

Glyphosate inhibition of ferric reductase activity in iron deficient sunflower roots

Levent Ozturk¹, Atilla Yazici¹, Selim Eker², Ozgur Gokmen¹, Volker Römheld³ and Ismail Cakmak¹

¹Faculty of Engineering and Natural Sciences, Sabanci University, TUR–34956 Istanbul, Turkey; ²Department of Soil Science, Faculty of Agriculture, Cukurova University, TUR–01330 Adana, Turkey; ³Institute of Plant Nutrition, University of Hohenheim, D–70593 Stuttgart, Germany

Summary

Author for correspondence:

Ismail Cakmak

Tel: +90 216 4839524

Fax: +90 216 4839550

Email: cakmak@sabanciuniv.edu

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- Iron (Fe) deficiency is increasingly being observed in cropping systems with frequent glyphosate applications. A likely reason for this is that glyphosate interferes with root uptake of Fe by inhibiting ferric reductase in roots required for Fe acquisition by dicot and nongrass species.
- This study investigated the role of drift rates of glyphosate (0.32, 0.95 or 1.89 mM glyphosate corresponding to 1, 3 and 6% of the recommended herbicidal dose, respectively) on ferric reductase activity of sunflower (*Helianthus annuus*) roots grown under Fe deficiency conditions.
- Application of 1.89 mM glyphosate resulted in almost 50% inhibition of ferric reductase within 6 h and complete inhibition 24 h after the treatment. Even at lower rates of glyphosate (e.g. 0.32 mM and 0.95 mM), ferric reductase was inhibited. Soluble sugar concentration and the NAD(P)H oxidizing capacity of apical roots were not decreased by the glyphosate applications.
- To our knowledge, this is the first study reporting the effects of glyphosate on ferric reductase activity. The nature of the inhibitory effect of glyphosate on ferric reductase could not be identified. Impaired ferric reductase could be a major reason for the increasingly observed Fe deficiency in cropping systems associated with widespread glyphosate usage.

Key words: chlorosis, ferric reductase, glyphosate, iron deficiency, sunflower (*Helianthus annuus*).

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Introduction

Iron (Fe) deficiency chlorosis is an important micronutrient deficiency problem in crop production because it diminishes growth, yield and nutritional quality of crops resulting in serious economic and health implications. Large investments are currently being made to correct Fe deficiency chlorosis. It is estimated that the annual cost of overcoming Fe deficiency is as high as 80–100 M Euros in the Mediterranean region (Abadia *et al.*, 2004) and 120 M USD in the north central soybean-growing area of the USA (Hansen *et al.*, 2004). Iron deficiency also represents an important nutritional and health

concern in animal and human nutrition, resulting in severe health complications in people, particularly in developing countries (Welch & Graham, 2004). A major reason for the widespread occurrence of Fe deficiency in humans is attributed to the consumption of food crops containing low levels of Fe as a result of various soil and genetic factors.

Iron deficiency in plants commonly occurs in soils that provide only low chemical and spatial Fe availability to plant roots. Soil, climatic and plant factors have been reported to reduce the availability and root uptake of Fe. Poorly aerated, cool and calcareous or alkaline soils are characteristic soils where Fe deficiency often occurs in plants (Marschner *et al.*, 1986;

Inskeep & Bloom, 1987). Plants grown in such soils have developed several adaptive mechanisms to improve Fe availability in the rhizosphere and to maintain sufficient root uptake of Fe. These adaptive root responses to Fe-deficient soil conditions differ among plant species. Dicots and nongrass monocot species (Strategy-I plants) induce distinct root morphological and physiological responses to Fe deficiency (Römheld, 1987; Marschner & Römheld, 1994; Schmidt, 1999). An increased capacity of roots to reduce Fe-chelates by an inducible ferric reductase enzyme at the plasma membrane is the most characteristic response of Strategy-I plants to Fe deficiency. Reduction of Fe(III) to Fe(II) at the root surfaces is an obligatory process in Fe acquisition of Strategy-I plants (Chaney *et al.*, 1972; Römheld & Marschner, 1986; Robinson *et al.*, 1999). Acidification of the rhizosphere by a proton-pumping ATPase is another characteristic root response of Strategy-I plants to Fe deficiency and contributes to increased solubility of Fe in the rhizosphere (Marschner *et al.*, 1986). Any impairment or alteration in activity of these root mechanisms consequently impairs Fe nutrition and induces leaf chlorosis, as shown in plants grown in high bicarbonate soils (Alcantara *et al.*, 2000), or subjected to low temperature (Schmidt & Steinbach, 2000), heavy metals (Barton *et al.*, 2000) or ethylene inhibitors (Lucena *et al.*, 2006).

Iron deficiency chlorosis is also becoming increasingly prevalent in cropping systems receiving frequent or prolonged applications of glyphosate (*N*-phosphonomethyl glycine). Glyphosate is the most extensively used herbicide in the world. Its usage is increasing with the widespread cultivation of glyphosate-resistant transgenic crops and the adoption of no-tillage cropping systems (Cerdeira & Duke, 2006). This increasing usage of glyphosate represents a potential threat to nontarget plants because of glyphosate drift and accumulation in soils. Up to 10% of the applied rate of glyphosate can drift off target and result in damage to nontarget plants, such as depressing nitrogen (N)-fixing capacity and disturbing nitrate assimilation (Koger *et al.*, 2005; Bellaloui *et al.* 2006; Buehring *et al.*, 2007). Foliar applied glyphosate to target plants is also effective in depressing root uptake of manganese of the nontarget plants, indicating glyphosate transfer from target to nontarget plants via the rhizosphere (Neumann *et al.*, 2006).

Impairment of Fe nutrition in plants seems to be an adverse effect of frequent glyphosate applications. Field observations in many parts of the USA show that widespread occurrence and increased severity of Fe deficiency chlorosis is associated with frequent applications of glyphosate. Application of Fe fertilizers has been shown to reduce the severity of chlorosis and improve grain yield (Franzen *et al.*, 2003; Hansen *et al.*, 2004; Jolley *et al.*, 2004). These observations are in accord with the recent findings that applying 1.89 mM glyphosate (6% of the recommended dose) significantly decreases root uptake and almost completely inhibits root-to-shoot transport of Fe in sunflower plants within 12 h after glyphosate treatment (Eker *et al.*, 2006).

Based on these results and field observations, it may be hypothesized that glyphosate restricts root uptake of Fe, by inhibiting the development of root adaptation mechanisms such as the root-cell ferric reductase activity. The objective of the present study was therefore to investigate the effect of glyphosate on root ferric reductase activity of sunflower plants grown under Fe deficiency conditions. By inhibiting the activity of the enolpyruvyl-shikimate-3-phosphate synthase (EPSPS), glyphosate causes accumulation of shikimate in plants (Shaner *et al.*, 2005; Cerdeira & Duke, 2006). Shikimate is, therefore, an important physiological indicator for the glyphosate activity and glyphosate-related damage in plants (Mueller *et al.*, 2003; Shaner *et al.*, 2005; Neumann *et al.*, 2006). To determine the degree of the glyphosate accumulation in roots after foliar application, shikimate concentrations of roots were measured. Since glyphosate application to leaves decreases sucrose export into the sink organs (e.g. roots) (Geiger *et al.*, 1999), and a possible sugar shortage in the roots may limit ferric reductase activity (Schmidt, 1999), the effect of glyphosate on the sugar concentration of roots was also studied. To our knowledge, this is the first study reporting the effects of glyphosate on ferric reductase enzyme under Fe deficiency.

Materials and Methods

Plant growth

Sunflower (*Helianthus annuus* L. cv. TR-3080) plants were grown hydroponically in a glasshouse equipped with an evaporative cooling system (23–26 : 20–22°C day/night) under natural daylight during the summer season in 2007 (location: 0°53'24.5" N, 029°22'46.7" E). Seeds were germinated in Perlite moistened with saturated CaSO₄ solution for 5 d and five uniformly selected seedlings were then transplanted to plastic nutrient solution culture pots containing 2.7 l of continuously aerated nutrient solution. The nutrient solution (pH 5.5, unbuffered) contained: 0.75 mM K₂SO₄, 2 mM Ca(NO₃)₂, 1 mM MgSO₄, 0.25 mM KH₂PO₄, 0.1 mM KCl, 1 μM MnSO₄, 1 μM ZnSO₄, 10 μM H₃BO₃, 0.1 μM CuSO₄, and 0.01 μM (NH₄)₆Mo₇O₂₄. Initially, Fe-deficient plants were supplied with 0.2 μM Fe-ethylenediaminetetraacetic acid (EDTA) for 5 d to avoid development of severe Fe-deficiency stress. The concentration of Fe for Fe-sufficient plants was 100 μM and applied as Fe-EDTA. Six days after transferring seedlings to the nutrient solution, Fe was withheld from the solution of Fe-deficient plants for 5 d. After 11 d of growth in nutrient solution under Fe deficiency, leaves started to show slight chlorosis. With the onset of leaf chlorosis, glyphosate treatments were applied as described below.

Foliar glyphosate applications

In all foliar applications, glyphosate formulated as Roundup Ultra (active ingredient (ai): 480 g l⁻¹ N-[phosphonomethyl]glycine

isopropylamine salt; Monsanto Ltd, Adana, Turkey) was used. The sublethal doses used for simulating foliar glyphosate drift were 1, 3, and 6% of the recommended application rate (for narrow- or broad-leaf annual weeds), as shown on the product label (i.e. 1.44 kg ha⁻¹ ai glyphosate applied with 200 l of water ha⁻¹: 31.55 mM glyphosate as active ingredient), which is equivalent to 0.32, 0.95, and 1.89 mM glyphosate, respectively. Glyphosate solutions were freshly prepared before foliar treatment. Application was made to the leaves using a plastic hand-sprayer. Glyphosate was sprayed until all leaves became wet (nearly 1.5 ml per plant), but without any run-off.

Root ferric reductase activity

To quantify ferric reductase activity of roots, intact plants were incubated in 5 mM 2-(*N*-morpholino) ethanesulfonic acid (MES) buffer (pH 5.75) containing 100 µM Fe-EDTA and 200 µM ferrozine (3-[2-Pyridyl]-5,6-diphenyl-1,2,4-triazine-4',4''-disulfonic acid sodium salt) for 3 h at 24°C under dark conditions, and the resulting chromophore was measured at 562 nm ($E = 27.9 \text{ mM}^{-1} \text{ cm}^{-1}$) (Stookey, 1970). Root Fe(III) reduction capacity was also visually demonstrated by fixing the roots of intact plants in 1% agar (w : v) containing the same chemical composition used in the ferrozine test.

Measurement of leaf chlorophyll and iron concentrations

Changes in chlorophyll (SPAD value) were measured on the youngest expanded leaves before harvest using a chlorophyll meter (Minolta SPAD-502, Japan). Leaves were dried, ground, and digested using a microwave digestion system (MarsExpress; CEM Corp., Matthews, NC, USA) before analysing for Fe by inductively coupled plasma optical emission spectrometry (ICP-OES) (Vista-Pro Axial; Varian Pty Ltd, Mulgrave, Australia).

Measurement of shikimic acid and soluble sugars

Shikimic acid concentrations were measured in apical parts of roots (3–4 cm) and in young leaves and shoot tips according to a modification of the method of Cromartie & Polge (2002). Fresh leaf and root tissues were extracted in 0.25 N HCl at a ratio of 1 : 10 (w : v) and then centrifuged at 15 000 *g* for 15 min at +4°C. The resulting supernatant was further diluted with 0.25 N HCl at a ratio of 1 : 10 (v : v) and directly used in the colorimetric assay. Aliquots of 200 µl of 1 : 10 diluted samples were mixed with 400 µl of reaction solution (a solution of 0.25% periodic acid and 0.25% Na meta-periodate) and incubated at room temperature for 1 h to oxidize the shikimic acid in the samples. Finally 400 µl of quenching solution (0.6 M NaOH and 0.22 M Na₂SO₃) was added to form a yellow chromophore, which correlates directly with the amount of shikimic acid in the aqueous solution.

Shikimic acid concentration was quantified by measuring the absorbance of the assay samples at 380 nm by using a standard curve of 0–100 µM shikimic acid (S5375; Sigma, St Louis, MO, USA) and presented on a fresh weight basis.

Apical root parts (generally 3–4 cm) were used for analysis of soluble sugars by using the anthrone method (Yemn & Wills, 1954). Fresh root samples were extracted with 80% ethanol and centrifuged at 15 000 *g* for 10 min. The supernatant obtained was treated with the anthrone solution (7.73 mM anthrone in 28 N H₂SO₄) and incubated in a 90°C water bath for 20 min. After cooling to room temperature, absorbance of the green chromophore was measured at 600 nm and the concentration of soluble sugars was quantified by using D-glucose as a standard.

NAD(P)H oxidation

Apical root parts were also used for measurement of NAD(P)H oxidizing capacity. Fresh root tissues were ground in 25 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) buffer (pH 7.5) and centrifuged at 15 000 *g* for 15 min at +4°C. The supernatant obtained was used for measurement of NAD(P)H oxidation at 340 nm ($E = 6.2 \text{ mM}^{-1} \text{ cm}^{-1}$), and the activity was calculated as nmol of NAD(P)H oxidized per minute and gram of fresh weight.

For the statistical treatments, see legends of the tables and figures.

Results

When compared with Fe-sufficient control plants (i.e. 100 µM Fe), the shoot and root growth of plants with the low Fe treatment were only slightly reduced after 12-d of growth in nutrient solution (Table 1). As expected, Fe-sufficient plants had higher levels of Fe and chlorophyll (SPAD values) than the Fe-deficient plants. Consistent with reduced SPAD values, plants receiving the low Fe treatment developed leaf chlorosis on newly developing leaves. A clear reduction in the pH value of the nutrient solution of the Fe-deficient plants began at day 10 and by day 12, the pH of the nutrient solution was 4.2 compared with 6.8 for plants supplied with sufficient Fe (Table 1).

With the onset of leaf chlorosis, glyphosate was sprayed on shoots at different rates (i.e. 0.32, 0.95 or 1.89 mM). Shoot and root growth were not affected by increasing glyphosate applications (Table 2). The selected drift treatments of glyphosate also had no influence on chlorophyll concentration within a 24 h period (Table 2). However, there was a severe inhibition of ferric reductase activity of the roots (Fig. 1). Glyphosate depressed ferric reductase activity of Fe-deficient plants by nearly 50% within 6 h after the glyphosate treatment (Fig. 1a). Ferric reductase activity was even more severely inhibited at 12 h and 24 h after the glyphosate treatment, and was lower than in Fe-sufficient control plants. The glyphosate-dependent

	Dry matter production		Fe concentration		Chlorophyll (SPAD)	Solution pH
	Shoot	Root	Shoot	Root		
	(mg per plant)		$(\mu\text{g Fe g}^{-1} \text{ DW})$			
Control	244 ± 33	64 ± 11	221 ± 48	1595 ± 130	34.4 ± 1.2	6.8 ± 0.1
Fe deficiency	196 ± 23	52 ± 10	41 ± 8	62 ± 17	16.4 ± 2.9	4.2 ± 0.1

Results are means ± SD of six independent replications.

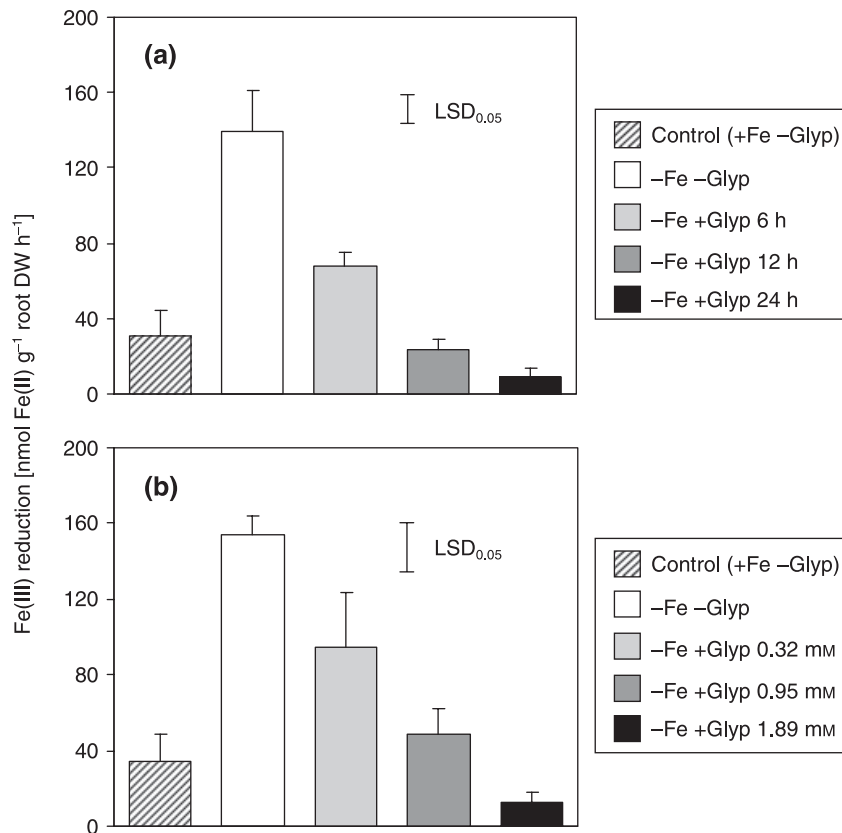


Table 1 Shoot and root dry matter production, iron (Fe) concentration, chlorophyll level in primary leaves and nutrient solution pH of sunflower plants (*Helianthus annuus* cv. TR-3080) grown with a sufficient (control, 100 μM Fe-EDTA) or a deficient Fe supply (0.2 μM Fe-EDTA for the first 6 d and then without further Fe treatment).

Fig. 1 Time- (a) and concentration-dependent (b) inhibition of ferric reductase activity (means ± SD, $n = 6$) in iron (Fe)-deficient sunflower plants (*Helianthus annuus* cv. TR-3080) as influenced by glyphosate (Glyp) treatments. Time-dependent changes (a) in ferric reductase activity were measured at 6 h, 12 h and 24 h after 1.89 mM glyphosate treatment, whereas concentration-dependent changes (b) were measured at 24 h after 0.32, 0.95 and 1.89 mM glyphosate treatments (corresponding to 1, 3 and 6% of the recommended rate for weed control, respectively). Plants were grown with sufficient (+Fe, 100 μM Fe-ethylenediaminetetraacetic acid (Fe-EDTA)) or deficient (-Fe) supply of Fe for 12 d in nutrient solution in a glasshouse. Vertical bar indicates least significant difference ($\text{LSD}_{0.05}$) at $P = 0.05$ probability level.

decreases in ferric reductase activity were also dose-dependent so that as the rate of glyphosate increased from 0 to 1.89 mM there was a sharp, proportional decrease in reductase activity of the roots (Fig. 1b). Application of glyphosate as low as 0.32 mM appeared to inhibit reductase activity, but this effect was not significant (Fig. 1b).

The high sensitivity of ferric reductase to foliar-applied glyphosate was confirmed using a visual staining technique. The formation of red colour on and around roots caused by the formation of Fe(II)-ferrozine complex as result of Fe(III) reduction was clearly inhibited by increasing rates of glyphosate (Fig. 2).

Acidification of the growth medium is another reaction of plants to Fe deficiency. The solution pH of Fe-deficient plants without glyphosate dropped from an initial pH of 5.5 to pH

4.2, while Fe-sufficient control plants increased the pH to 6.8. In contrast to ferric reductase, solution pH was not consistently changed in Fe-deficient plants treated with glyphosate under the experimental conditions used (data not shown).

Shikimate increased fourfold in young leaves and threefold in apical parts of roots within 6 h after the glyphosate application compared with the control. Leaf and root concentrations of shikimate were even higher at 24 h after the glyphosate treatment (Fig. 3a). Shikimate accumulation in roots and leaves had a distinct dose-dependency. As glyphosate increased from 0.32 mM to 1.89 mM, shikimate in young leaves increased from 0.9 $\mu\text{mol g}^{-1}$ FW to 11.5 $\mu\text{mol g}^{-1}$ FW and from 0.3 $\mu\text{mol g}^{-1}$ FW to 4.6 $\mu\text{mol g}^{-1}$ FW in roots (Fig. 3b).

Apical parts of roots were also used to determine sugar concentrations and NADPH oxidation. Iron-deficient plants had

Table 2 Shoot and root dry matter production and chlorophyll concentration of sunflower plants (*Helianthus annuus* cv. TR-3080) grown in nutrient solution with a sufficient (+Fe, 100 μ M Fe-ethylenediaminetetraacetic acid (Fe-EDTA)) or deficient Fe supply for 12 d under glasshouse conditions

Treatments	Dry matter production		Chlorophyll (SPAD)
	Shoot (mg per plant)	Root	
Control (+Fe –Glyp)	226 \pm 30	61 \pm 13	39.2 \pm 1.9
–Fe –Glyp	208 \pm 16	58 \pm 8	21.2 \pm 1.0
–Fe +Glyp 0.32 mM	202 \pm 16	55 \pm 8	21.3 \pm 0.7
–Fe +Glyp 0.95 mM	208 \pm 15	56 \pm 11	21.7 \pm 1.4
–Fe +Glyp 1.89 mM	194 \pm 23	53 \pm 9	21.8 \pm 2.3
LSD _{0.05}	24	12	2.3

Glyphosate was sprayed when plants were 11 d old, and harvesting of plants was realized 24 h after glyphosate application. Results are means \pm SD of six independent replications (LSD_{0.05}: least significant difference at $P = 0.05$ probability level).

a higher concentration of soluble sugars (Fig. 4a) and tended to show a greater capacity for NADPH oxidation (Fig. 4b) in roots when compared with Fe-sufficient plants. Applying glyphosate at increasing rates tended to increase both sugar concentrations and NADPH oxidation in the roots (Fig. 4).

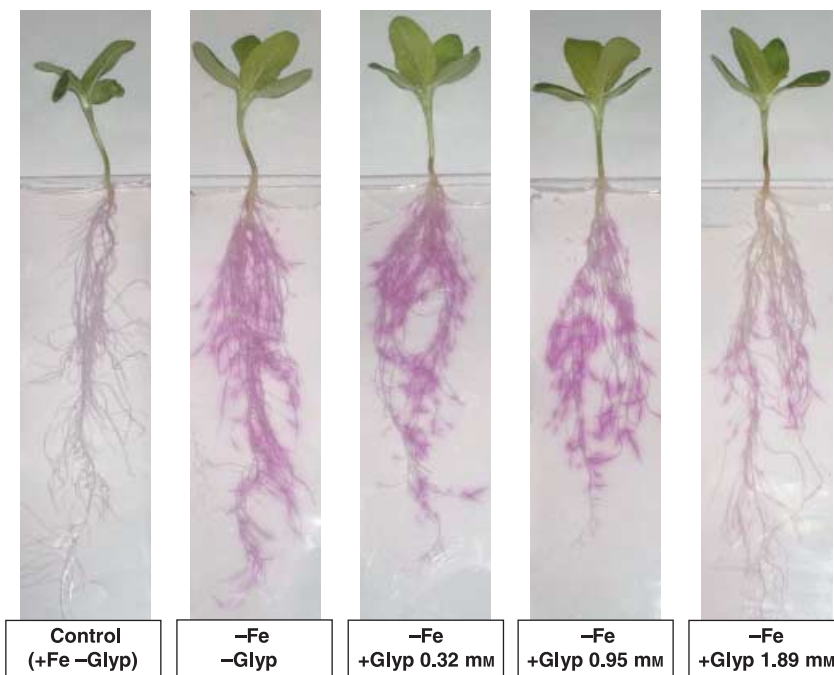
Discussion

The present study documents the high sensitivity of ferric reductase in sunflower roots to foliar-applied glyphosate (Fig. 1).

The inhibitory effect of glyphosate on ferric reductase occurred at very low, subherbicidal concentrations of glyphosate (i.e. 1–6% of the widely recommended glyphosate dose for weed control) corresponding to reported field drift rates of glyphosate (Ellis *et al.*, 2003; Koger *et al.*, 2005; Bellaloui *et al.*, 2006). Foliar application of glyphosate at 1.89 mM (e.g. 6% of the widely recommended glyphosate dose for weed control) resulted in almost 50% inhibition of reductase activity within 6 h and nearly complete inhibition 24 h after exposure to glyphosate (Fig. 1). Such substantial inhibition of ferric reductase occurred before any adverse effects of glyphosate on shoot or root growth were observed (Table 2).

The mechanism for such substantial and rapid inhibition of ferric reductase activity by glyphosate was not studied, but could be related to a sudden shortage of energy needed to maintain root growth and initiate reductase activity. However, this was not confirmed by root concentrations of soluble sugars, which were not influenced by the glyphosate treatments (Fig. 4a). Both NADPH and NADH have been suggested as the source of electrons for the reduction of Fe(III) to Fe(II) in roots of different plant species (Sijmons *et al.*, 1984; Schmidt & Schuck, 1996; Schmidt, 1999). Glyphosate may affect the flow of electrons to Fe(III) by interfering with NAD(P)H oxidation. However, our results, showed that glyphosate did not interact with NADPH oxidation, and even tended to increase it (Fig. 4b). Similar findings, as shown in Fig. 4b for NADPH, were also determined for NADH (data not shown). The results obtained from root extracts appear to exclude the possibility that a shortage of sugars or any impairment in NAD(P)H oxidation capacity of root cells was responsible for glyphosate-induced inhibition of ferric reductase activity. However, further

Fig. 2 Visualization of root iron (Fe) reduction in sunflower plants (*Helianthus annuus* cv. TR-3080) using the ferrozine test in agar medium. The ferrozine test was performed 24 h after 0.32, 0.95 and 1.89 mM glyphosate (Glyp) treatments (corresponding to 1, 3 and 6% of the recommended rate for weed control, respectively). Plants were grown with sufficient (+Fe, 100 μ M Fe-ethylenediaminetetraacetic acid (Fe-EDTA)) or deficient (–Fe) supply of Fe for 12 d in nutrient solution in a glasshouse.



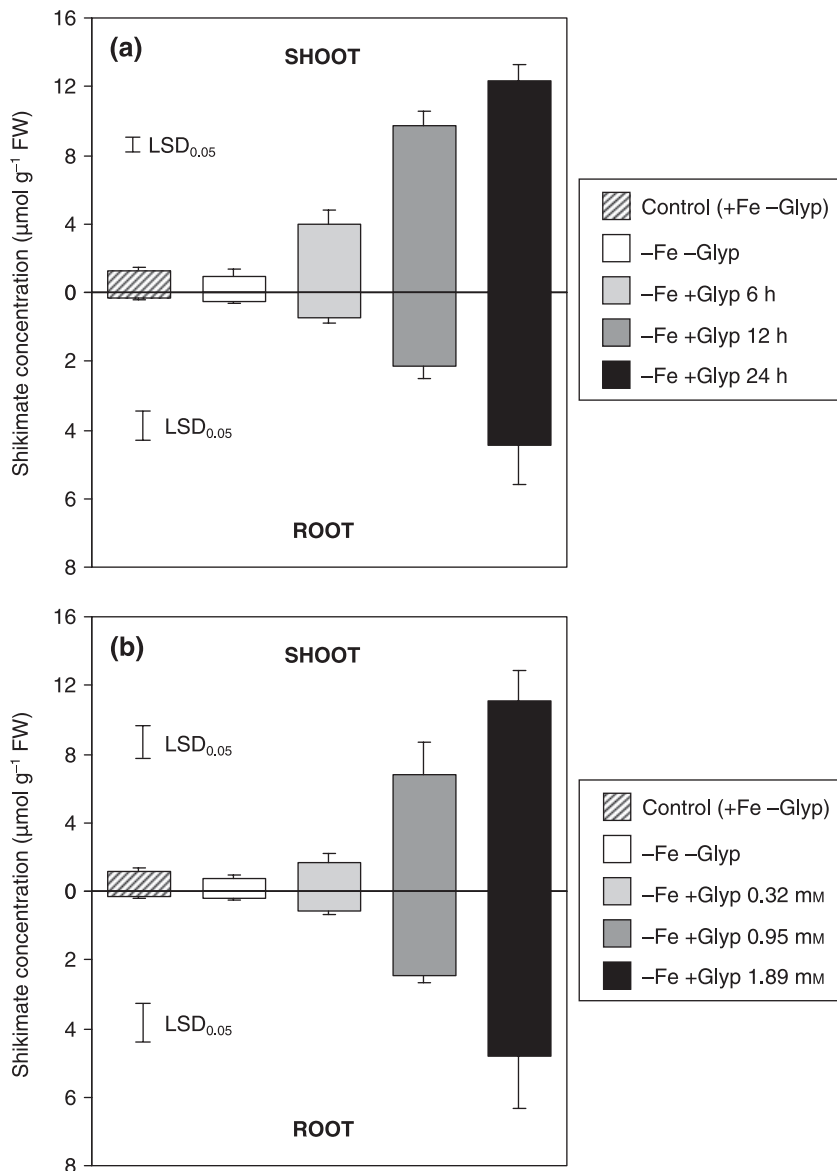


Fig. 3 Time- (a) and concentration-dependent (b) changes in shikimate concentrations (means \pm SD, $n = 6$) in shoots and roots of iron (Fe)-deficient sunflower plants (*Helianthus annuus* cv. TR-3080) as influenced by glyphosate (Glyp) applications. Time-dependent changes (a) in shikimate concentration were measured at 6 h, 12 h and 24 h after 1.89 mM glyphosate treatment, whereas concentration-dependent changes (b) were measured at 24 h after 0.32, 0.95 and 1.89 mM glyphosate treatments (corresponding to 1, 3 and 6% of the recommended rate for weed control, respectively). Plants were grown with sufficient (+Fe, 100 μ M Fe-ethylenediaminetetraacetic acid (Fe-EDTA)) or deficient (-Fe) supply of Fe for 12 d in nutrient solution in a glasshouse. Vertical bar indicates least significant difference (LSD_{0.05}) at $P = 0.05$ probability level.

detailed studies are needed to elucidate the mechanism of inhibition of ferric reductase enzyme by glyphosate.

The rapid reduction in ferric reductase activity following the glyphosate treatment (Fig. 1), suggests that glyphosate or its degradation products may form insoluble stable Fe-complexes that are not useful or not available for reduction by ferric reductase. Glyphosate has been reported to possess a high affinity and chelating capacity for Fe and other metals, resulting in the formation of poorly soluble glyphosate-metal complexes or insoluble precipitates (Motekaitis & Martell, 1985; Subramaniam & Hoggard, 1988; Barja *et al.*, 2001; Barrett & McBride, 2005). Formation of such insoluble complexes of glyphosate with Fe or Mn is a major reason for the loss of herbicidal effectiveness when glyphosate is prepared in 'hard water' containing metals (Bernards *et al.*, 2005). These results suggest that glyphosate or its degradation product(s) can diminish the availability

of Fe(III) for the reductase enzyme by forming insoluble complexes. Recent reports of marked decreases in root uptake and root-to-shoot transport of Fe in sunflower plants were also attributed to the Fe-complexing ability of glyphosate (Eker *et al.*, 2006). Alternatively, glyphosate may inhibit reductase activity directly through an unknown mechanism.

As mentioned earlier, high shikimate concentrations in glyphosate-treated plant tissues are a good indicator of phytotoxicity due to inhibition of EPSPS. Foliar-applied glyphosate is rapidly translocated to actively growing tomato and spinach shoots and roots, and accounts for up to 16% of the dry weight of these tissues (Schulz *et al.*, 1990). In the current study with sunflower plants, foliar applied glyphosate was also translocated rapidly and accumulated in meristematic tissues such as apical root parts. Shikimate concentrations in the apical parts of roots increased fourfold within 6 h after glyphosate

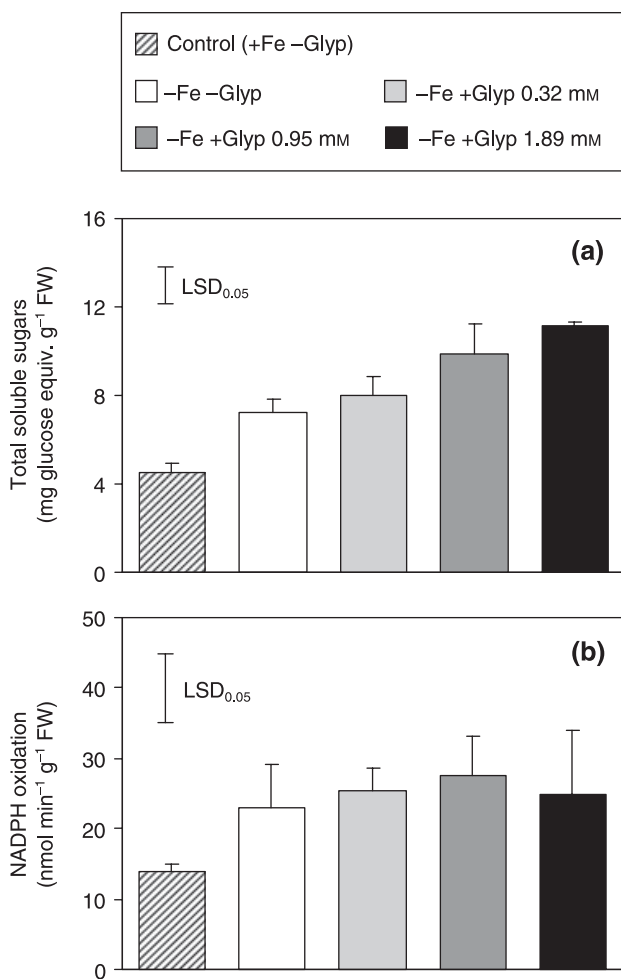


Fig. 4 Effect of glyphosate (Glyp) applications (0.32, 0.95 and 1.89 mM Glyp corresponding to 1, 3 and 6% of the recommended rate for weed control) on concentration of soluble sugars (a) and NADPH oxidation (b) in apical root parts of sunflower plants (*Helianthus annuus* cv. TR-3080) grown with sufficient (+Fe: 100 μ M Fe-ethylenediaminetetraacetic acid (Fe-EDTA)) or deficient (-Fe) supply of Fe for 12 d in nutrient solution in a glasshouse. Results are means \pm SD of six independent replications. Vertical bar indicates least significant difference (LSD_{0.05}) at $P = 0.05$ probability level.

treatment (Fig. 3a). Assuming that root and leaf water content is 90%, it is estimated that as much as 12 mM shikimic acid accumulated in young shoots and 5 mM in roots (Fig. 3a). Apical roots are the plant parts in which ferric reductase is particularly localized and expressed under Fe deficiency (Marschner *et al.*, 1986; Marschner & Römheld, 1994). High expression of ferric reductase activity in root tips can also be seen in Fig. 2. The colocalization of ferric reductase and glyphosate in the same root parts facilitates the rapid interaction of glyphosate with ferric reductase. Similar to ferric reductase, nitrogenase and nitrate reductase activities (both Fe-containing enzymes) in soybean plants are significantly depressed after foliar application of glyphosate (Zablotowicz & Reddy, 2004; Bellaloui *et al.*, 2006). Glyphosate, at 1.25 mM, resulted in a

33% reduction in nitrogenase activity within 24 h (De Maria *et al.*, 2006); the reason for the decrease is unrelated to protein damage or a shortage of carbohydrates (energy). Complexing of the protein bound Fe with glyphosate might explain the high sensitivity of such Fe-containing enzymes to glyphosate. Young active nodules are also important accumulation sites of glyphosate (Bellaloui *et al.*, 2006; De Maria *et al.*, 2006).

Although acidification of the rhizosphere by Fe-deficiency-induced H⁺ release from roots is a characteristic of Strategy-I plants to stress (Römheld & Marschner, 1986), these results showed that ferric reductase activity was more sensitive to glyphosate than was the release of protons from roots into solution. Apparently, glyphosate does not interfere with the proton pumping ATPase activity of root cells for 24 h after the treatment. New experiments should be designed to follow H⁺ efflux from intact roots after glyphosate treatment. There are, however, controversial results in the literature on the effects of glyphosate on ATPase activity in different cell systems (Lockau & Pfeffer, 1982; Lopez-Brana *et al.*, 1984; Peixoto, 2005 and references therein).

In conclusion, the results presented in this study showing that glyphosate is especially inhibitory to ferric reductase complement the recently published report (Eker *et al.*, 2006) that glyphosate exerts a strong inhibitory influence on ferric reductase activity of Fe-deficient roots and impairs the uptake and translocation of Fe in plants. These impairments could be a major reason for the increasingly observed Fe deficiency chlorosis in cropping systems associated with widespread glyphosate usage as reported for different parts of the USA (Franzen *et al.*, 2003; Jolley *et al.*, 2004). Such strong interference of glyphosate with root uptake and root-to-shoot transport of Fe in crop plants may represent a potential threat to human and animal nutrition because of possible reduction of Fe in edible plants parts (e.g. seed/grain).

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References

- Abadia J, Alvarez-Fernandez A, Rombola AD, Sanz M, Tagliavini M, Abadia A. 2004. Technologies for the diagnosis and remediation of Fe deficiency. *Soil Science and Plant Nutrition* 50: 965–971.
- Alcantara E, Romera FJ, Canete M, de la Guardia MD. 2000. Effects of bicarbonate and iron supply on Fe(III) reducing capacity of roots and leaf chlorosis of the susceptible peach rootstock 'Nemaguard'. *Journal of Plant Nutrition* 23: 1607–1617.
- Barja BC, Herzage J, dos Santos Afonso M. 2001. Iron (III)-phosphonate complexes. *Polyhedron* 20: 1821–1830.
- Barrett KA, McBride MB. 2005. Oxidative degradation of glyphosate and aminomethylphosphonate by manganese oxide. *Environmental Science and Technology* 39: 9223–9228.

- Barton LL, Johnson GV, O'Nan AG, Wagener BM. 2000. Inhibition of ferric chelate reductase in alfalfa roots by cobalt, nickel, chromium, and copper. *Journal of Plant Nutrition* 23: 1833–1845.
- Bellaloui N, Reddy KN, Zablotowicz RM, Mengistu A. 2006. Simulated glyphosate drift influences nitrate assimilation and nitrogen fixation in nonglyphosate-resistant soybean. *Journal of Agricultural and Food Chemistry* 54: 3357–3364.
- Bernards ML, Thelen KD, Muthukumar RB. 2005. Glyphosate interaction with manganese in tank mixtures and its effect on glyphosate absorption and translocation. *Weed Science* 53: 787–794.
- Buehring WN, Massey JH, Reynolds DB. 2007. Shikimic acid accumulation in field-grown corn (*Zea mays*) following simulated glyphosate drift. *Journal of Agricultural and Food Chemistry* 55: 819–824.
- Cerdeira AL, Duke SO. 2006. The current status and environmental impacts of glyphosate-resistant crops: a review. *Journal of Environmental Quality* 35: 1633–1658.
- Chaney RL, Brown JC, Tiffin LO. 1972. Obligatory reduction of ferric chelates in iron uptake by soybeans. *Plant Physiology* 50: 208–213.
- Cromartie TH, Polge ND. 2002. Method of detecting shikimic acid. United States Patent 6482654. (http://www.braindex.com/patent_pdf/patents/US6482654.pdf)
- De Maria N, Becerril JM, Garcia-Plazaola JL, Hernandez A, De Felipe MR, Fernandez-Pascual M. 2006. New insights on glyphosate mode of action in nodular metabolism: role of shikimate accumulation. *Journal of Agricultural and Food Chemistry* 54: 2621–2628.
- Eker S, Ozturk L, Yazici A, Erenoglu B, Römhelt V, Cakmak I. 2006. Foliar-applied glyphosate substantially reduced uptake and transport of iron and manganese in sunflower (*Helianthus annuus* L.) plants. *Journal of Agricultural and Food Chemistry* 54: 10019–10025.
- Ellis JM, Griffin JL, Linscombe SD, Webster EP. 2003. Rice (*Oryza sativa*) and corn (*Zea mays*) response to simulated drift of glyphosate and glufosinate. *Weed Technology* 17: 452–460.
- Franzen DW, O'Barr JH, Zollinger RK. 2003. Interaction of a foliar application of iron HEDTA and three postemergence broadleaf herbicides with soybeans stressed from chlorosis. *Journal of Plant Nutrition* 26: 2365–2374.
- Geiger DR, Shieh WJ, Fuchs MA. 1999. Causes of self-limited translocation of glyphosate in *Beta vulgaris* plants. *Pesticide Biochemistry and Physiology* 64: 124–133.
- Hansen NC, Jolley VD, Naeve SL, Goos RJ. 2004. Iron deficiency of soybean in the North Central U.S. and associated soil properties. *Soil Science and Plant Nutrition* 50: 983–987.
- Inskip WP, Bloom PR. 1987. Soil chemical factors associated with soybean chlorosis in calciquolls of western Minnesota. *Agronomy Journal* 79: 779–786.
- Jolley VD, Hansen NC, Shiffler AK. 2004. Nutritional and management related interactions with iron-deficiency stress response mechanisms. *Soil Science and Plant Nutrition* 50: 973–981.
- Koger CH, Shaner DL, Krutz LJ, Walker WW, Buehring N, Henry WB, Thomas WE, Wilcut JW. 2005. Rice (*Oryza sativa*) response to drift rates of glyphosate. *Pest Management Science* 61: 1161–1167.
- Lockau W, Pfeffer SA. 1982. A cyanobacterial ATPase distinct from the coupling factor of photophosphorylation. *Zeitschrift für Naturforschung C* 37: 658–664.
- Lopez-Brana I, Delibes A, Garcia-Olmedo F. 1984. Effects of glyphosate on isolated maize mitochondria. *Journal of Experimental Botany* 35: 905–911.
- Lucena C, Waters BM, Romera FJ, Garcia MJ, Morales M, Alcantara E, Perez-Vicente R. 2006. Ethylene could influence ferric reductase, iron transporter, and H⁺-ATPase gene expression by affecting FER (or FER-like) gene activity. *Journal of Experimental Botany* 57: 4145–4154.
- Marschner H, Römhelt V. 1994. Strategies of plants for acquisition of iron. *Plant and Soil* 165: 261–274.
- Marschner H, Römhelt V, Kissel M. 1986. Different strategies in higher plants in mobilization and uptake of iron. *Journal of Plant Nutrition* 6: 695–713.
- Motekaitis RJ, Martell AE. 1985. Metal chelate formation by *N*-phosphonomethylglycine and related ligands. *Journal of Coordination Chemistry* 14: 139–149.
- Mueller TC, Massey JH, Hayes RM, Main CL, Stewart CN. 2003. Shikimate accumulates in both glyphosate-sensitive and glyphosate-resistant horseweed (*Conyza canadensis* L. Cronq.). *Journal of Agricultural and Food Chemistry* 51: 680–684.
- Neumann G, Kohls S, Landsberg E, Souza KSO, Yamada, T, Roemheld V. 2006. Relevance of glyphosate transfer to nontarget plants via the rhizosphere. *Journal of Plant Diseases and Plant Protection* 20: 963–969.
- Peixoto F. 2005. Comparative effects of the roundup and glyphosate on mitochondrial oxidative phosphorylation. *Chemosphere* 61: 1115–1122.
- Robinson NJ, Procter CM, Connolly EL, Guerinot ML. 1999. A ferric-chelate reductase for iron uptake from soils. *Nature* 397: 694–697.
- Römhelt V. 1987. Different strategies for iron acquisition in higher plants. *Plant Physiology* 70: 231–234.
- Römhelt V, Marschner H. 1986. Evidence for a specific uptake system for iron phytosiderophores in roots of grasses. *Plant Physiology* 80: 175–180.
- Schmidt W. 1999. Mechanisms and regulation of reduction-based iron uptake in plants. *New Phytologist* 141: 1–26.
- Schmidt W, Schuck C. 1996. Pyridine nucleotide pool size changes in iron-deficient *Plantago lanceolata* roots during reduction of external oxidants. *Physiologia Plantarum* 98: 215–221.
- Schmidt W, Steinbach S. 2000. Sensing iron – A whole plant approach. *Annals of Botany* 86: 589–593.
- Schulz A, Munder T, Holländer-Czytko H, Amrhein N. 1990. Glyphosate transport and early effects on shikimate metabolism and its compartmentation in sink leaves of tomato and spinach plants. *Zeitschrift für Naturforschung* 45: 529–534.
- Shaner DL, Nadler-Hassar T, Henry WB, Koger CH. 2005. A rapid *in vivo* shikimate accumulation assay with excised leaf discs. *Weed Science* 53: 769–774.
- Sijmons PC, Vandenbriel W, Bienfait HF. 1984. Cytosolic NADPH is the electron-donor for extracellular Fe-III reduction in iron-deficient bean roots. *Plant Physiology* 75: 219–221.
- Stookey LL. 1970. Ferrozine – a new spectrophotometric reagent for iron. *Analytical Chemistry* 42: 779–781.
- Subramaniam V, Hoggard PE. 1988. Metal complexes of glyphosate. *Journal of Agricultural and Food Chemistry* 36: 1326–1329.
- Welch RM, Graham RD. 2004. Breeding for micronutrients in staple food crops from a human nutrition perspective. *Journal of Experimental Botany* 55: 353–364.
- Yemn EW, Wills AJ. 1954. The estimation of carbohydrate in plant extract by anthrone. *Biochemical Journal* 57: 508–514.
- Zablotowicz RM, Reddy KN. 2004. Impact of glyphosate on the *Bradyrhizobium japonicum* symbiosis with glyphosate-resistant transgenic soybean: a minireview. *Journal of Environmental Quality* 33: 825–831.