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REGULAR ARTICLE

Glyphosate-induced impairment of plant growth and micronutrient status in glyphosate-resistant soybean (*Glycine max* L.)

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Abstract This investigation demonstrated potential detrimental side effects of glyphosate on plant growth and micronutrient (Mn, Zn) status of a glyphosate-resistant (GR) soybean variety (*Glycine max* cv. Valiosa), which were found to be highly dependent on the selected growth conditions. In hydroponic experiments with sufficient Mn supply [0.5 μM], the GR cv. Valiosa produced similar plant biomass, root length and number of lateral roots in the control treatment without glyphosate as compared to its non-GR parental line cv. Conquista. However, this was associated with 50% lower Mn shoot concentrations in cv. Conquista, suggesting a higher Mn demand of the transgenic cv. Valiosa under the selected growth conditions. Glyphosate application significantly inhibited root biomass production, root elongation, and lateral root formation of the GR line, associated with a 50% reduction of Mn shoot concentrations. Interestingly, no comparable effects were detectable at low Mn supply [0.1 μM]. This may indicate Mn-dependent differences in the intracellular transformation of glyphosate to the toxic metabolite aminomethylphosphonic acid (AMPA) in the two isolines. In soil culture experiments conducted on a calcareous loess sub-soil of a Luvisol (pH 7.6) and a highly weathered Arenosol (pH 4.5), shoot biomass production and Zn leaf concentrations of the GR-variety were affected by glyphosate applications on the Arenosol but not on the calcareous Loess sub-soil. Analysis of micronutrient levels in high and low molecular weight (LMW) fractions (80% ethanol extracts) of young leaves revealed no indications for internal immobilization of micronutrients (Mn, Zn, Fe) by excessive complexation with glyphosate in the LMW phase.

Keywords Glyphosate · Glyphosate-resistant soybean (*Glycine max* L.) · Micronutrient acquisition · Micronutrient utilisation

Abbreviations

AMPA	aminomethylphosphonic acid
cv.	cultivar
GM	genetically modified
GR	glyphosate-resistant
LMW	low molecular weight
HMW	high molecular weight

Introduction

Due to low production costs and high herbicidal efficiency, glyphosate is the most widely used wide-

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spectrum herbicide in the world (Baylis 2000; Service 2007). Glyphosate acts as a non-selective total herbicide by inhibiting the shikimate pathway responsible for the biosynthesis of aromatic amino acids and phenolic compounds (Hernandez et al. 1999), thereby causing impairment of general metabolic processes, such as protein synthesis and photosynthesis (de María et al. 2005; Geiger et al. 1986). Glyphosate also affects the micronutrient status of plants (Eker et al. 2006; Neumann et al. 2006). Field observations in Brazil and the US report that frequent applications of glyphosate may directly or indirectly induce iron (Fe), zinc (Zn), and manganese (Mn) deficiencies in glyphosate-resistant (GR) as well as non-GR plants (Huber 2006; Jolley et al. 2004; Huber and McCay-Buys 1993).

Hydroponic experiments demonstrated that even low levels (1.25–6% of the recommended dosage, comparable to levels in non-target drift) of glyphosate caused a pronounced decline in acquisition, root uptake and root-to-shoot translocation of radio-labeled Fe, Zn, and Mn in non-GR sunflower (Ozturk et al. 2008; Eker et al. 2006). Neumann et al. (2006) demonstrated that glyphosate applied exclusively to GR soybean leaves, impaired Mn uptake of non-GR sunflower seedlings cultivated simultaneously in the same pot, suggesting an inhibition of micronutrient uptake by root to root transfer of glyphosate. On the other hand, even growth-stimulating effects of sublethal doses of glyphosate have been reported in some cases (Wagner et al. 2003).

Calcium and cationic micronutrients in spray solutions reduce the herbicidal effectiveness of glyphosate due to the formation of glyphosate-metal complexes (Bernards et al. 2005a; Bailey et al. 2002). Iron and Mn in spray solutions are known to inhibit glyphosate herbicidal activity by limiting absorption and translocation of glyphosate in treated leaves (Bernards et al. 2005b).

Since glyphosate toxicity has multiple direct and indirect effects on susceptible plants, an assessment of mechanisms underlying the impairment of the micronutrient status is difficult. However, observations of micronutrient deficiencies in GR plants suggests detrimental effects of glyphosate independent of direct toxicity. These effects may comprise (1) reduced availability of cationic micronutrients in soils due to external or internal complexation with glyphosate, or due to toxic side effects on certain rhizosphere microorganisms, with functions in micronutrient (particularly Mn) mobilization (Huber 2006; Neumann et al. 2006); and (2) intracellular accumulation of phytotoxic glyphosate metabolites, such as aminomethylphosphonic acid (AMPA) in GR plants (Reddy et al. 2004; Nandula et al. 2007).

In the present research, experiments were conducted under controlled conditions to study the effect of glyphosate on shoot and root dry matter production, patterns of root growth and morphology, and the nutritional status of Fe, Mn, and Zn in GR soybean plants (*Glycine max* L. cv. Valiosa). To assess potential effects on uptake and internal utilization of micronutrients, independent of external factors determining their solubility and plant availability in soils (e.g. binding forms, pH, redox conditions, microbial activity), one set of experiments was performed in hydroponic culture. The impact of soil factors was investigated in a greenhouse study using two contrasting soils (acidic Arenosol, calcareous Loess sub-soil) in rhizoboxes equipped with root observation windows.

To assess a possible physiological immobilization of the investigated micronutrients in young leaves of glyphosate-treated plants by metal complexation with glyphosate (Sprankle et al. 1975), leaf tissue was extracted with 80% ethanol to separate the low-molecular weight (LMW) soluble fraction containing potential metal complexes with glyphosate, from high-molecular weight (HMW) compounds. After glyphosate application, the formation of stable LMW metal complexes with glyphosate may limit the availability of micronutrients for interactions in the HMW fraction. This will consequently lead to alterations in micronutrient distribution between the HMW and LMW fractions.

The experiments were conduced with the GR soybean cv. Valiosa and the non-GR parental line cv. Conquista. Inclusion of both lines allowed the investigation of potential effects of the transgenic modification on plant growth, development and micronutrient status, independent of glyphosate application (Gordon 2007).

Materials and methods

Plant material and growth conditions

Soybean (*G. max* L.) seeds of the GR cv. BSR Valiosa RR and of the non-GR, parental line cv. BR-16

Conquista were used in all experiments. BSR Valiosa RR was derived from the crossing of cv. BR-16 Conquista with one genotype possessing the glyphosate-tolerance gene. With an initial crossing and five retro-crossings, it was estimated that the index of the paternal recurrent (Conquista) is 0.984%, suggesting that cv. BSR Valiosa RR possesses about 98.4% of Conquista genes (Neylson Arantes, Embrapa, Brazil, personal communication).

Two soil culture experiments in “rhizoboxes” (equipped with root observation windows) and two studies in hydroponics were conducted. Seeds of both cultivars were sterilized for 5 min in 30% H_2O_2, soaked for 5 h in 10 mM $CaSO_4$ and germinated in upright position for 3 days in an incubator at 24°C in rolls of filter paper (MN 710, Macchery & Nagel, Düren, Germany) soaked with 2.5 mM $CaSO_4$.

Two contrasting soils were used in the soil experiments: a calcareous, loamy sub-soil of a Luvisol (pH ($CaCl_2$) 7.6; C_{org} [%] <0.3) and a sandy acidic Ap-horizon of an Arenosol (pH ($CaCl_2$) 4.5; C_{org} [%] 0.16). Calcium chloride-diethylenetriamine penta-acetic acid (CAT)-extractable micronutrient concentrations (VDLUFA 2004) [mg kg^{-1} soil]: Mn=7.4, Fe=369, Zn=0.8, B=0.9 and Cu 0.5 for the Arenosol and Mn=15, Fe=7.8, Zn=0.6, B=0.2 and Cu=0.7 for the calcareous Loess subsoil.

Soils were sieved through a 2 mm mesh and then fertilized with 100 mg N kg^{-1} soil as $Ca(NO_3)_2$, 50 mg K kg^{-1} soil as K_2SO_4, 50 mg Mg kg^{-1} soil as $MgSO_4$, and 80 mg P kg^{-1} soil as $Ca(H_2PO_4)_2$. The calcareous, loamy subsoil was additionally supplied with 7.3 mg Fe-EDTA kg^{-1} soil. After fertilization, the soils were sieved again to guarantee homogeneous distribution of the fertilizers. Previous measurements showed no profound changes in soil pH after identical fertilizer application to the two soils. Two seedlings of cv. Conquista or cv. Valiosa were transplanted into rhizoboxes (40×20×2 cm) filled with each 3 kg of fertilized soil and soil moisture was adjusted to 70% of the soil water-holding capacity. Plants were grown under greenhouse conditions with an average day/night temperature of 20–22/14–16ºC. Water loss was determined gravimetrically and replaced by daily applications of de-ionized water. A 14/10 h day/night light regime was guaranteed by additional lighting with fluorescent lamps (Osram HQL-R 400 W, Osram, Munich, Germany).

Hydroponic experiments were performed in a growth chamber under controlled environmental conditions with a light/dark regime of 14/10 h at 26/24°C, light intensity of 220 µmol m^{-2} s^{-1} at canopy height, provided by fluorescent lamps (Osram HQL-R 400, Osram, Munich, Germany) and 60% relative humidity. Six seedlings of cv. Conquista or cv. Valiosa were transferred to plastic pots (diameter: 18 cm, depth: 16 cm) containing 2.8 L continuously aerated nutrient solution containing 2 mM $Ca(NO_3)_2$, 0.25 mM KH_2PO_4, 0.7 mM K_2SO_4, 0.1 mM KCl, 0.5 mM $MgSO_4$, 20 µM Fe-EDTA, 10 µM H_3BO_3, 0.5 µM $ZnSO_4$, 0.2 µM $CuSO_4$ and 0.01 µM $(NH_4)_6Mo_7O_{24}$. Mn-supply varied between 0.5 µM (sufficient) and 0.1 µM (marginal) $MnSO_4$.

Glyphosate applications

The glyphosate formulation Roundup® UltraMax (Monsanto Agrar, Düsseldorf, Germany) containing 450 g L^{-1} N-[phosphonomethyl]glycine isopropylamine salt as the active ingredient was used in all experiments. Two concentrations of spray solutions were prepared according to the product label at 2 and 4 L Roundup® UltraMax in 200 L spray solution per hectare (equivalent to 28.4 and 56.8 mM of active ingredient), as recommended by the manufacturer against most annual or perennial weed species. Field application rates in pot experiments were performed according to recommendations for small scale glyphosate applications obtained from Monsanto (personal communication) and resulted in glyphosate doses of 9.6 and 19.2 µg cm^2 of pot surface area. In all experiments, glyphosate was applied with a hand-held sprayer. To achieve a manageable volume of spray solution, the initial glyphosate spray-solution was diluted 1:10 resulting in application volumes between 3 and 6 mL per pot. In all experiments, the freshly prepared glyphosate solution was sprayed on foliage only of the GR soybean cv. Valiosa. The sprayed solution did not cause run-off from leaves. Glyphosate applications were performed at 7 days after transfer into nutrient solution in the experiments conducted in hydroponics and at 14 and 37 days after transplanting to the rhizoboxes in the soil culture experiments. Due to the long time period between first application and harvest, two applications of glyphosate were performed in the soil experiments.

Plant growth measurements

During the experiments in rhizoboxes, root growth was documented by repeated drawing of roots visible along the root observation windows on plastic films. Patterns of root elongation of plants grown in soil culture and root growth and root morphology of plants grown in nutrient solution were subsequently analyzed using the WinRhizo Pro®, (Regent Instruments, Quebec, Canada) digital imaging software.

At harvest, plants were separated into roots, old leaves, and the youngest leaves, and biomass was recorded. Young leaves were frozen in liquid nitrogen. Fresh weights of all plant parts (roots and shoot) were determined at harvest and dry weights of roots and old shoots were determined after oven-drying at 60°C.

Analysis of mineral nutrients

Two hundred milligram of dried young leaf material was ashed in a muffle furnace at 500°C for 5 h. After cooling, the samples were extracted twice with 2 mL of 3.4 M HNO_3 until dryness to precipitate SiO_2. The ash was dissolved in 2 mL of 4 M HCl, subsequently diluted ten times with hot deionized water, and boiled for 2 min. After addition of 0.1 mL Cs/La buffer to 4.9 mL ash solution, Fe, Mn and Zn concentrations were measured by atomic absorption spectrometry (UNICAM 939, Offenbach/Main, Germany).

To assess a potential intracellular complexation of micronutrients by glyphosate in soil-grown plants, young leaves were homogenized in liquid nitrogen and extracted with 80% ethanol to separate the low molecular weight fraction from macromolecules. The extracts were centrifuged to remove insoluble HMW—plant material and the supernatant, containing LMW—compounds was evaporated to dryness on a heating plate. The dried residues were ashed in a muffle furnace at 500°C for 5 h and analyzed as described above for total micronutrient concentration.

The distribution of micronutrients (Mn, Zn, Fe) between 80% ethanol-soluble (LMW) and insoluble (HMW) fractions was calculated, based on the difference of total micronutrient concentration in the leaf tissue and the micronutrients detected in the soluble fraction.

Statistics

Both soil experiments were conducted in a completely randomized block design with four replicates per treatment. Nutrient solution experiments were conducted in a completely randomized block design with three (first experiment) or four (second experiment) replicates per treatment. Analysis of variance and the Tukey test for detection of significant differences were performed using the SigmaStat-software (Jandel Scientific, Sausalito, CA, USA).

Plant greenhouse culture did not allow exactly reproducible culture conditions. Therefore, one representative set of reproducible data obtained in both replications of the experiments in soil culture and hydroponics is presented.

Results

Studies in hydroponics

Dry matter production of the GR cv. Valiosa was comparable with the parental line Conquista in hydroponic culture both at high [0.5 μM] and low levels [0.1 μM] of Mn. In contrast, glyphosate significantly reduced root dry matter of cv. Valiosa at 0.5 μM Mn but not at 0.1 μM (Table 1). Similar trends were also detected for shoot biomass of glyphosate-treated plants although the differences

Table 1 Root and shoot dry matter production of the glyphosate-resistant soybean cv. Valiosa and the non-resistant parental line Conquista, grown for 2 weeks in hydroponic culture with sufficient [0.5 μM] and low [0.1 μM] Mn supply

Mn supply	0.1 μM		0.5 μM	
	Dry matter production (mg DM pot^{-1})			
Treatment	Root	Shoot	Root	Shoot
Conquista	218 a	1,002 a	201 a	1,009 a
Valiosa −Gly	181 a	1,027 a	164 a	960 a
Valiosa +Gly	176 a	990 a	156 b	906 a
Valiosa ++Gly	160 a	927 a	107 b	847 a

Foliar glyphosate application was performed only with cv. Valiosa using two application levels (+ Gly=28.4 mM and ++ Gly=56.8 mM) at 7 days after transfer to nutrient solution. Data represent means of three independent replicates. For each column, statistically significant differences at $P<0.05$ are indicated by different characters.

were not significant (Table 1). Root morphology of cv. Valiosa was significantly altered by glyphosate application, with a decline of root elongation by approximately 30% and reduced development of lateral roots (Fig. 1).

At the low level of Mn supply [0.1 µM], Mn concentrations in young leaves of all investigated plants ranged close to the critical level for Mn deficiency [20 µg g^{-1} DM], although the Mn concentration and total Mn content of cv. Conquista were approximately 20% higher than in cv. Valiosa (Fig. 2). At sufficient supply of Mn [0.5 µM] in the absence of glyphosate, internal Mn concentrations increased above the critical level in both cultivars but the transgenic cv. Valiosa accumulated approximately twice as much Mn in young leaves as its non-transgenic parent cv. Conquista. In contrast, glyphosate decreased the Mn concentration and total Mn in leaves by approximately 50–60% in cv. Valiosa relative to Valiosa not treated with glyphosate.

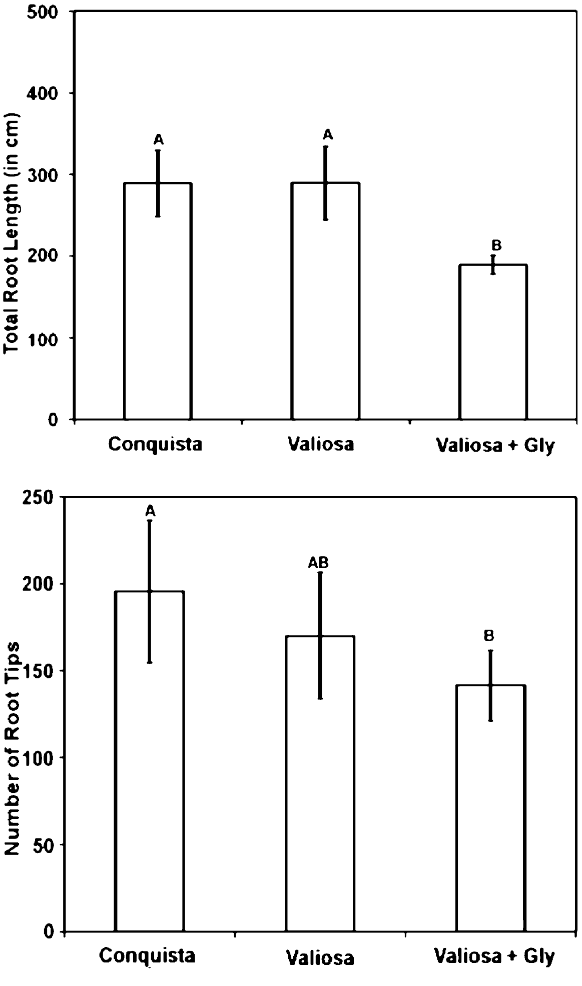

Fig. 1 Root length (above) and number of root tips (below) of the glyphosate-resistant soybean cv. Valiosa and its non-resistant parental line cv. Conquista after 2 weeks of growth in hydroponic culture with sufficient [0.5 µM] or low [0.1 µM] Mn supply. Foliar glyphosate application was performed only with cv. Valiosa using a glyphosate concentration of 28.4 mM at 7 days after transfer to nutrient solution. Data represent means and standard deviations of three independent replicates. Statistically significant differences at $P<0.05$ are indicated by different *characters*

Studies in soil culture

Shoot biomass of the two soybean cultivars was generally lower on the calcareous loess sub-soil compared with the acidic Arenosol, while root biomass remained largely unaffected (Fig. 3). Glyphosate reduced shoot biomass of the GR cv. Variosa on the acidic Arenosol but not on the calcareous sub-soil. There were no significant glyphosate effects on root biomass (Fig. 3) or root elongation on both soils.

Glyphosate significantly reduced the concentration of Zn in young leaves of cv. Valiosa (Table 2) in two independent replications of the experiment, while no significant differences were detectable for Mn (Table 2). In both cultivars, Zn leaf tissue concentrations were generally higher and Mn concentrations generally lower on the Arenosol than on the calcareous sub-soil, while Fe levels were comparable on both soils (Table 2).

The proportion of micronutrients in the LMW fraction (soluble fraction) ranged from 2.0–6.5% for Mn, 30–45% for Zn and 10–20% for Fe of the total concentration. There were no significant micronutrient differences in the 80% ethanol-soluble LMW fraction of young leaves obtained from glyphosate-treated and non-treated control plants in soil culture (Table 2).

Discussion

Plant growth

During the last decade, transgenic expression of the bacterial 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) gene has been employed as a strategy to confer glyphosate resistance to soybean and

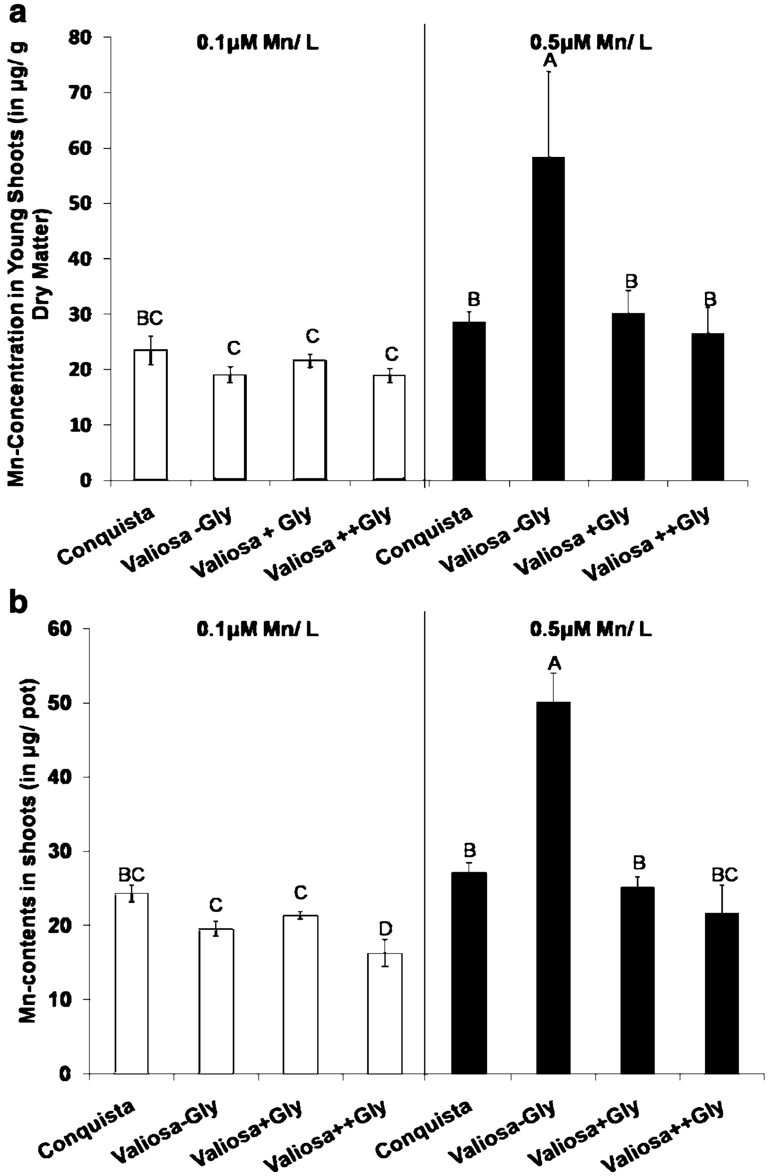

Fig. 2 Manganese concentration (**a**) and total Mn content (**b**) in young leaves of the glyphosate-resistant soybean cv. Valiosa and its non-resistant parental line cv. Conquista after 2 weeks of growth in hydroponic culture with sufficient [0.5 µM] or low [0.1 µM] Mn supply. Foliar glyphosate application was performed only with cv. Valiosa, using glyphosate concentrations of 28.4 mM (+ Gly) and 56.8 mM (++ Gly) at 7 days after transfer to nutrient solution. Data represent means and standard deviations of three independent replicates. Statistically significant differences at $P<0.05$ are indicated by different *characters*

various other crop species (Cerdeira and Duke 2006). Although GR soybean cultivars have been demonstrated to be 50 times less sensitive to glyphosate toxicity than non-resistant varieties (Nandula et al. 2007), various studies and field observations reported growth depressions, chlorosis, leaf necrosis and micronutrient deficiencies after glyphosate applications with the recommended dosage (Duke et al. 2003; Jolley et al. 2004; Reddy et al. 2004). This has been frequently attributed to detrimental effects of AMPA, a phytotoxic metabolite of glyphosate, and to ingredients and surfactants of the glyphosate formulation (Reddy et al. 2004; Nandula et al. 2007). Under field conditions, AMPA residues were detected in leaves, stems and seeds of glyphosate-treated GR soybean (Duke et al. 2003; Arregui et al. 2003), while in most plant species in planta conversion of glyphosate to AMPA was considered as marginal (Duke 1988). A particularly high ability for glyphosate degradation was reported for soybean cell cultures (Komossa et al. 1992). High variability in the expression of glyphosate toxicity in GR soybean was assigned to differences of the plant physiological status, genotype, and to environmental factors with

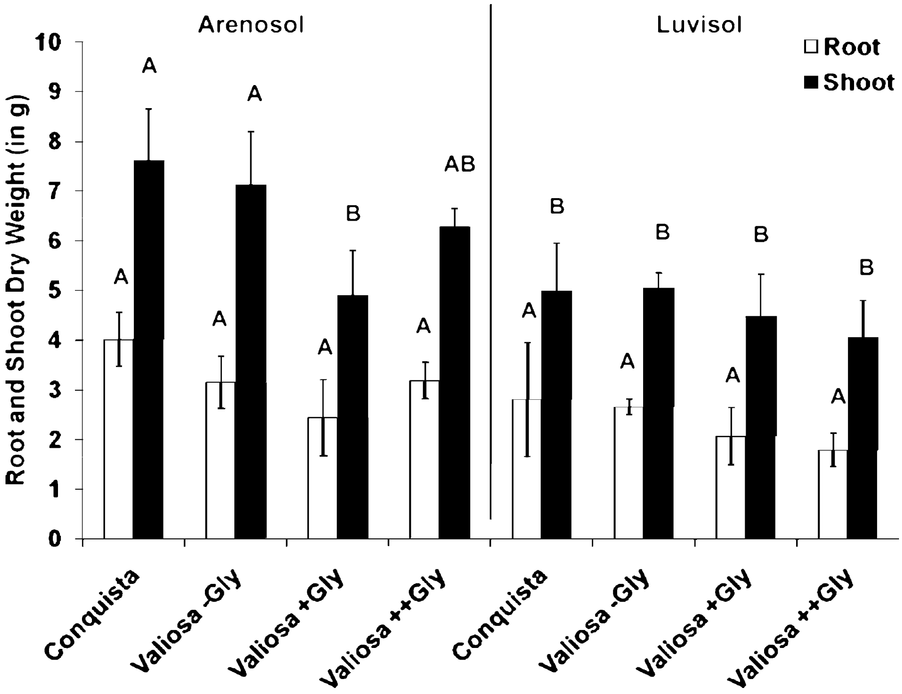

Fig. 3 Root-, and shoot biomass production of the glyphosate-resistant soybean cv. Valiosa and its non-GR parental line cv. Conquista at 42 days of growth on an acidic Arenosol (*left*) or a calcareous Luvisol subsoil (*right*). Foliar glyphosate application was performed only with cv. Valiosa with glyphosate concentrations of 28.4 mM (+ Gly) and 56.8 mM (++ Gly) at application intervals of 14 and 37 days after transplanting. Data represent means and standard deviations of four independent replicates. Statistically significant differences $P < 0.05$ are indicated by different *characters* for each plant organ

impact on glyphosate turn-over (Reddy et al. 2004), but the underlying mechanisms are still largely unknown.

Accordingly, in the present study, glyphosate-induced depressions of plant growth in the GR soybean cultivar Valiosa were strongly dependent on the selected culture conditions (Figs. 1 and 3, Table 1). In a hydroponic culture experiment, designed to study effects on growth and micronutrient status of the plants, independent of external factors determining the solubility and plant availability of micronutrients in soils, glyphosate application induced an inhibition of root growth in plants supplied with full nutrient sufficient Mn but not under conditions of low [0.1 µM] Mn supply (Fig. 1, Table 1). Assuming that AMPA toxicity is responsible for the growth depression (Reddy et al. 2004), this may indicate that the enzymatic conversion of glyphosate to AMPA in GR soybeans requires a certain level of external Mn supply, which was insufficient in the low Mn treatment.

In soil culture, shoot biomass production declined by approximately 15–30% in glyphosate treated plants grown on an acidic Arenosol but not on a calcareous Loess sub-soil, while root biomass was not significantly affected (Fig. 3). Therefore, the differences in plant responses to glyphosate treatments on the two contrasting soils and in the different culture systems suggest an important role of the physiological status or the developmental stage of the plants (17 DAT in hydroponics versus 47 DAT in soil culture) as factors determining e.g. the rates of internal glyphosate degradation or the sensitivity to AMPA toxicity.

Growth inhibition was associated with a selective decline of Mn concentrations in young shoots of plants grown in hydroponics (Fig. 2) and of Zn in plants grown in soil culture (Table 2). However, no visible symptoms of micronutrient limitation were detectable and the tissue concentrations did not drop below the critical deficiency levels (Mn 20; Zn 30, Fe 30–40 µg g^{-1} DM; Bennett 1993; Reuter and Robinson 1997). These findings suggest that the decline of the micronutrient concentration was a consequence rather than the cause of impaired plant growth induced by glyphosate application.

Interestingly, at high levels of Mn supply [0.5 µM in the nutrient solution] without glyphosate application, the transgenic cv. Valiosa accumulated twice the concentrations and shoot contents of Mn compared with the parental line cv. Conquista (Fig. 2), while other micronutrients, such as Zn and Fe remained unaffected (data not shown). This may be a conse-

Table 2 Micronutrient (Mn, Zn, Fe) concentrations in the 80% ethanol-soluble (LMW) and insoluble fractions (HMW) obtained from young leaves of the glyphosate-resistant soybean cv. Valiosa and the non-GR parental isoline Conquista, grown for 42 days in rhizoboxes under greenhouse conditions on an acidic Arenosol (left) and a calcareous Luvisol subsoil (right)

	Arenosol				Luvisol			
	Conqista	Valiosa	Val. +Gly	Val. ++Gly	Conquista	Valiosa	Val. +Gly	Val. ++Gly
Soluble Mn [μg g^{-1} DM]	1.2a (±0.2)	0.9a (±0.2)	1.3a (±0.2)	1.2a (±0.4)	5.1b (±2.3)	5.7b (±1.3)	5.6b (±1.1)	6.4b (±1.5)
Insoluble Mn [μg g^{-1} DM]	53.2a (±2.7)	48.3a (±7.6)	53.6a (±4.6)	56.3a (±5.1)	85.7b (±18.4)	81.6b (±8.5)	93.0b (±16.0)	92.9b (±15.0)
Total Mn [μg g^{-1} DM]	54.4a (±2.9)	49.2a (±7.6)	54.9a (±4.4)	57.5a (±4.8)	90.9b (±20.6)	87.4b (±9.5)	98.7b (±16.6)	99.4b (±14.4)
Soluble Zn [μg g^{-1} DM]	41.3a (±5.7)	28.1b (±7.7)	27.5b (±6.3)	22.2b (±9.9)	28.1a (±8.9)	27.7a (±3.7)	30.3a (±4.9)	32.4a (±2.6)
Insoluble Zn [μg g^{-1} DM]	85.5a (±35.0)	68.6a (±18.2)	39.1b (±2.6)	38.2b (±10.8)	40.5a (±11.2)	35.1a (±1.8)	35.8a (±7.3)	38.0a (±9.7)
Total Zn [μg g^{-1} DM]	126.8a (±35.2)	96.8a (±25.5)	66.6b (±8.5)	68.8b (±16.7)	68.6a (±15.8)	62.8a (±3.2)	66.3a (±4.1)	70.4a (±8.8)
Soluble Fe [μg g^{-1} DM]	21.6a (±8.6)	14.2a (±5.3)	27.2b (±6.8)	13.6a (±3.8)	16.1a (±4.8)	15.1a (±5.2)	18.5a (±9.6)	20.9a (±11.7)
Insoluble Fe [μg g^{-1} DM]	134.4a (±42.8)	124.9a (±22.8)	114.8a (±25.7)	114.8a (±18.8)	122.8a (±25.0)	124.2a (±23.5)	105.2a (±29.7)	111.7a (±36.4)
Total Fe [μg g^{-1} DM]	155.9a (±44.9)	139.1a (±22.5)	141.9a (±28.2)	128.4a (±21.9)	138.9a (±24.0)	139.2a (±24.7)	123.7a (±22.7)	132.5a (±33.5)

Foliar glyphosate application was performed only with cv. Valiosa using two application levels (+ Gly=28.4 mM and ++ Gly=56.8 mM) and two application intervals at 14 and 37 days after transplanting. Data represent means and standard deviations of four independent replicates. In each row, statistically significant differences $P<0.05$ are indicated by different characters.

quence of higher uptake and/or root to shoot translocation of the easily available Mn in the nutrient solution culture system and reflect a selectively higher Mn demand (up to 50%) reported for some GR soybean varieties also in field observations (Gordon 2007). However, the reasons for this effect of the transgenic modification of the EPSPS gene are currently unknown. After glyphosate application, the reduced root development of the transgenic cv Valiosa (Fig. 1, Table 1) may be no longer sufficient to match the increased Mn demand of this variety, resulting in the observed decline of Mn accumulation in the shoot tissue (Fig. 2).

In the soil culture experiments, soil analysis surprisingly revealed a similar or even lower availability for Zn and Mn (VDLUFA 2004) on the acidic Arenosol as compared with the calcareous Loess sub-soil. Obviously, low absolute levels of these micronutrients in the highly weathered Arenosol superimposed the effects of increased micronutrient solubility, expected by the low pH of the Arenosol. Although soil analysis (VDLUFA 2004) revealed similar Zn levels in both soils (0.8 and 0.6 mg kg^{-1} in the Arenosol and the Loess sub-soil, respectively), glyphosate application induced a decline of shoot Zn in cv. Valiosa, grown on the Arenosol but not on the calcareous soil. This may indicate a selective impairment of mechanisms for Zn acquisition or translocation by glyphosate application, restricted to the growth conditions on acidic Arenosol. Glyphosate released into the rhizosphere by roots of GR soybean (Neumann et al. 2006) and also AMPA as major phytotoxic metabolite of glyphosate in soils (Giesy et al. 2000) may be differentially adsorbed and inactivated in the two soils with different properties. Accordingly, Neumann et al. (2006) demonstrated that glyphosate released by roots of GR soybean, exerted phytotoxic effects on co-cultivated non-GR sunflower on the acidic Arenosol but not on the calcareous loess sub-soil. Obviously, on the highly weathered Arenosol with low buffering capacity, glyphosate was sufficiently available in the soil solution for interactions with the roots of sunflower as a non-target plant. High Ca^{2+} concentrations in the calcareous sub-soil (30% $CaCO_3$) may lead to rapid complexation and immobilization of glyphosate (Gauvrit et al. 2001; Schönherr and Schreiber 2004) to make it unavailable for plant roots and to protect it from conversion to AMPA, which can exert phytotoxic effects even to GR soybean (Reddy et al. 2004) Recently, Wang et al. (2008) reported increased Zn adsorption on goethite in presence of glyphosate at pH values <5.0. Similarly, root exudation of glyphosate may limit Zn availability in the rhizosphere of the glyphosate-treated GR soybean plants on the Fe-rich Arenosol with pH 4.5.

After foliar application, glyphosate is rapidly translocated to young growing tissues of roots and shoots where it can accumulate in millimolar concentrations (Feng et al. 1999; Hetherington et al. 1999). Therefore, a possible internal inactivation of micronutrients in young leaves via formation of glyphosate-metal complexes, unavailable for plant metabolism, was also investigated. The well-documented ability of glyphosate to form stable complexes with metal cations such as Al, Fe, Zn, Mn and Ca (Sprankle et al. 1975) may thereby induce internal micronutrient deficiencies, although total micronutrient leaf concentrations are in the sufficiency range. However, micronutrients in the 80% ethanol-soluble LMW fraction of young leaves obtained from glyphosate-treated and non-treated control plants in soil culture were not significantly different (Table 2). This suggests that at least in the rhizobox experiments of this study, there was no increased partitioning or immobilisation of micronutrients in the LMW fraction by complexation with glyphosate, which could limit the availability of micronutrients for their physiological function in membrane stabilisation and enzyme interactions in the HMW fraction of young leaves (Cakmak 2000). However, a possible micronutrient immobilization in the root tissue by complexation with glyphosate, which may limit the translocation of micronutrients to the shoots still needs to be investigated.

Conclusions

Glyphosate application at the recommended dosage can exert negative side-effects on plant growth and micronutrient status under some conditions, even in transgenic, glyphosate-resistant GR soybean. The differential expression of these effects in different culture systems (hydroponics, soil culture) and on different soils suggests a strong interrelationship with growth conditions and environmental factors. The development of strategies to avoid these negative side effects requires further attention to characterize responsible factors and to investigate underlying mechanisms of action and their degree of expression under field conditions.

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