

Relevance of glyphosate transfer to non-target plants via the rhizosphere

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Summary

There is a common understanding that the widely used herbicide glyphosate is easily degraded and adsorbed in soils and thus, harmless for use in agriculture. We can demonstrate, however, that this conclusion is wrong and dangerous for farmers because in former risk assessments the behaviour of glyphosate in the rhizosphere was not properly considered.

In nutrient solution, rhizobox and pot experiments we can show that foliar applied glyphosate to target plants is released into the rhizosphere after a fast translocation from shoots to roots. In the rhizosphere glyphosate can obviously be stabilized long enough to achieve negative effects on non-target plants. Such a negative side effect is for example inhibited acquisition of micronutrients such as Mn, but also Zn, Fe and B, which are involved in plant own disease resistance mechanisms.

From this glyphosate transfer from target to non-target plants (e.g. from weed to trees in orchards) we predict an increase in disease problems, particularly on soils with low micronutrient availability as already reported in the USA. In view of plant and soil health, we urgently call for a re-assessment of glyphosate as herbicide.

Keywords: Glyphosate, root exudation, rhizosphere, micronutrients

Zusammenfassung

Einfluss des Rhizosphären-Transfers von Glyphosat auf Nicht-Zielpflanzen

Aufgrund des schnellen mikrobiellen Abbaus und der Sorption in Böden wird die Anwendung des weit verbreiteten Herbizids Glyphosat in der Regel als problemlos für die landwirtschaftliche Praxis betrachtet. Unsere Arbeiten weisen jedoch darauf hin, dass diese Schlussfolgerungen keine uneingeschränkte Gültigkeit besitzen, da in den bisherigen Risikobewertungen Rhizosphärenprozesse offensichtlich nicht ausreichend berücksichtigt wurden.

In Nährlösungsexperimenten, Minrhizotron- und Topfversuchen konnte gezeigt werden, dass Glyphosat nach Blattapplikation schnell vom Spross in die Wurzeln der Zielpflanzen verlagert und anschließend in die Rhizosphäre abgegeben wird. In der Rhizosphäre kann Glyphosat offenbar lange genug stabilisiert werden, um Nicht-Zielpflanzen negativ zu beeinflussen. Bei solchen negativen Nebeneffekten handelt es sich z.B. um eine gehemmte Aneignung von Mikronährstoffen, wie Mn aber auch von Zn, Fe und B, die an pflanzeigenen Krankheitsresistenzmechanismen beteiligt sein können.

Als Folgen dieses Glyphosat-Transfers auf Nicht-Zielpflanzen (z.B. bei der Unkrautbekämpfung im Obstbau) wird ein Anstieg der Krankheitsanfälligkeit, besonders auf Böden mit verminderter Verfügbarkeit von Mikronährstoffen, prognostiziert, wie dies Feldbeobachtungen in den USA bereits gezeigt haben. Im Interesse der Gesundheit von Pflanzen und Böden erscheint eine Neubewertung des Risikopotentials von Glyphosatanwendungen dringend geboten.

Stichwörter: Glyphosat, Wurzelexsudate, Rhizosphäre, Mikronährstoffe

Introduction

Glyphosate is the most widely-used systemic herbicide in agricultural practice. It is characterized by high efficiency and low production costs. Glyphosate acts via inhibition of the shikimate pathway for the biosynthesis of aromatic amino acids and phenolic compounds. Negative side effects on non-target organisms are generally considered as marginal, due to rapid microbial degradation and immobilisation in soils. However, despite of this widely accepted and positive evaluation of environmental risks, an increasing number of more recent observations suggest a relationship between prolonged glyphosate application and negative effects reported for various non-target organisms in agro-ecosystems. These observations comprise: (1) increased sensitivity to plant diseases, associated with a low Mn-, and Fe-nutritional status, (2) increased nematode infections, (3) inhibition of root growth, possibly induced by glyphosate interactions with the calcium metabolism, (4) reduced honey production due to limited synthesis of flavonoids as flower pigments, and (5) reduced biological nitrogen fixation (HUBER and MCCAY-BUYS, 1993; KING *et al.* 2001; KREMER *et al.* 2001).

In this study we investigated the potential transfer of foliar applied glyphosate, released from roots of target plants (glyphosate-resistant and glyphosate-sensitive soybean cultivars, *Brachiaria brizantha*) to non-treated indicator plants (sunflower, coffee seedlings) simultaneously cultivated in hydroponics and in soil culture systems. Uptake of glyphosate by non-treated plants was detected by measuring intracellular shikimate accumulation as a physiological indicator in consequence of glyphosate-induced inhibition of shikimate turn-over. Effects on Mn uptake and the soil Mn reducing potential were investigated.

Materials and methods

Germination and pre-culture of test plants

Seeds of soybean (*Glycine max* L., cv. BRSMG68; Nidera A8000 RR) and Sunflower (*Helianthus annuus* L. cv. TR 6149 SA) were surface sterilized for 10 min in a 30 % H₂O₂ solution and subsequently germinated in the dark at 25 °C for 4 days in rolls of filter paper (MN710, Macchery & Nagel, Düren, Germany) moistened with 60 mL of 2.5 mM CaSO₄ solution. Thereafter the seedlings were cultivated for one day in a growth chamber with 16/8 h day/night cycle at a light intensity of 150 μmol m⁻² s⁻¹, a 25 °C/20 °C day/night temperature regime and a relative humidity of 60 % and subsequently transferred to nutrient solution or planted into rhizoboxes.

Nutrient solution experiment

At 5 days after sowing (DAS) seven seedlings of Round-up- sensitive soybean (cv. BRSMG 68) and three seedlings of sunflower (cv. TR 6149 SA) were cultivated together in pots with each 2.5 L of continuously aerated nutrient solution containing 2 mM Ca(NO₃)₂; 0.7 mM K₂SO₄; 0.1 mM KCl; 0.1 mM KH₂PO₄; 0.5 mM MgSO₄; 30 μM Fe-EDTA; 10 μM H₃BO₃; 0.5 μM MnSO₄; 0.5 μM ZnSO₄; 0.2 μM CuSO₄; 0.01 μM (NH₄)₆Mo₇O₂₄ in a growth chamber under controlled conditions (see above). At 7 DAS the nutrient solution was replaced and the soybean plants (fully expanded primary leaves) were treated with glyphosate leaf-spray applications. The plants were subsequently cultivated for additional 6 days with sequential harvests of each one sunflower seedling at 2 d, 4 d and 6 d after the glyphosate treatment for measurements of ⁵⁴Mn uptake and intracellular shikimate accumulation.

Rhizobox and pot experiment

At 5 DAS, two Round-up- resistant soybean (cv. Nidera A8000 RR) seedlings and one seedling of sunflower were transplanted together into rhizoboxes filled with 300 g of an acidic sandy Arenosol from West Africa (pH CaCl₂: 4.5; C_{org}: 0.16 %; P_{CAL}: 7 ppm) or with a calcareous loess sub soil of a Luvisol (pH CaCl₂: 7.6; C_{org}: < 0.3 %; P_{CAL}: 5 ppm). Basal fertilization was performed with N: 100 mg kg soil⁻¹ as Ca(NO₃)₂; K: 150 mg/kg soil⁻¹ as K₂SO₄; Mg: 50 mg/kg soil⁻¹ as MgSO₄; P: 80 mg P/kg soil⁻¹ as Ca(H₂PO₄)₂. For the calcareous soil FeEDTA (20 μmol kg soil⁻¹) was supplied additionally. For

inoculation with a vital microflora, 20 % (w/w) of a fresh field soil from Hohenlohe was mixed with the test soils. Soil moisture level was adjusted to 15 % (Arenosol) and 20 % (calcareous soil), respectively and adjusted by gravimetric determination every two days. The experiment was performed with five replicates for each soil. When the primary leaves were fully expanded (10 DAS), soybean plants were treated with glyphosate foliar applications and sunflower plants were harvested at 17 DAS for determination of intracellular shikimate accumulation. The Mn-reducing potential of the soils was visualized with MnO₂-impregnated filter paper according to the method described by ENGELS *et al.* (2000).

In a similar pot experiment, citrus seedlings (*Citrus limon* L. cv. Limao cravo) were grown in greenhouse culture for 9 months on the Arenosol (5.5 kg per plant). After 48 d co-cultivation with 40 target plants of *Brachiaria brizantha* (Hochst Ex A. Rich), *Brachiaria* weed was either manually removed (control) or sprayed with glyphosate. Citrus plants were harvested at 4 weeks after glyphosate application.

Glyphosate treatments

Glyphosate applications were performed twice with a sprayer onto the surface of the primary leaves of the soybean seedlings. Roundup-Ultra (Monsanto, St. Louis, USA) was diluted as recommended by the manufacturer (1 l / 200 l⁻¹ deionized water) to obtain a glyphosate concentration of 28.4 mM. In the nutrient solution experiment, glyphosate was applied with 0 %, 5 %, 50 % and 100 % (v/v) of the recommended concentration. In the rhizobox experiment, 0 % and 100 % (v/v) were foliar applied. Each treatment consisted of 5 replicates arranged in a randomised block design. To avoid leaf contamination of the non-target plants, the sunflower seedlings were removed from the nutrient solution pots during glyphosate spraying of the soybean target plants. The lids of the culture pots were sealed with strips of filter paper to prevent contamination of the nutrient solution. In rhizobox culture, leaves of sunflower plants were protected from glyphosate contamination by polyethylene bags covering the shoots during the application period. During the subsequent culture period, contact between shoots of target and non-target plants was avoided by covering the shoots of the sunflower seedlings with transparent tubes of plastic foil.

In the citrus pot experiment, *Brachiaria* weed was removed manually by cutting or by foliar application of 0 %, 100 % and 400 % of the recommended glyphosate concentration (28.4 mM) with six replicates per treatment. Citrus plants were protected from aerial glyphosate co-contamination by covering the shoots with transparent plastic tubes during 10 d after the glyphosate treatment.

⁵⁴Mn uptake studies

At 2, 4 and 6 days after the glyphosate treatments, sunflower plants were transferred for 1 h to 500 mL of continuously aerated nutrient solution without micronutrients (4 replicates with each one plant per harvest date), followed by a labelling period of 4 h with nutrient solution containing 1 μM MnSO₄ and a ⁵⁴Mn activity of 1 μCi l⁻¹. Subsequently, the roots were washed for 10 min with 10 μM NaEDTA. The washing-solution was removed by filter papers and root and shoot dry weight was recorded after drying at 70 °C. Plant material was ashed at 500 °C and ⁵⁴Mn activity was determined by liquid scintillation counting.

Determination of shikimate

Fresh root and shoot material washed with deionised water was carefully dried with filter paper and homogenized to a fine powder using liquid nitrogen. Extraction was performed by grinding the homogenized plant with 5 % ortho-phosphoric acid (1 ml 100 mg⁻¹ fresh weight) using mortar and pestle. Insoluble material was removed by centrifugation (5 min at 12 000 g) and the supernatant was used for HPLC analysis after appropriate dilution with the HPLC mobile phase. Separation of the organic acids was conducted on a reversed-phase C-18 column (GROM-SIL 120 ODS-5 ST, particle size 5 μm; length 250 mm, ID 4.6 mm), with a guard column (length 20 mm, ID 4.6 mm; GROM, Herrenberg, Germany) with the same column material. A sample volume of 20 μL were injected into the isocratic flow (0.5 mL min⁻¹) of the eluent (18 mM KH₂PO₄, pH 2.25, 35 °C), and detected spectrophotometrically at 215 nm. Identification and quantification of shikimate was conducted by comparing the retention times and peak areas with a known standard.

Results

Glyphosate transfer in hydroponic culture

Leaf spray applications of glyphosate (Roundup-Ultra) solutions with 0 %, 5 %, 50 % and 100 % of the recommended concentration (28.4 mM glyphosate) to soybean seedlings in the primary leaf stage induced shikimate accumulation in leaf tissue and particularly in roots of non-treated sunflower seedlings. Increased tissue concentrations of shikimate in the non-target plants were detected already 2 days after glyphosate application at 50 % and 100 % of the recommended glyphosate concentration and increased continuously within the 6 d culture period (Tab. 1).

Tab. 1: Intracellular shikimate accumulation [nmol g^{-1} fresh weight] in non-target plants (sunflower) as physiological indicator for glyphosate transfer via the roots of foliar-treated soybean target plants during simultaneous cultivation in hydroponics. One representative plant was analyzed for each glyphosate concentration and each harvest date.

Tab. 1: *Intrazelluläre Shikimat Akkumulation [nmol g^{-1} Frischmasse] in Nicht-Zielpflanzen (Sonnenblume) als physiologischer Indikator für einen Glyphosat-Transfer über die Wurzeln von Zielpflanzen (Soja) nach Glyphosat-Blattapplikation während gemeinsamer Kultur in Nährlösung. Für jede Glyphosatkonzentration und jeden Erntezeitpunkt wurde eine repräsentative Pflanze analysiert.*

Organ / days after treatment	0 % of the recommended glyphosate concentration [28.4 mM]	5	50	100
<u>Shoot</u>				
2d	46	55	136	340
6d	61	99	895	1513
<u>Roots</u>				
2d	0	146	92	2673
6d	57	32	6233	11455

This was associated with a significant reduction of ^{54}Mn uptake into the shoots of non-target sunflower plants, particularly expressed 4 days after glyphosate application (Fig. 1).

Glyphosate transfer in soil culture

Glyphosate leaf application to glyphosate-resistant Roundup Ready soybean seedlings induced shikimate accumulation in the root tissue of non-treated sunflower seedlings when the plants were grown simultaneously in an acidic sandy soil (Arenosol) but no effects were observed on a calcareous loess sub-soil (Fig. 2).

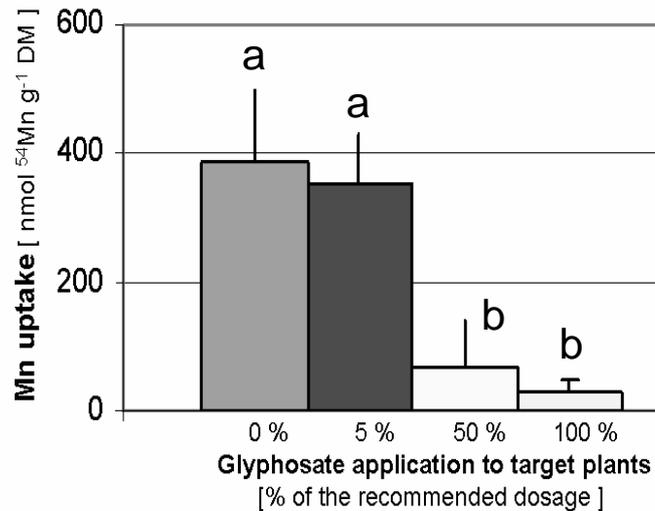


Fig. 1: ⁵⁴Mn in shoots of non-target plants (sunflower), simultaneously cultivated in hydroponics with soybean target plants at 4 days after foliar glyphosate application. 4 replicates per treatment. Significant differences (one-way Anova, p = 0.05) are marked by different characters.

Abb. 1: ⁵⁴Mn im Sprossgewebe von Nicht-Zielpflanzen (Sonnenblume) in gemeinsamer Nährlösungskultur mit Soja-Zielpflanzen, 4 Tage nach Glyphosat-Blattapplikation. 4 Wiederholungen pro Behandlung. Signifikante Unterschiede (einfaktorielle Varianzanalyse, p = 0,05) sind mit unterschiedlichen Buchstaben gekennzeichnet.

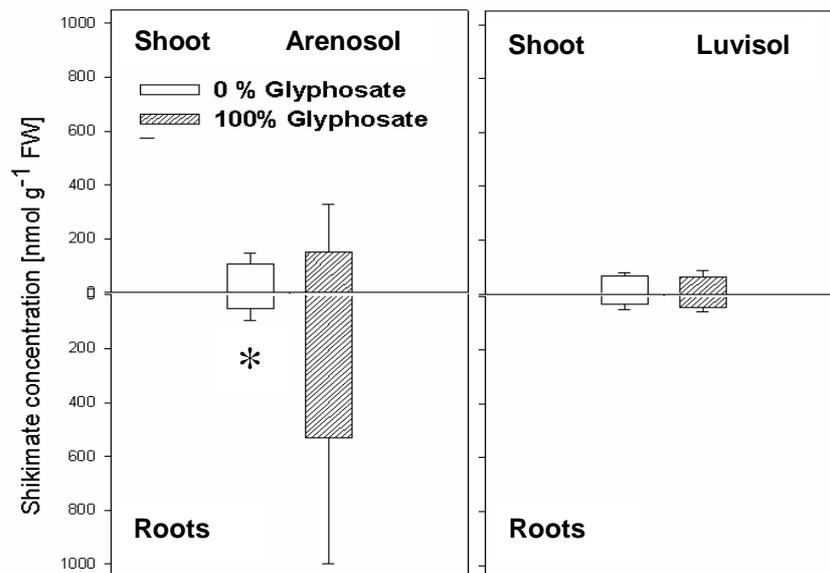


Fig. 2: Intracellular shikimate accumulation [nmol g⁻¹ fresh weight] in non-target plants (sunflower), as physiological indicator for glyphosate transfer via the roots of foliar treated Round-up-resistant soybean target plants during simultaneous cultivation on an acidic Arenosol or a calcareous loess sub-soil (Luvisol). 5 replicates per treatment. Significant differences between glyphosate treatments (t-test, p = 0.05) are marked by an asterisk.

Abb. 2: Intrazelluläre Shikimat-Akkumulation [nmol g⁻¹ Frischmasse] in Nicht-Zielpflanzen (Sonnenblume), als physiologischer Indikator für einen Glyphosat-Transfer über die Wurzeln Round-up-resistenter Soja Zielpflanzen nach Glyphosat-Blattapplikation während gemeinsamer Kultur auf einem Arenosol oder einem kalkhaltigen Loess Unterboden (Luvisol). 5 Wiederholungen pro Behandlung. Signifikante Unterschiede zwischen den Glyphosatbehandlungen (t-Test, p = 0,05) sind mit Stern gekennzeichnet.

The Mn-reducing potential of the Arenosol, indicated by decolouration of a Mn oxide-impregnated filter paper which was placed onto soil surface (ENGELS *et. al.* 2000), was lower in the variant with glyphosate-treated plants compared with the untreated control. In contrast, no differences of glyphosate treatments could be observed on the calcareous sub-soil and the Mn-reducing potential was generally low (Fig. 3).

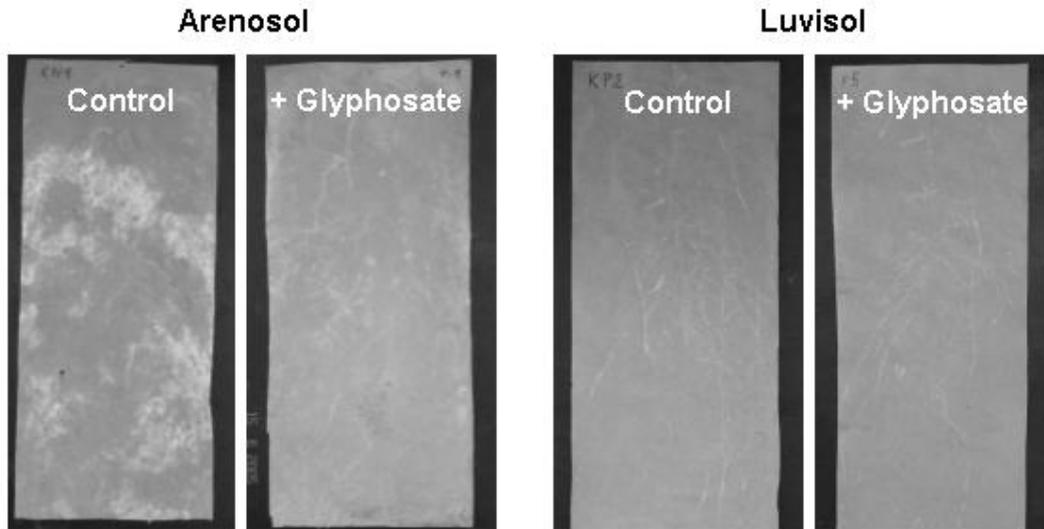


Fig. 3: Manganese-reducing potential of soils used for rhizobox culture of sunflower and Round-up resistant soybean with and without foliar glyphosate application. Manganese reduction was detected by decolouration of MnO₂ impregnated filter paper, which was placed onto the soil surface for 48 h at 5 d after the glyphosate treatment.

Abb. 3: Mangan-reduzierendes Potenzial der Böden, die für die Rhizoboxkultur von Sonnenblume und Round-up resistenten Sojapflanzen, mit oder ohne Blattapplikation von Glyphosat, verwendet wurden. Die Manganreduktion wurde durch die Entfärbung von MnO₂-imprägnierten Filterpapieren dargestellt, die 5 Tage nach der Glyphosatapplikation für 48 Stunden auf die Bodenoberfläche aufgelegt wurden.

In a similar pot experiment. with citrus seedlings (*Citrus lemon* L., cv. Limao cravo), grown for 9 months on the Arenosol, removal of simultaneously cultured *Brachiaria* weed by foliar glyphosate applications (28.4 mM and 113.6 mM), significantly increased shikimate concentrations in the root tissue of the citrus plants, detectable even four weeks after the glyphosate treatments. (Tab. 3).

Tab. 2: Intracellular shikimate accumulation [$\mu\text{g g}^{-1}$ fresh weight] in roots of non-target plants (*Citrus*), as physiological indicator for glyphosate transfer via the roots of foliar treated *Brachiaria brizantha* target plants during simultaneous cultivation on an acidic Arenosol, 4 weeks after the glyphosate treatments. Significant differences (one-way Annova, $p = 0.05$) are marked by different characters.

Tab. 2: Intrazelluläre Shikimat Akkumulation [$\mu\text{g g}^{-1}$ Frischmasse] in Nicht-Zielpflanzen (*Citrus*), als physiologischer Indikator für einen Glyphosat-Transfer über die Wurzeln von *Brachiaria brizantha* als Zielpflanzen, 4 Wochen nach Glyphosat-Blattapplikation während gemeinsamer Kultur auf einem sauren Arenosol. Signifikante Unterschiede (einfaktorielle Varianzanalyse, $p = 0,05$) sind mit unterschiedlichen Buchstaben gekennzeichnet.

	0 %	100 %	400 %
	% of the recommended glyphosate concentration [28.4 mM]		
Shikimate concentration [$\mu\text{g g}^{-1}$ fresh weight]	3.93 a	5.40 b	5.31 b

Discussion

The results clearly demonstrate a release of glyphosate via the roots of target plants, which can be subsequently taken up by non-treated plants, exerting inhibitory effects on the shikimate pathway (Tab. 1; Fig. 2), on uptake of micronutrients (Mn, Fig. 1), and plant growth. The release of glyphosate may occur from damaged roots of dying target plants but can be also released as exudates from undamaged roots of glyphosate-resistant GM crops (Fig. 2).

Plant to plant transfer of glyphosate is detectable both in hydroponics (Tab. 1) but also in soil culture (Fig. 2; Tab. 2) and seems to be particularly expressed in acid sandy soils. An associated inhibition of soil Mn reduction (Fig. 3) may be related with inhibitory effects of glyphosate on Mn-reducing microorganisms, such as *Pseudomonades* (MARSCHNER *et al.* 1997), known to express a shikimate pathway. In the calcareous soil however, glyphosate released from the roots of target plants may be immediately precipitated in the rhizosphere by the high Ca levels in the soil solution, preventing rapid uptake by non-target organisms. Therefore, no negative effects on non-target plants or on soil Mn-reduction could be observed on the calcareous loess subsoil (Fig. 2). It remains to be established whether the postulated glyphosate fixation in the rhizosphere soil leads to a stabilization with a potential risk of later remobilisation by the activity of roots or microorganisms. The pot experiment with removal of *Brachiaria* weed in the culture vessels of citrus plants by foliar application of glyphosate suggests a maintainance of the glyphosate effect on non-target plants (Citrus) for at least 4 weeks after the Glyphosate treatment, indicated by increased shikimate accumulation in citrus roots (Tab. 2).

The interspecific glyphosate transfer from weed to non-target organisms investigated in this study may at least partially explain the increasing number of field observations, suggesting a relationship of long-term glyphosate application and negative effects on growth and disease resistance of crop plants (FERNANDEZ *et al.* 2005). These observations demonstrate that, in face of the widespread and increasing use of Glyphosate in agricultural practice, research on the fate of Glyphosate in the rhizosphere and on long-term effects on non-target organisms is urgently required to improve the management of glyphosate applications in agricultural practice.

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