.66**	239.4
Giza 6	Control	22.65 ± 1.80	100	60.50 ± 4.54	100
	LD	36.55 ± 2.33*	161.4	67.00 ± 4.84	110.7
	HD	46.72 ± 2.98**	206.3	106.75 ± 5.45*	176.5

Table 4. Effect of glyphosate treatments on ascorbate peroxidase (APX) and superoxide dismutase (SOD) activities of *Arachis hypogaea* (L. *cv*. Giza 5 and Giza 6) leaves. The values are means of four replicates ± SD. * – significant at $P<0.05$; **– significant at $P<0.01$.

Cultivar	Treatment	APX [nmol g^{-1}(FM) h^{-1}]	[%]	SOD [nmol g^{-1}(FM) h^{-1}]	[%]
Giza 5	Control	8.33 ± 1.09	100	12.44 ± 1.47	100
	LD	13.58 ± 1.34*	163.0	25.56 ± 2.56**	205.5
	HD	29.38 ± 1.25**	352.7	46.62 ± 3.34**	374.8
Giza 6	Control	10.63 ± 1.59	100	18.94 ± 2.41	100
	LD	19.40 ± 1.30**	182.5	32.50 ± 3.94**	171.6
	HD	38.17 ± 2.38**	359.1	42.84 ± 2.56**	226.2

Table 5. Effect of glyphosate treatments on malondialdehyde (MDA), H_2O_2, and total antioxidant activity using DPPH scavenging assay (%) of *Arachis hypogaea* (L. *cv*. Giza 5 and Giza 6) leaves. The values are means of four replicates ± SD. * – significant at $P<0.05$; **– significant at $P<0.01$.

Cultivar	Treatment	MDA [μmol g^{-1}(FM)]	[%]	H_2O_2 [nmol g^{-1}(FM)]	[%]	DPPH	[%]
Giza 5	Control	12.04 ± 5.56	100	50.32 ± 1.49	100	37.83 ± 13.98	100
	LD	23.66 ± 9.22*	196.4	55.12 ± 2.44**	109.5	47.93 ± 2.69	126.7
	HD	33.98 ± 8.02**	282.1	68.74 ± 2.26**	136.6	60.07 ± 6.33*	158.8
Giza 6	Control	33.33 ± 4.84	100	56.39 ± 2.33	100	42.56 ± 13.01	100
	LD	36.77 ± 1.12*	110.3	78.40 ± 2.46**	139.0	43.84 ± 10.19	103.0
	HD	43.01 ± 12.65**	129.0	85.17 ± 3.52**	151.0	54.58 ± 13.91*	128.3

activities, demonstrating that oxidative stress can be induced by glyphosate (Kielak *et al.* 2011).

Accumulations of some metabolites are another way protecting against oxidative stress within the plant cell. Among these metabolites, MDA is the most important. MDA is an indicator of lipid peroxidation, however, biological effects of MDA on plant functions are not yet known (Yamauchi *et al.* 2008). In this experiment, increased amounts of MDA were detected in the glyphosate-treated leaves indicating larger peroxidation and oxidative stress. Moreover, Ahsan *et al.* (2008) found that glyphosate application generates H_2O_2, resulting in peroxidation and destruction of lipids in rice (*Oryza sativa*) leaves. MDA causes adverse effects in plants, thus, free MDA has been determined in various sources as an oxidative stress marker (Yamauchi *et al.* 2008, Harfouche

et al. 2009). MDA, a product of lipid peroxidation, has been seen to be greatly accumulated after stress (Anjum *et al.* 2008). In addition, it has been recently reported that maize leaves exposed to glyphosate had increased lipid peroxidation, glutathione (GSH), free proline content, and ion flux, suggesting that along with the inhibition of its specific target site, the glyphosate action also leads to oxidative stress in plants which is most probably the secondary effect of the blocked shikimate pathway (Sergiev *et al.* 2006).

An increased MDA (lipid peroxidation) and H_2O_2 contents and activation of antioxidant enzymes (SOD, CAT, and GPX) were found (Sergiev *et al.* 2006, Miteva *et al.* 2010). The changes in the antioxidant activity in response to glyphosate treatments were confirmed by analyzing the TAA using DPPH free radical scavenging assay of leaf extracts. The TAA increased in both G5 and G6 and the increase in G5 was higher than that of the G6 leaves in comparison with the control. Similar results were obtained in case of maize plants treated with clethodim herbicide (Radwan 2012). This confirmed changes in the antioxidant status and accumulation of antioxidants in response to glyphosate application. Antioxidants, in interaction with DPPH, either transfer an electron or hydrogen atom to DPPH, thus neutralizing its free radical character (Naik *et al.* 2003).

To conclude, the peanut plants were negatively affected by glyphosate application even under low doses. Chlorosis, yellowing, and curling of leaf edges were the most noticeable morphological glyphosate-application symptoms. Oxidative stress due to glyphosate caused the decline in pigments, proteins, and free amino acid contents. Pronounced changes in the antioxidant activity were detected. These changes were proved through monitoring the POD, CAT activities, MDA content, and from analyzing the TAA of leaf extracts.

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