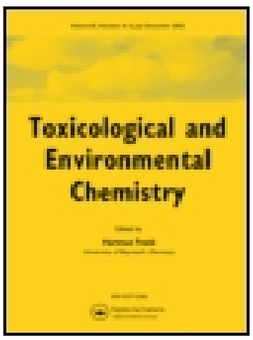

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Mitigation of glyphosate-based herbicide toxicity in maize (*Zea mays* L.) seedlings by ascorbic acid

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Abstract

The toxicity of glyphosate at 3.6 mg L^{-1} to maize seedlings raised from un-treated seeds and the effectiveness of seed pretreatment by soaking in 0.25 mmol L^{-1} ascorbic acid solution for mitigation of toxicity were evaluated in hydroponic culture. Glyphosate dramatically reduced growth of roots and photosynthetic pigments in the leaves but increased protein content in the leaves. Superoxide dismutase activity and ascorbic acid concentration in the roots were increased, and guaiacol peroxidase activity was unaffected. Pretreatment with ascorbic acid improved the dry mass of the roots and shoots, increased the protein content in roots and leaves, and significantly decreased activity of guaiacol peroxidase in roots. The positive effect of ascorbic acid treatment was not associated with more efficient functioning of the antioxidative system.

Keywords: glyphosate; maize; antioxidants; seeds pretreatment; ascorbic acid

Introduction

Experimental studies with various plants have shown that application of ascorbic acid (AsA) may reduce deleterious effects induced by various environmental stress conditions (Dolatabadian, Modarres Sanavy and Sharifi 2009; Farooq et al. 2013; Noman et al. 2015; Akram, Shafiq and Ashra 2017) but there is a lack of information on herbicides. Use of herbicides in agricultural and non-agricultural systems increases environment contamination and the risk of impairment of non-target plants (Latinen et al. 2006; Latinen, Rämö and Siimes 2007; Aktar, Sengupta and Chowdhury 2009; Bott et al. 2011).

Glyphosate (*N*-phosphonomethylglycine) is the most widely used broad-spectrum, non-selective herbicide, extensively applied to growing genetically modified crops, and glyphosate residues in soil may affect subsequently cultivated plants (Latinen et al. 2006; Tesfamariam at al. 2009). Glyphosate can reach the soil via foliar wash-off, undirected spray drift and by exudation from roots or decomposition of treated plant residues (Tesfamariam et al. 2009). Concentrations in soil may vary from μg kg^{-1} to mg kg^{-1} (Latinen et al. 2006; Rose at al. 2017; Silva et al. 2018). Glyphosate, apart from the primary effect (the inhibition of 5-enolpyruvyl shikimate-3-phosphate synthase), causes many secondary effects such as the disruption in photosynthesis, mineral nutrition, water uptake, interactions between plants and microorganisms, and also leads to oxidative stress (Sacała, Demczuk and Grzyś 1999, 2011; Ahsan et al. 2008; Cakmak at al. 2009; Johal and Huber 2009; Zabiole et al. 2011; Gomes et al. 2014).

To remove reactive oxygen species and reduce oxidative damage, plants possess very efficient antioxidative mechanisms. AsA is one of the crucial antioxidants but also plays a vital role in the stress perception, redox homeostasis and regulation of plant responses under normal and abiotic stress conditions (Akram, Shafiq and Ashra 2017).

The aim of this study was to examine the response of maize seedlings to glyphosate present in a nutrient medium and evaluate whether AsA can improve plant tolerance to the herbicide.

Materials and methods

Plant material, seed treatments and growth conditions

Maize seeds (*Zea mays* L. variety Kosmo 230), 50 g, obtained from Nasiona Kobierzyc (Kobierzyce, Poland) were soaked in 1 L 0.25 mmol L^{-1} AsA solution or 1 L distilled water (control) at room temperature for 2 h. For germination, 80 soaked seeds were placed in a plastic box with a lid (12.5 × 22.5 × 4 cm) on filter paper moistened with distilled water at 28 °C for 48 h in darkness. Twelve uniformly germinated seeds were transferred into 1.2 L glass container filled with nutrient solution containing (in mmol L^{-1}): 3 $Ca(NO_3)_2$, 2 KNO_3, 1 $MgSO_4$, 1 KH_2PO_4, 0.2 ethylenediaminetetraacetic acid iron(III)-sodium salt and microelements (in μmol L^{-1}): 46 H_3BO_3, 18 $MnCl_2$, 0.8 $ZnSO_4$, 0.5 Na_2MoO_4, 0.3 $CuSO_4$, pH 6.5±0.1. Glyphosate as iso-propylamine salt of N-phosphonomethyl glycine was added to the nutrient medium at a concentration of 3.6 mg L^{-1} (Glifocyd®, 360 g L^{-1} iso-propylamine salt of N-phosphonomethyl glycine, Business Group of Bayer Crop Science, Nowa Sarzyna, Poland). Plants grew under controlled conditions: 16 h photoperiod (220 μmol·m^{-2}·s^{-1}) at 26/20 °C day/night, 60-65 % relative humidity.

All experiments (assessment of growth parameters and biochemical analyses) were repeated four times. Spectrophotometric assays were conducted with an UV-Vis spectrophotometer (Evolution 600, Thermo Fisher Scientific, Cambridge, UK).

Growth parameters and water content

After 7 days of cultivation, plants were harvested and separated into roots and shoots and their lengths and fresh weights were measured. After that, the plant organs were dried and the dry weight was determined. The water content was calculated on a fresh weight basis as the difference between the fresh weight and the dry weight divided by the fresh weight and multiplied by 100%.

Determination of photosynthetic traits

The photosynthetic pigments and chlorophyll fluorescence parameters were assayed in the second leaf of maize. The ratio F_v/F_m (F_v – variable fluorescence, F_m – maximal fluorescence), representing the maximum photochemical efficiency of photosystem II (PS II), was measured by using a portable fluorometer (OS-30p, Opti-Sciences Inc, Hudson, New Hampshire, USA). Photosynthetic pigments were extracted with 80% acetone. The absorbance of the obtained extracts was recorded at 470, 647, 663 nm and the concentrations of total chlorophyll (chlorophyll a + chlorophyll b) and carotenoids were calculated using Lichtenthaler equations (1987).

Activity of selected antioxidant enzymes

Plant material (0.5 g of second leaves and 1.0 g roots) was homogenized in pre-chilled mortar 0.05 mol L^{-1} phosphate buffer pH=7.0 containing 0.002 mol L^{-1} ethylenediaminetetraacetic acid disodium salt. After centrifugation (12000 × g for 10 min at 4 °C) the supernatant was used for the determination of enzyme activity and protein concentration.

Superoxide dismutase (SOD) activity was assayed using the photochemical nitro blue tetrazolium method (Beuchamp and Fridovich 1971).

Guaiacol peroxidase (GPOX) activity was measured by monitoring the rate of oxidation of guaiacol at 470 nm for 2 min (Nakano and Asada 1981). The enzyme activity was expressed as the change in absorbance (ΔA_{470}) mg^{-1} protein min^{-1}.

Concentration of soluble protein and AsA

Protein content in the enzyme extracts was assayed by the method of dye binding Coomassie Brilliant Blue according to Bradford (1976). AsA was extracted and estimated according the procedure described earlier (Sacała 2017).

Statistical analysis

The experiment was arranged as a completely randomized design with two factors. The first factor was the growth conditions (optimal – control, without glyphosate and with added glyphosate) and second was the ascorbic acid level (0 and 0.25 mmol L^{-1}). The presented data are the means of four independent replications. The data were subjected to a two-way variance analysis using the statistical package Statistica version 13.1 (StatSoft Polska, Cracow, Poland). Means were compared using the Duncan post-hoc test and statistical differences among treatments were determined based on least significant differences (LSD) at a significance level of p=0.05.

Results and discussion

Growth parameters and photosynthetic traits

Glyphosate dramatically reduced the fresh and dry weight of shoots and roots, but a greater reduction was observed in the fresh weight of organs than the dry weight (Figure 1). The fresh and dry mass of roots accounted for only 27 % and 42 % of the control. Shoot growth was less inhibited constituted 38 % and 54 % of the values observed in the control plants.

A negative effect of glyphosate applied to the rooting medium was also observed in experiments on other non-target plants: sunflower, tomato, maize and cucumber (Cornish 1992; Sacała, Demczuk and Grzyś 1999, 2011; Tesfamariam et al. 2009). Tesfamarian et al. (2009) showed that direct application of glyphosate to the soil or as a residue in the root

tissues of target weeds strongly impaired the growth and biomass production of non-target plants. This effect was particularly evident when plants were cultivated immediately after applying the herbicide.

The applied AsA did not modify the fresh weight of examined organs but caused a statistically significant increase in the dry weight of seedlings (Figure 1). The observed increases amounted to 22 % and 32 % for the roots and shoots, respectively, compared to plants raised from non-treated seeds. A positive effect of AsA was also observed for the length of the roots. Improved root growth is a very important effect that allows plants to uptake minerals and water more efficiently, thus promoting further growth. The results demonstrate that the pre-sowing AsA treatment of seeds may help young seedlings to manage glyphosate stress and grow better in these conditions.

Glyphosate caused a disruption of the water status in plant tissues and 3.6 % and 2.4 % loss of water content was found in the roots and shoot, respectively, compared to the control plants (Table 1). The exogenous application of AsA did not reverse the negative effect.

Adverse environmental conditions usually cause disruptions in photosynthesis and a reduction in photosynthetic pigments. Applying glyphosate resulted in a substantial decrease (23 % compared to the control) in photosynthetic pigments (Table 1) and the application of AsA did not change this trend.

Most of the data in the literature shows that applying glyphosate resulted in a decrease in the chlorophyll content in plants (Sacała, Demczuk and Grzyś 1999; Moldes et al. 2008; Reddy, Bellaloui and Zablotowicz. 2010; Kielak et al. 2011; Zabiole et al. 2011). The noticed decline in chlorophyll content may be due to an inhibition of chlorophyll biosynthesis. Kitchen, Witt and Rieck (1981) showed that glyphosate inhibits δ-aminolevulinic acid synthesis, causing a blockage of the synthesis of chlorophyll and other porphyrins. Some researchers claim that glyphosate may act indirectly by immobilizing Mg and other cations and consequently leading to a deficiency in plants (Cakmak et al. 2009; Zabiole et al. 2011). The level of carotenoids also decreased in response to the application of glyphosate, thus limiting their protective effect (Table 1).

A marked decrease in the chlorophyll concentration did not affect the Fv/Fm ratio under all examined conditions indicating that plants maintained the integrity and functionality of PS II (Table 1). This positive reaction may results from the ability of maize seedlings to maintain a high level of protein and ascorbic acid in the leaves under glyphosate stress (Table 2). Data in the literature concerning the Fv/Fm ratio are inconclusive and some researchers claim that

this parameter displays a relatively low sensitivity to herbicides (Juneau, Baosheng and Deblois 2007).

Protein content and antioxidative activity

The concentration of soluble protein increased by 10 % in the leaves of maize grown under glyphosate (Table 2). In response to environmental stresses, plants synthesise different classes of proteins that protect cells against stress-induced damage (Rodziewicz et al. 2014). Leaves that were not directly exposed to glyphosate functioned relatively properly. Protein synthesis was not disrupted and the AsA content and activity of the examined enzymes did not change compared to the control plants (Table 2). The application of AsA resulted in an increased protein content in the roots and leaves of maize treated by glyphosate. This positive interaction shows that AsA promotes protein synthesis and might partially explain the observed improvement in plant growth.

AsA is a crucial non-enzymatic antioxidant but plays also an important role in other physiological processes and can improve plant tolerance to abiotic stresses (Akram, Shafiq and Ashra 2017). Glyphosate caused a marked increase in the AsA content in roots (28 % increase compared to the control) and AsA application did not modify this parameter (Table 2). An increased level of AsA might be essential for the protection of plants against oxidative damage and in improving stress tolerance.

Among the enzymes involved in the antioxidative defence system, SOD and peroxidases are very important. In the leaves of maize, the activity of these enzymes did not change under glyphosate treatment, whereas in the roots SOD activity increased almost 2.5-fold and pre-treatment of seeds resulted in a marked decrease in GPOX activity in maize roots, particularly in glyphosate treated plants. The decrease in GPOX activity, concomitant with the increase in SOD activity and the unavoidable increase in the production of hydrogen peroxide (H_2O_2), suggests that GPOX doesn't play a crucial role in H_2O_2 detoxification. Moldes et al. (2008) indicated that GPOX activity increased in soybean leaves treated by glyphosate, whereas in the roots it was unaffected. The activation of antioxidant enzymes in plants exposed to glyphosate was also demonstrated by other researchers (Sergiev et al. 2006; Miteva, Ivanov and Alexieva 2010).

Conclusions

The glyphosate present in the rooting medium considerably affected the growth and metabolism of maize seedlings. AsA significantly improved the length of roots and dry weight of seedlings treated by glyphosate. The increased protein level in plants raised from AsA treated seeds and exposed to glyphosate suggests that AsA promotes protein synthesis,

and consequently increases plant tolerance to the herbicide. The results demonstrate that exogenously applied AsA may mitigate phytotoxicity of glyphosate but this effect is not connected with better functioning of the antioxidative system.

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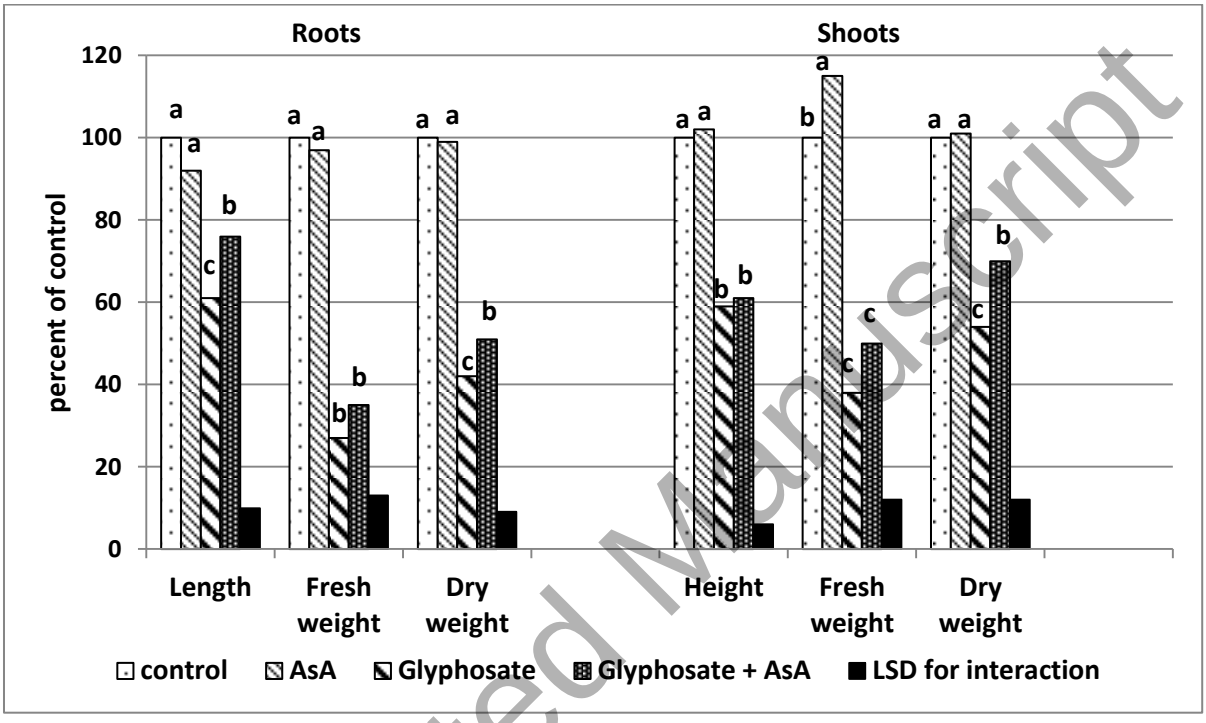

Figure 1. The influence of glyphosate and AsA pretreatment of seeds on growth parameters of roots and shoots of 7-day old maize. Control – are seedlings grown without glyphosate and non-pretreated with AsA.

Data are means of four independent replications. The data with different letter are significantly different according to Duncan post-hoc test at a significance level of p=0.05. Results are expressed as percent of control.

Table 1. The influence of glyphosate and AsA (0.25 mmol L^{-1}) pretreatment of seeds on photosynthetic pigments concentrations, Fv/Fm ratio and water content in 7-day old maize seedlings. Control – are seedlings grown without glyphosate and non-pretreated with AsA.

Treatments	AsA [mmol L^{-1}]	Total chlorophyll concentration [mg g^{-1} fresh weight]	Carotenoid concentration [mg g^{-1} fresh weight]	Fv/Fm	Water content [%]	
					Shoots	Roots
Control	0	2.37±0.044^{a}	0.373±0.0015^{a}	0.736±0.004^{a}	91.6±0.052^{a}	94.4±0.056^{a}
	0.25	2.21± 0.105^{a}	0.342±0.017^{b}	0.741±0.006^{a}	91.5 ±0.074^{a}	94.6±0.076^{a}
Glyphosate	0	1.83±0.027^{b}	0.270±0.0023^{c}	0.715± 0.015^{a}	89.2±0.068^{b}	90.9± 0.064^{b}
	0.25	1.78±0.033^{b}	0.289±0.005^{c}	0.736±0.009^{a}	89.1±0.085^{b}	91.0±0.082^{b}
Mean for treatments	Control	2.29^{a}	0.357^{a}	0.739^{a}	91.5^{a}	94.5^{a}
	Glyphosate	1.81^{b}	0.280^{b}	0.726^{a}	89.2^{b}	91.0^{b}
Mean for AsA	0	2.10^{a}	0.321^{a}	0.726^{a}	90.4^{a}	92.7^{a}
	0.25	2.00^{a}	0.316^{a}	0.739^{a}	90.3^{a}	92.8^{a}
$LSD_{0.05}$ for treatments(I)		0.133	0.020	0.021	0.155	0.166
$LSD_{0.05}$ for AsA (II)		0.133	0.020	0.021	0.155	0.166
$LSD_{0.05}$ for interaction (I×II)		0.188	0.028	0.028	0.219	0.231

Data presented are means of four independent replications ± standard error. Significant mean differences were determined using the Duncan post-hoc test at a significance level of p=0.05 and marked with different letters.

Table 2. The influence of glyphosate and AsA (0.25 mmol L^{-1}) pretreatment of seeds on some biochemical parameters in leaves and roots of 7-day old maize seedlings. Control – are seedlings grown without glyphosate and non-pretreated with AsA.

Treatments	ASA [mmol L^{-1}]	SOD activity [unit mg^{-1} protein min^{-1}]		GPOX activity [ΔA_{470} mg^{-1} protein min^{-1}]		AsA content [mg g^{-1} fresh weight]		Protein content [mg g^{-1} fresh weight]	
		Leaves	Roots	Leaves	Roots	Leaves	Roots	Leaves	Roots
Control	0	1.27±0.058^{a}	1.18±0.051^{d}	0.73±0.021^{b}	15.2±0.362^{ab}	0.945±0.046^{ab}	0.454±0.024^{c}	8.72±0.088^{c}	5.14±0.252^{bc}
	0.25	1.31±0.094^{a}	1.58±0.044^{c}	0.87±0.023^{a}	13.5±0.738^{b}	0.888±0.022^{b}	0.499±0.030^{bc}	8.56±0.221^{c}	5.76±0.439^{ab}
Glyphosate	0	1.33±0.058^{a}	2.85±0.038^{a}	0.70±0.021^{b}	16.8±0.729^{a}	1.096±0.071^{a}	0.581±0.001^{a}	9.60±0.387^{b}	4.44±0.227^{c}
	0.25	1.21±0.205^{a}	2.15±0.094^{b}	0.72±0.033^{b}	9.3±1.234^{c}	1.068±0.065^{a}	0.545±0.013^{ab}	10.81±0.323^{a}	6.11±0.247^{a}
Mean for treatments	Control	1.29^{a}	1.38^{b}	0.80^{a}	14.4^{a}	0.917^{b}	0.477^{b}	8.64^{b}	5.45^{a}
	Glyphosate	1.27^{a}	2.50^{a}	0.71^{b}	13.1^{a}	1.082^{a}	0.563^{a}	10.21^{a}	5.27^{a}
Mean for AsA	0	1.30^{a}	2.01^{a}	0.72^{b}	16.0^{a}	1.021^{b}	0.517^{a}	9.16^{a}	4.79^{b}
	0.25	1.26^{a}	1.87^{b}	0.80^{a}	11.4^{b}	0.978^{b}	0.522^{a}	9.69^{a}	5.94^{a}
$LSD_{0.05}$ for treatments (I)		0.301	0.132	0.054	1.80	0.120	0.046	0.607	0.663
$LSD_{0.05}$ for AsA (II)		0.301	0.132	0.054	1.80	0.120	0.046	0.607	0.663
$LSD_{0.05}$ for interaction (I×II)		0.425	0.187	0.077	2.55	0.171	0.068	0.859	0.938

Data presented are means of four independent replications ± standard error. Significant mean differences were determined using the Duncan post-hoc test at a significance level of p=0.05 and marked with different letters.