Glyphosate inhibition of ferric reductase activity in iron deficient sunflower roots

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Received: *14 September 2007*
Accepted: *12 November 2007*

Summary

• 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*).

New Phytologist (2008) **177**: 899–906

doi: 10.1111/j.1469-8137.2007.02340.x

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 $CaSO_4$ 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 K_2SO_4, 2 mM $Ca(NO_3)_2$, 1 mM $MgSO_4$, 0.25 mM KH_2PO_4, 0.1 mM KCl, 1 μM $MnSO_4$, 1 μM $ZnSO_4$, 10 μM H_3BO_3, 0.1 μM $CuSO_4$, and 0.01 μM $(NH_4)_6Mo_7O_{24}$. 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 ($E = 27.9$ mM^{-1} 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 Na_2SO_3) 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 H_2SO_4) 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 ($E = 6.2$ mM^{-1} 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			
	Shoot	Root	Shoot	Root	Chlorophyll (SPAD)	Solution pH
	(mg per plant)		(μg Fe g^{-1} 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-ethylenediaminetetraacetic acid (Fe-EDTA)) or a deficient Fe supply (0.2 μM Fe-EDTA for the first 6 d and then without further Fe treatment).

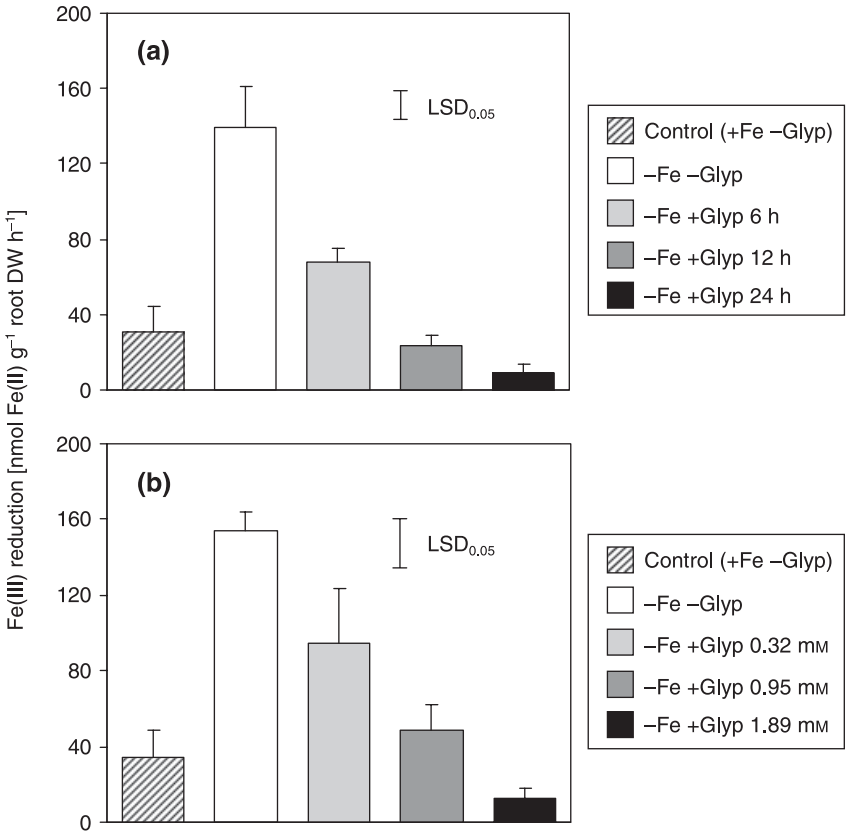

Fig. 1 Time- (a) and concentration-dependent (b) inhibition of ferric reductase activity (means ± SD, $n = 6$) in iron (Fe)-deficient sunflower plants (*Helianthus annuus* cv. TR-3080) as influenced by glyphosate (Glyp) treatments. Time-dependent changes (a) in ferric reductase activity were measured 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 ($LSD_{0.05}$) 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 μmol g^{-1} FW to 11.5 μmol g^{-1} FW and from 0.3 μmol g^{-1} FW to 4.6 μmol 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 μM Fe-ethylenediaminetetraacetic acid (Fe-EDTA)) or deficient Fe supply for 12 d under glasshouse conditions

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

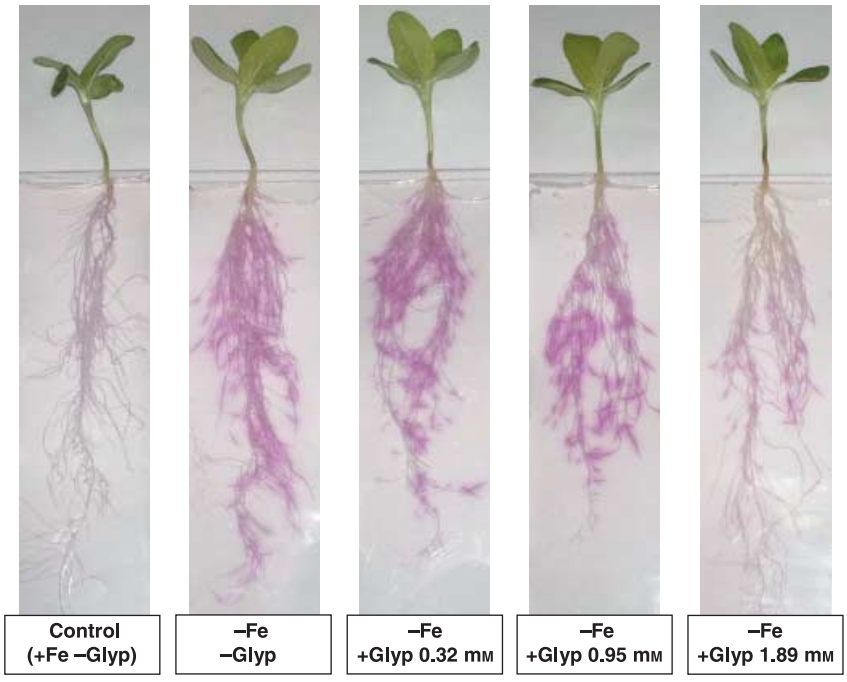

Fig. 2 Visualization of root iron (Fe) reduction in sunflower plants (*Helianthus annuus* cv. TR-3080) using the ferrozine test in agar medium. The ferrozine test was performed 24 h after 0.32, 0.95 and 1.89 mM glyphosate (Glyp) treatments (corresponding to 1, 3 and 6% of the recommended rate for weed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

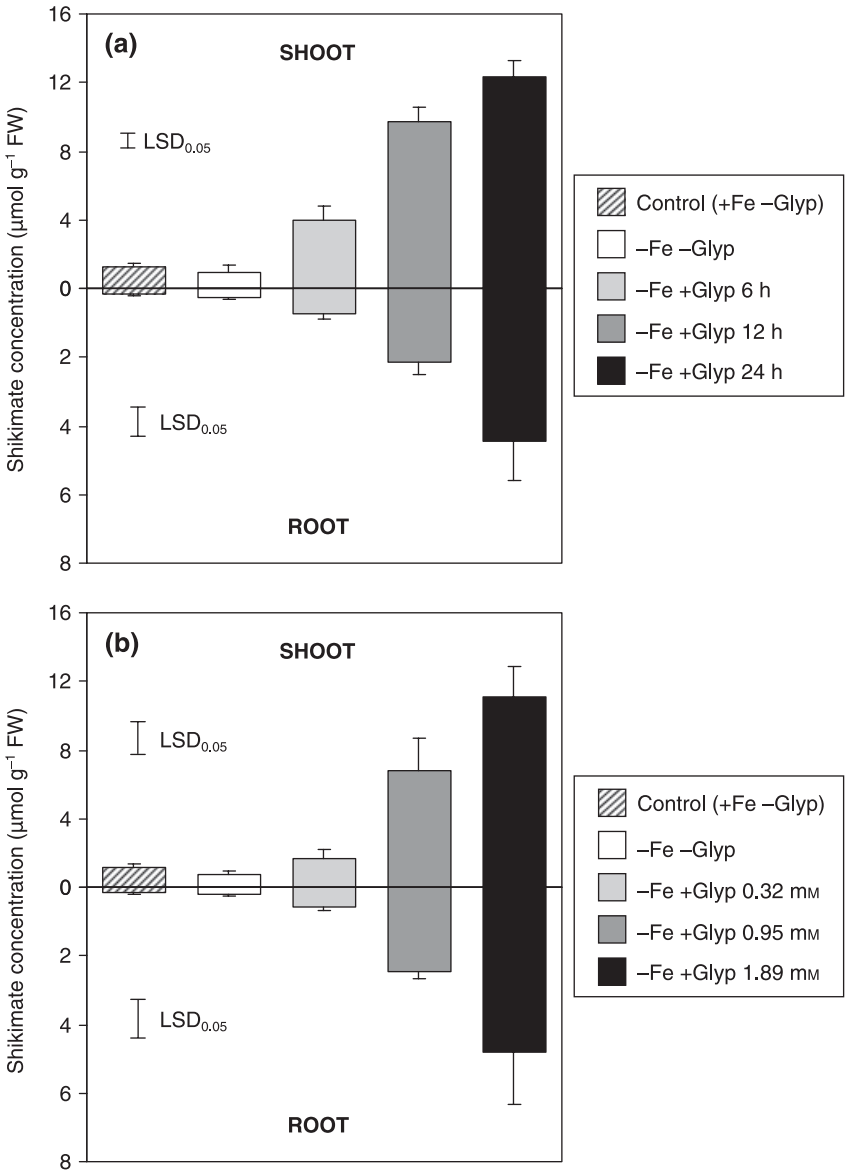

Fig. 3 Time- (a) and concentration-dependent (b) changes in shikimate concentrations (means ± SD, $n = 6$) in shoots and roots of iron (Fe)-deficient sunflower plants (*Helianthus annuus* cv. TR-3080) as influenced by glyphosate (Glyp) applications. Time-dependent changes (a) in shikimate concentration 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 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 ($LSD_{0.05}$) 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

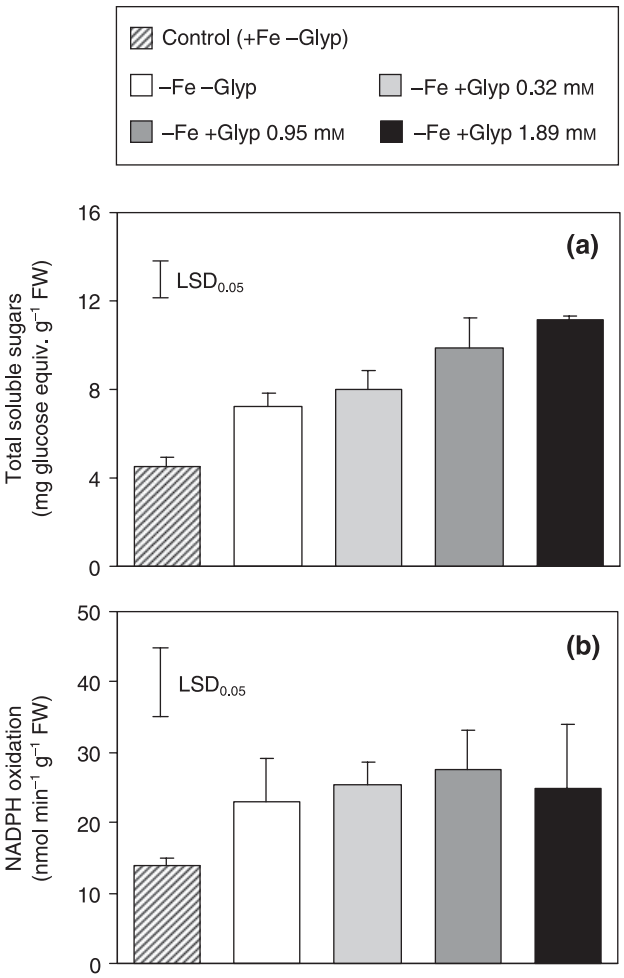

Fig. 4 Effect of glyphosate (Glyp) applications (0.32, 0.95 and 1.89 mM Glyp corresponding to 1, 3 and 6% of the recommended rate for weed control) on concentration of soluble sugars (a) and NADPH oxidation (b) in apical root parts of sunflower plants (*Helianthus annuus* cv. TR-3080) grown with sufficient (+Fe: 100 μM Fe-ethylenediaminetetraacetic acid (Fe-EDTA)) or deficient (–Fe) supply of Fe for 12 d in nutrient solution in a glasshouse. Results are means ± 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).

Acknowledgements

We thank Prof. Don Huber (Purdue University, IN, USA) and Ernest A. Kirkby (Leeds University, UK) for constructive comments on the manuscript and correction of the English text.

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