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Rhizosphere processes as determinants for glyphosate damage of non-target plants

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1 Summary-Zusammenfassung

1.1 Summary

Due to low production costs and high herbicidal efficiency, glyphosate is the most widely used wide-spectrum herbicide. Glyphosate acts as a non-selective, total herbicide by inhibiting the biosynthesis of aromatic amino acids. Apart from glyphosate drift contamination, risks of glyphosate toxicity to crop plants and other non-target organisms are generally considered as marginal, because glyphosate is almost instantaneously inactivated by adsorption to the soil matrix and rapid microbial/chemical degradation in the soil solution.

However, in the recent past, an increasing number of yet unexplained observations on significant damage of crop plants have been reported in the literature and by farmers, suggesting gaps in the risk assessment, with respect to the fate glyphosate in the rhizosphere and the interaction with rhizosphere processes.

According to these observations, the aim of present study was a systematic evaluation of potential rhizosphere effects of glyphosate, including direct toxicity, risks of re-mobilisation by fertiliser application, potential role of pathogens and allelopathic compounds, and interactions with micronutrients, both in glyphosate-sensitive and transgenic glyphosate-resistant crops.

A series of field trials in reduced soil tillage cropping systems as well as green-house experiments on soils with contrasting properties with sunflower, winter wheat and soybean, consistently revealed a close clausal relationship between crop damage and (a) short waiting times between glyphosate application on target weeds and subsequent sowing of crops and (b) the density and speed of decay of glyphosate-treated weeds. The results suggested that damage of crop plants is induced by a rhizosphere transfer of glyphosate from weeds to subsequently sown crops. This transfer might take place by contact contamination due to exudation of glyphosate from living roots of treated weeds and/or release during decomposition of the root residues.

A comparison between phytotoxic effects of glyphosate and aminomethylphosphonic acid (AMPA) as major metabolite of glyphosate in soils, revealed high toxicity in case of root exposure to glyphosate, but not to AMPA. By contrast, a significant decline of germination was induced by seed exposure to AMPA, while germination was not affected by glyphosate treatments. The observed differences in sensitivity to glyphosate and AMPA in different stages of plant development may explain variable symptoms of crop damage under field conditions, ranging from growth depressions and chlorosis to reduced field emergence.

The results of the present study further suggest that risks for crop damage associated with rhizosphere transfer of glyphosate are additionally influenced by a range of environmental factors, such as growth season (spring or fall application), temperature, soil moisture, redox potential of soils and soil microbial activity. These factors might shorten or prolongate the time window for crop damage of glyphosate contact contamination in the rhizosphere under field conditions.

Model experiments investigating the sensitivity of different plant species to glyphosate root exposure, revealed significant differences between winter wheat, maize and soybean in terms of glyphosate-induced plant damage but also in their ability for recovery from glyphosate damage suggesting marked genotypic differences in the expression of damage symptoms also under field conditions.

In agreement with previous investigations, results of the present study indicated a rapid inactivation of glyphosate by adsorption to the soil matrix. Glyphosate adsorption in soils seem to be mainly mediated by the phosphonate group of the molecule in a way similar to the adsorption of inorganic phosphate. Accordingly glyphosate re-mobilisation is possible via ligand exchange by phosphate application. The results of the present study have demonstrated for the first time that depending on soil properties also the application of fertiliser phosphate is able to re-mobilise glyphosate in sufficient quantities to mediate crop damage in pot experiments. This finding suggest, that re-mobilisation of glyphosate potentially by fertiliser P or root-induced chemical modifications for P and Fe mobilisation needs to be considered as additional potential rhizosphere pathway for glyphosate damage to non-target plants.

Field trials and model experiments under soil and hydroponic conditions consistently revealed a significantly impaired nutritional status of glyphosate-sensitive but also glyphosate-resistant crops. However, depending on the culture conditions different mineral nutrients were affected by the glyphosate treatments and plant damage was not related with a certain nutrient deficiency. These findings suggest that damaged root growth, induced by glyphosate toxicity, rather than specific interactions with certain mineral nutrients are responsible for the observed impairment of nutrient acquisition.

In conclusion, results of the present study highlight that risks for crop damage associated with glyphosate toxicity in the rhizosphere can be substantial and is influenced by factors such as waiting time after herbicide application, weed density, cropping systems, fertilizer management, genotypic differences, and probably also environmental factors including temperature, soil moisture, and soil microbial activity.
The independency between these factors is so far not entirely clear but should be investigated in future studies. Nevertheless, results of present study suggest that risks could be minimized by simple management tools such as the consideration of waiting times between application of glyphosate and sowing of crops particularly in case of high weed densities and alternation of herbicides to reduce not only risk for remobilization of glyphosate but also problems associated to the selection of glyphosate-resistant weeds.

1.2 Zusammenfassung

Auf der Organo-Phosphatverbindung Glyphosat ([N-Phosphonomethyl] Glycine) beruhende Produkte wie Roundup, Clinic, Touchdown etc. sind wegen niedriger Herstellungskosten, hoher Wirksamkeit und vergleichsweise guter biologischer Abbaubarkeit die weltweit am häufigsten eingesetzten Herbizide. Glyphosat wirkt durch die Hemmung der Biosynthese aromatischer Aminosäuren als Totalherbizid und damit generell phytotoxisch. Die Anwendung wird aufgrund des schnellen mikrobiellen Abbaus und der starken Adsorption in Böden in der Regel als problemlos für die landwirtschaftliche Praxis betrachtet. Somit kommt dem Glyphosateinsatz eine kontinuierlich steigende Bedeutung zu. Durch die Einführung transgener, Glyphosat-resistenter Nutzpflanzen, wie z.B. Soja, Mais, Raps, Baumwolle, Zuckerrübe und Weizen, sowie durch die zunehmende Bedeutung von Anbausystemen mit reduzierter Bodenbearbeitung ist vorhersehbar, dass der Einsatz von Glyphosat weltweit und künftig auch in Europa noch weiter ansteigen wird.

Im Gegensatz zur propagierten Unbedenklichkeit der Glyphosat-Anwendung häufen sich jedoch in jüngerer Zeit weltweite Beobachtungen von signifikanten Schäden an Kulturpflanzen. Die beobachteten Schäden lassen einen Zusammenhang mit der Anwendung von Glyphosat vermuten und weisen häufig auf Interaktionen mit Rhizophärenprozessen hin, die bislang nur ansatzweise verstanden sind, sodass die Sicherheitsbewertung für Glyphosatanwendungen in der Praxis, trotz umfangreicher Voruntersuchungen, bei weitem noch nicht als abgeschlossen betrachtet werden kann.

Vor diesem Hintergrund bestand das Ziel der hier vorliegenden Promotionsarbeit in einer systematischen Untersuchung der möglichen Schädigung von Kulturpflanzen durch Glyphosatexposition im Wurzelraum. Dabei lag der Schwerpunkt der Untersuchungen auf der Aufklärung der relevanten Transferpfade, insbesondere der Bedeutung von Pflanzenrückständen behandelter Unkrautpflanzen als Glyphosatspeicherpools mit nachfolgender Glyphosatfreisetzung und der Bedeutung einer möglichen Remobilisierung von an der Bodenmatrix adsorbiertem Glyphosat. Darüber hinaus sollte die Möglichkeit einer Glyphosat-induzierten Einschränkung der Mikronährstoffversorgung bei Glyphosat-resistenten und nicht Glyphosat-resistenten Pflanzen untersucht werden.

In einer Reihe von Feldversuchen in Anbausystemen mit reduzierter Bodenbearbeitung in enger Zusammenarbeit mit betroffenen Landwirten und in kontrollierten Modellversuchen unter Gewächshausbedingungen auf Böden mit unterschiedlichen Charakteristika zeigte sich bei Weizen, Sonnenblumen und Sojabohnen übereinstimmend ein enger kausaler Zusammenhang zwischen Pflanzenschäden und (i) den Wartezeiten nach einer Glyphosatanwendung und der Aussaat der Kulturpflanzen, und (ii) der Dichte des behandelten Unkrautbestandes bzw. der Geschwindigkeit des Absterbens der behandelten Unkrautpflanzen. Diese Ergebnisse weisen auf eine Speicherung von Glyphosat in der organischen Substanz absterbender bzw. bereits abgestorbener Unkrautpflanzen und hier insbesondere im Wurzelmaterial hin, bei dessen mikrobiellem Abbau es zu einer Freisetzung von Glyphosat und zur Schädigung der Folgekultur kommen kann. Die Ergebnisse weisen auch darauf hin, dass die mit einem Rhizosphären-Transfer von Glyphosat verbundenen

Risiken für Kulturpflanzen stark von Umweltfaktoren, wie Temperatur, Bodenfeuchte, Redoxpotenzial und mikrobieller Aktivität abhängig sind, da diese die Abbaugeschwindigkeit der organischen Substanz in Böden bestimmen. Vergleichende Untersuchungen von Glyphosat und seinem primären Abbauprodukt Aminomethyl-Phosphonsäure (AMPA) bezüglich ihrer toxischen Wirkung im Wurzelraum zeigten im Falle einer kurzzeitigen Exposition von Weizenkeimlingen ausschließlich für Glyphosat eine signifikante Schädigung der Pflanzen. Im Gegensatz dazu verursachte AMPA, aber nicht Glyphosat, nach einer kurzzeitigen Exposition von Weizensamen eine signifikante Hemmung der Keimrate. Diese Ergebnisse deuten an, dass unter Feldbedingungen unterschiedliche Schadbilder auftreten können, je nachdem ob toxische Mengen an Glyphosat oder AMPA oder beider Substanzen in einem sensitiven Stadium der Pflanzenentwicklung vorliegen.

Darüber hinaus konnten in weiteren Versuchen Pflanzenart-spezifische Unterschiede in der Empfindlichkeit gegenüber einer Glyphosat-Wurzelexposition gezeigt werden, sodass auch genotypische Unterschiede bei der Ausprägung von Schadsymptomen im Feld in Erwägung gezogen werden müssen.

In den Versuchen der vorliegenden Arbeit zeigten sich übereinstimmend mit den Ergebnissen zahlreicher anderer Studien deutliche Hinweise für eine rasche Inaktivierung von Glyphosat durch Adsorption an die Bodenmatrix die zum Großteil auf der Interaktion der Phosphonatgruppe im Glyphosatmolekül mit kationischen Phosphatbindungsplätzen in Böden beruht. Jedoch konnte in Modellversuchen unter Gewächshausbedingungen gezeigt werden, dass durch die Applikation von anorganischen Phosphatdüngern über Austauschadsorption in Abhängigkeit von der Bodenart Schäden an Kulturpflanzen durch Glyphosat-Remobilisierung induziert werden können. Eine derartige kurzzeitige Remobilisierung von Glyphosat muss also als weiterer möglicher Risikofaktor für Pflanzenschäden in Erwägung gezogen werden.

In den Versuchen der hier vorliegenden Studie verursachte die Applikation von Glyphosat in der Regel eine signifikante Einschränkung der Mineralstoffversorgung der Testpflanzen. Dabei zeigte sich jedoch weder ein klarer Zusammenhang zwischen Glyphosatapplikation und der Induktion eines bestimmten Nährstoffmangels, noch eine eindeutige Beziehung zwischen der Intensität Glyphosat-induzierter Pflanzenschäden und der Nährstoffversorgung. Diese Ergebnisse sprechen dafür, dass die Verminderung der Versorgung von Kulturpflanzen mit mineralischen Nährstoffen eher auf einer generellen Beeinträchtigung des Wurzelwachstums durch Glyphosattoxizität als auf Glyphosatinteraktionen mit spezifischen kationischen Mineralstoffen beruht.

Zusammenfassend weisen die Ergebnisse der vorliegenden Studie auf ein nicht unerhebliches Risiko für Schäden an Kulturpflanzen durch Glyphosat aus den Rückständen behandelter Unkrautpflanzen oder durch Remobilisierung adsorbierter Glyphosatrückstände hin,, das jedoch maßgeblich von Umweltfaktoren beeinflusst wird. Die genauen Zusammenhänge zwischen Glyphosat-induzierten Schäden an Kulturpflanzen und diesen biotischen und abiotischen Faktoren konnten nicht vollständig aufgeklärt werden und bedürfen weiterer Untersuchungen. Dennoch erscheinen nach den Ergebnissen der vorliegenden Studie

Maßnahmen, wie die Beachtung von Wartezeiten zwischen Vorsaatapplikation von Glyphosat und Aussaat insbesondere bei dichten Unkrautbeständen und die wechselnde Verwendung verschiedener Maßnahmen zur Unkrautbekämpfung geeignet, um Risiken von Glyphosatschäden an Kulturpflanzen weitgehend zu minimieren und darüber hinaus auch der Bildung von Resistenzen entgegenzuwirken.

2 General introduction

Weeds are economically the most important of all pests with respect to sales of pesticides and limitations to crop yields. In United States herbicide sales represent more than two thirds of pesticides used annually (Pimentel *et al*., 1991), and almost half of the $21 billion worldwide pesticide market (Belcher, 1989).
Since its commercial introduction in 1974, glyphosate (N-(phosphonomethyl)glycine), the active ingredient of systemic, broad-spectrum, non-selective post-emergence herbicides, has become by any measure the world's best-selling agrochemical compound and the most extensively applied herbicide in history of agriculture. Glyphosate is used for weed control in agriculture, horticulture, silviculture, along roadsides and railways, private gardens and even aquatic systems (AquaMaster™). In agriculture, glyphosate is particularly used in cropping systems with genetically modified glyphosate-resistant plants, but also before sowing of crops in cropping systems with high weed pressure (e.g. no-/minimal tillage systems).

High phytotoxic unit activity, high mobility and rapid translocation within plants leading to the effective control of essentially all annual perennial weed plants are frequently mentioned as important characteristics of glyphosate-containing herbicides. Moreover, low toxicity for humans, other mammalians and insects and low risks for long-term accumulation in soils and/or groundwater pollution by leaching are cited as advantages of glyphosate. Even today glyphosate containing herbicides like Roundup® and many other herbicide brands are in terms of sale and applications the fastest growing agrochemicals worldwide.

2.1 Glyphosate discovery and chemical properties

2.1.1 Glyphosate discovery

As reported by Franz *et al*., (1997) glyphosate was found during a screening of over 300 tertiary aminomethylphosphonic acids derived from various primary and secondary amines for herbicidal effects (Moedritzer and Irani, 1966). However, initial attempts to find tertiary aminomethylphosphonic acids with high herbicidal activity failed. Only two produced compounds showed some herbicidal activity leading to the introduction of glyphosine, as a sugar cane ripening agent (Polaris ®, Monsanto Co.). Contrary to the general trend that metabolism reduces toxicity it was hypothesised that degradation of glyphosine and the 2nd identified compound might give rise to a common metabolite with high herbicidal activity. Glyphosate was among the possible metabolites of the two compounds and was found to have extremely high herbicidal activity (Franz, 1985).

2.1.2 Physical and chemical properties of glyphosate

Chemically, glyphosate is an organic acid derivate of the amino acid glycine and phosphonic acid. According to Knuuttila and Knuuttila (1979) glyphosate exists as a zwitterionic species 1a in solid state. The empirical formula is $C_3H_8NO_5P$, and the structural formula is as follows (Fig. 2.1):

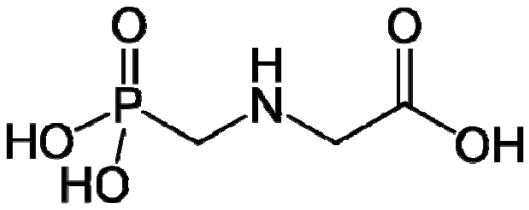

Fig. 2.1: Structural formula of glyphosate (N-(phosphonomethyl)glycine)

The relative molecular mass of glyphosate is 169.07. Physical and chemical properties of glyphosate are summarised in Table 2.1.

Tab. 2.1 Physical and chemical properties of glyphosate
(WHO, 1994)

Physical state	crystalline powder
Colour	white
Odour	none
Melting point	184.5 °C, decomposition at 187 °C
Boiling point	n.a.
Specific gravity (density)	1.704 (at 20 °C)
Vapour pressure	< 1 x 10-5 Pa (at 25 °C)
Solubility in water	10-100 mg/litre (at 20 °C)
Henry's law constant	< 7 x 10-11
Octanol-water partition coefficient (log Kow)	-2.8
Surface tension	0.072 N/m 0.5% (w/v) at approx. 25 °C
pKa values	< 2, 2.6, 5.6, 10.6 Sprankle et al. (1975c)
Molar absorptivity	0.086 litre/mol per cm at 295 nm
Flammability	not flammable
Explosiveness	not explosive
pH	2.5 (1% solution)

Water solubility of glyphosate as N-(phosphonomethyl)glycine is relatively low. Due to its high polarity glyphosate is practically insoluble in organic solvents, for instance, ethanol, acetone and benzene (Franz, 1985). Glyphosate has a low acute toxicity for humans, other mammalians, birds and insects like bees. According to Franz *et al*. (1997) extensive studies

have shown no evidence of mutagenic, carcinogenic, teratogenic, or allergenic activity of glyphosate as N-(phosphonomethyl)glycine in numerous assays and tests.
However, toxicity of glyphosate to fishes and other aquatic organisms was observed (Tsui *et al.*, 2003; Relyea, 2005) and human/mammalian (geno)toxicity of glyphosate and/or its metabolites and surfactants in the herbicide solution are discussed in recent studies (Monroy *et al*., 2005; Gasnier *et al*., 2009; Benachour *et al*., 2009; Mañas *et al*., 2009)

In herbicides, glyphosate is usually formulated as a salt of the deprotonated acid of glyphosate and a cation. The salt formulations that have been utilised are isopropylamine (IAS), trimethylsulfonium (TMS), diammonium (DAS), and potassium (PS) (Hess, 1999). Regardless to the various products on market, the herbicide contains surfactants (e.g. polyoxyethalinealkly-tallowamin (POEA)) to enhance foliar penetration and water. The herbicide formulation is generally thermostable under normal conditions (-20 to 40° C), non-volatile, photostable, and generally non persistent in the soil even so half-life times of glyphosate in soils are reported to be variable depending soil and environmental conditions. Under agricultural soil conditions, half-life times range from 1-197 days but are typically less than 60 days (Giesy *et al*., 2000).

Glyphosate efficiently controls most annual and perennial weed species at rates ranging from 960-1920g active ingredient, respectively 2-4L of glyphosate as Roundup Ultra formulation ha^{-1}. Control of some perennial weeds and woody species requires greater herbicide rates. Plants cannot readily metabolise the herbicide to non-toxic compounds (Gottrup *et al*., 1976; Coupland and Caseley 1979). Currently, glyphosate is approved for use in more than 100 crops and controls more than 300 weed species. According to Franz (1985) glyphosate can efficiently control 76 of the world´s 78 worst weed species.
In contrast to other herbicides, emergence of glyphosate-resistant weed plants has not been an issue of concern for a long time. In fact glyphosate use in a pre-crop weed control application has been effective for more than three decades, with few occurrences of evolved glyphosate-resistant weed populations. One of the first reviews on the topic of glyphosate resistant weeds indicated that after 20 years of frequent glyphosate use there were no known cases of evolved glyphosate-resistant weeds (Dyer, 1994). According to Powles (2008) pre-crop glyphosate application before growing of non-resistant crops is for treated weed plants a short, intense selection event acting only on emerged plants. As weeds often emerge throughout a growing season, this imposes less overall selection pressure than long-term, soil-residual herbicides, which can exert selection over several months of the growing season. Similarly, Bradshaw *et al.* (1997) concluded that due to unique mode of action, low residual activity in soils and a low selection pressure on weed plants associated to the pre-crop application, evolution of glyphosate-resistant weeds was unlikely.
However, over the last 10-15 years and in particular with the adoption of glyphosate-resistant crop plants associated with repeated in-crop glyphosate application during the whole vegetation period, glyphosate-resistance in weed plants has emerged. According to Service (2007) the number of evolved glyphosate-resistant weed species has increased from 1 known weed species in 1996 to 16 known species in 2006 including among others highly competitive and economically damaging weeds like *Ambrosia artemissifolia*, *Ambrosia trifida*,

Amaranthus tuberculatus, *Amaranthus palmeri*, *Amaranthus rudis*, *Sorghum halepens, Conyza spp*. and *Euphorbia heterophylla* (Powles, 2008). Beside this, a number of other important weed genera and species including grass weeds such as *Digitaria*, *Setaria* and *Sorghum*, or dicotyledonous species such as *Chenopodium album* L., *Kochia* species and *Xanthium strumarium* L. are at risk of developing into evolved glyphosate-resistant weeds (Powles, 2008).
According to Service (2007) and Powles (2008), because of the emergence of glyphosate-resistant weeds, maintenance of diversity in weed management systems is crucial for glyphosate to be sustainable.

2.2 Glyphosate behaviour in plants and mode of action

2.2.1 Glyphosate behaviour in plants

In case of foliar application, glyphosate absorption occurs rapidly in most plant species. Glyphosate absorption in quackgrass *(Agropyron repens* L. Beauv.) occurs most rapidly within the first four hours after application, followed by limited absorption thereafter (Sprankle *et al*., 1975c; Majek, 1980). According to Caseley and Coupland (1985) glyphosate crosses the plant cuticle by diffusion via the hydrophilic pathway. After crossing the cuticle, transport away from the point of absorption is a limiting step (Majek, 1980).

Uptake of glyphosate into plant cells is inhibited by the plasma membrane, which is generally described as the major barrier to foliar absorbed herbicides. There is evidence that glyphosate uptake through the plasma membrane occurs through an active amino acid transport system (Fernandez and Bayer, 1977; Richard and Slife, 1979; Gougler and Geiger, 1981). Studies investigating glyphosate uptake in *Catharanthus roseus* L.- and broad bean (*Vicia faba* L.) cells indicated that glyphosate uptake is in part mediated by a phosphate transporter (Morin *et al*., 1997; Denis and Delrot, 1993). Investigations on the requirements of the Pi/glyphosate-transporter in *Catharanthus roseus* L.-cells revealed that the major elements increasing the cellular glyphosate uptake were calcium (Ca), magnesium (Mg), and the presence of iron (Fe) (Morin *et al*., 1997). Beside this, results of Anthelme and Marigo (1998) and Tilquin *et al*. (2000) indicate that glyphosate uptake into cells of *Catharanthus roseus* L. is possibly mediated by a Ca-dependent Fe/glyphosate cotransport, which can also act as Ca-dependent Fe/phosphate cotransport. This Ca-dependent Fe/glyphosate cotransport occurs in various plant cells indicating that Fe/glyphosate cotransport may be considered to be a general mechanism in plant cells (Tilquin *et al*., 2000). According to Tilquin *et al*. (2000), there is the possibility that one or two distinct transporters are required for iron- and glyphosate uptake by cells. However, if two transporters are involved, data indicate that they are most likely functionally related.
In sum, there is considerable evidence that glyphosate is one of the few herbicides that crosses the plasma membrane using an active transport system.

After movement of the herbicide to the cytoplasm, glyphosate is transported in leaves via the symplastic pathway. The long distance transport of glyphosate follows the same source-to-sink pattern as photo assimilates (Sprankle *et al*., 1975c; Wyrill and Burnside, 1976; Ahmadi *et al*., 1980; Bingham *et al*., 1980; Gougler and Geiger, 1981). Glyphosate is rapidly

translocated to stems, leaves and roots of the entire plant, finally accumulating preferentially in young growing tissues (Franz *et al*., 1997).
Thus, accumulation of glyphosate occurs in meristematic regions of the roots, shoots, rhizomes, tubers, stolons, etc. (areas of high growth activity) (Sprankle *et al*., 1975c; Bingham *et al*., 1980).

Beside this, Geiger *et al*. (1999) observed in case of foliar application of glyphosate on sugar beet (*Beta vulgaris* L.) clear indications for self-limited translocation of glyphosate. In this study export of glyphosate from treated leafs essentially stopped 10 hours after application in glyphosate-sensitive (GS) plants. In the study of Geiger *et al*. (1999) inhibition of carbon translocation and glyphosate translocation coincided, confirming that glyphosate export from treated leafs was inhibited by disruption of a process that drives carbon export. Similarly, also Hess (1999) reported self-limitation of glyphosate translocation from treated leafs caused by effects on carbon metabolism in source leaves (disruption of aromatic amino acid biosynthesis, increased diversion of carbon to shikimate pathway, reduction of carbon pool available for Calvin cycle, decreased starch synthesis and decreased export of triose to cytoplasm, decreased phloem transport of assimilates).

2.2.2 Glyphosate mode of action

Glyphosate is generally classified as an aromatic amino acid biosynthesis inhibitor, whose primary mechanism of action is the inhibition of the shikimic acid pathway (Hoagland and Duke, 1982; Cole, 1985; Duke and Hoagland, 1985). The herbicide is a competitive inhibitor of the 5-enolpyruvylshikimate-3-phosphate (EPSP) synthase enzyme in the shikimic acid pathway, leading to impaired conversion of shikimic acid to chorismic acid (Fig 2.2). Because of this effect, glyphosate phytotoxicity is causing the impairment of general metabolic processes, such as protein synthesis and photosynthesis (de María *et al*., 2005; Geiger *et al*., 1986).

According to Cole (1985), inhibition of this particular enzyme in the absence of a regulatory feedback mechanism in earlier parts of the pathway, leads to accumulation of the substrate of the inhibited enzyme (s. Fig. 2.2). An enormous accumulation of shikimic acid (shikimate) occurred in a variety of plants (Amrhein *et al*., 1980; Berlin and Witte, 1981). Inhibition of the shikimic acid pathway is a unique characteristic of glyphosate and was subsequently used in a vast number of studies as specific bio-indicator of glyphosate toxicity to plants (Roider *et al*., 2007; Brown *et al*., 2009; Cakmak *et al*., 2009; Ozturk *et al*., 2008; Eker *et al*., 2006; Miller *et al*., 2004; Henry *et al*., 2007; Lassiter *et al*., 2007; Norsworthy, 2004a; Reddy *et al*., 2010; Bellaloui *et al*. 2006, 2009; Gilreath *et al*., 2000a, 2000b; Neumann *et al.*, 2006). To date competitive inhibition of the 5-enolpyruvylshikimate-3-phosphate (EPSP) synthase enzyme appears to be the primary mode of action of the herbicide (Fig. 2.2).

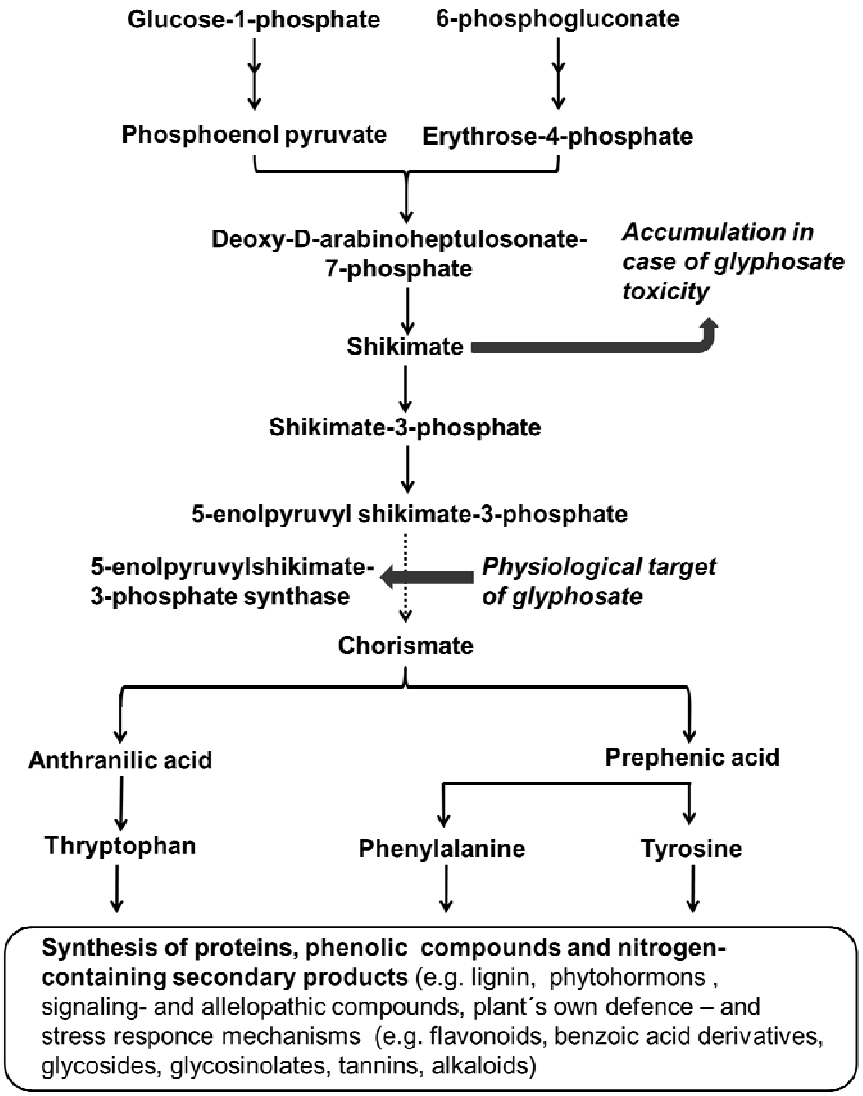

Fig. 2.2: Shikimate pathway of higher plants

Inhibition of the 5-enolpyruvyl shikimate-3-phosphate synthase (EPSPS) as physiological target of glyphosate and shikimate used as specific physiological indicator for glyphosate toxicity are highlighted.

The shikimate pathway is of key importance in linking primary and secondary metabolism in higher plants and is initiated by the condensation of phosphoenolpyruvate (PEP) with erythrose-4-phosphate. Major end-products of the pathway are the aromatic amino acids tryptophan, tyrosine, and phenylalanine essential for protein synthesis (Cornai and Stalker, 1986). In addition, phenylalanine is connected to secondary phenolic compound pathways via the important regulatory enzyme phenylalanine ammonialyase (PAL) to produce a diverse array of phenolic end-products like precursors of lignin, flavonoids, tannins, alkaloids and quinones (Hoagland and Duke, 1982; Duke and Hoagland, 1985). Interestingly, in gramineous plant species tyrosine can also serve as a substrate for these compounds.

Glyphosate-induced inhibition of trypthophan synthesis is directly connected to synthesis of auxin as phytohormone. Chorismate gives also directly rise to a number of phenolic compounds.

Secondary effects induced by glyphosate include: chloroplast disruption, chlorophyll and porphyrin synthesis reduction, decreased photosynthesis and respiration, inhibition of

enzymes of nitrate assimilation, reduced nucleic acid synthesis, inhibition of transpiration and of anthocyanin formation (Holländer and Amrheim, 1980; Cole, 1985). Inhibition of transpiration and photosynthesis were coincident, thus inhibition of transpiration is most likely not caused by an initial effect of glyphosate on photosynthesis (Shaner and Lyon, 1979; Cole, 1985). Effects of glyphosate on phytohormones are also frequently reported even so the underlying causes are not entirely understood. Whether the tryptophane pool for synthesis of auxin/indole-3-acetic acid (IAA) is directly limited by glyphosate-induced inhibition of aromatic amino acid synthesis is not known. *In planta*, after application of glyphosate both, increased as well as decreased IAA levels have been observed (Canal *et al*., 1987; Lee, 1984). Canal *et al*. (1987) attributed the increase in IAA to glyphosate-induced increased levels of gentisic acids as inhibitor of the IAA oxidase. On the other hand, as phenols have been shown to affect conjugation and oxidation of IAA in maize stems (Lee, 1980), it is suggested that the reported change in phenolic levels by glyphosate (Holländer and Amrhein, 1980; Berlin and Witte, 1981; Lee, 1982a, 1982b) may be related to the promotion of IAA metabolism. Also Devine *et al*. (1993) attributed increased metabolism of IAA in plants to altered phenolic compound content. Moreover, inhibition of IAA transport via increased ethylene synthesis has been reported (Cole, 1985; Lee and Dumas, 1983). Promotion of lateral bud growth/ increase in tillering following glyphosate application has been reported in a number of weed- (Lee, 1984) as well as crop plant species such as sorghum, and wheat (Baur *et al*., 1977). Such an effect has been attributed to a change in the auxin-cytokinin balance in the basal internodes through an inhibition of the basipetal transport of auxin by glyphosate (Baur, 1979a, 1979b).

Phosphonic acids are known as chelators of metal cations (Cater *et al*., 1967) and it has been argued that glyphosate may increase its mode of action by complexing biologically important di- and trivalent cations like Ca, Mg, Fe, manganese (Mn), zinc (Zn) and copper (Cu) within the cell or in the rhizosphere.

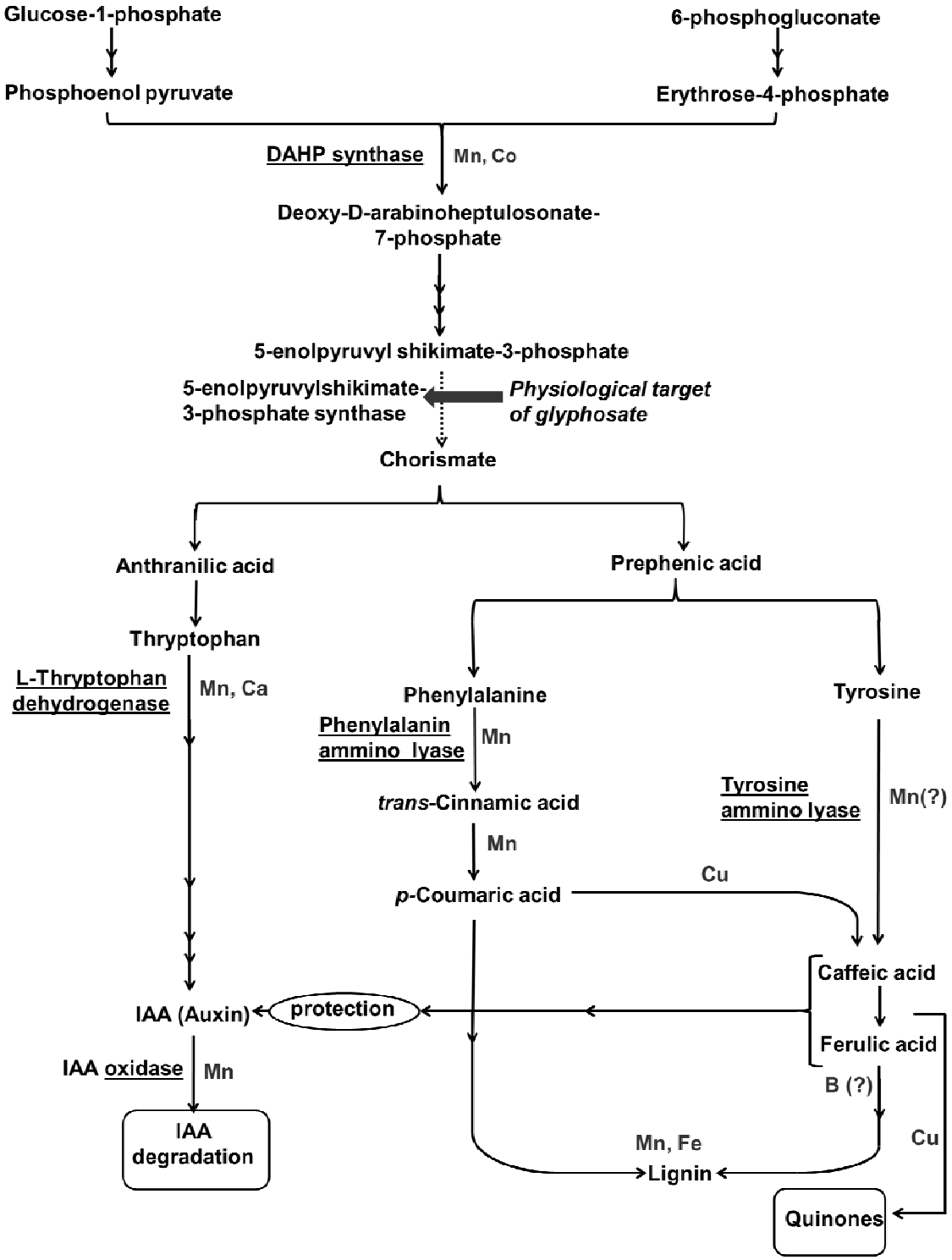

Fig. 2.3: Physiological steps in the shikimate pathway and subsequent pathways of secondary metabolism of phenolic compounds requiring specific (metal) cofactors

These steps may be negatively affected by direct or indirect interactions between glyphosate and plant availability of nutrients. Inhibition of the 5-enolpyruvyl shikimate-3-phosphate synthase (EPSPS) as physiological target of glyphosate, as well as cofactors of physiological steps are indicated.

Beside this, in several physiological steps of the shikimate pathway as well as in the secondary metabolism of phenolic compounds downstream of the aromatic amino acids tryptophane, tyrosine, and phenylalanine, nutrients such as Mn, Ca or Cu are required as cofactors (Fig. 2.3). In particular Mn is needed as cofactor of the Deoxy-D-arabinoheptulosonate-7-phosphate synthase (DAHPS) and the phenylalanine ammonialyase (PAL) both representing physiological bottlenecks in the shikimate – and secondary phenolic compound pathways (Fig. 2.3).
Therefore, impaired acquisition of (micro) nutrients caused either by direct interactions between glyphosate and specific nutrients or as secondary effect of phytotoxicity of glyphosate, potentially increases and prolongates the mode of action of glyphosate.

Even so there is a general agreement that the inhibition of the shikimic acid pathway by glyphosate is the primary cause for phytotoxicity, it is still unknown whether this effect on aromatic amino acid synthesis is the sole mechanism of action of glyphosate. Due to rapid absorption and translocation in the plant and lack of significant degradation/ metabolism, glyphosate may have multiple sites of action (Cranmer, 1988).

2.3 Glyphosate behaviour in soils

Compared to other pesticides, glyphosate possesses unique sorption characteristics in soil. Almost all other pesticides are moderately to weakly adsorbed in soils, mainly by soil organic matter (SOM), because most of these molecules are dominated by apolar groups, i.e. aliphatic and/or aromatic carbon, and often have only one functional group (Borggaard and Eberling, 2004; Schwarzenbach *et al*., 1993). In contrast, glyphosate, which is a small molecule with three polar functional groups (carboxyl, amino and phosphonate groups), is strongly adsorbed by soil minerals.

2.3.1 Glyphosate adsorption

Glyphosate has been suggested to adsorb to soils and minerals by ligand exchange through its phosphonic acid group in a way similar to the adsorption of phosphate (Piccolo *et al*., 1992, 1994; Piccolo and Celano, 1994; Nicholls and Evans, 1991; Hance, 1976; Hill, 2001; Gimsing and Borggaard, 2002a, 2002b, 2007; Gimsing *et al*., 2004, 2007; Borggaard and Gimsing, 2008).
Numerous studies showed that the main soil sorption sites of glyphosate in soils are found on surfaces of aluminium and iron (Fe) oxides, poorly ordered aluminium (Al) silicates (allophane/imogolite) and edges of layer silicates. Gerritse *et al*. (1996) found that the adsorption of glyphosate strongly increased with increasing Fe and Al contents in soils and decreased with increasing soil organic matter. In line with this, evaluation of glyphosate adsorption on three top soils with different characteristics in terms of cation exchange capacity, textural fraction and amorphous Fe and Al oxides revealed that the interaction of glyphosate with soils was mainly governed by amorphous Fe and Al oxides and organic matter (Morillo *et al*., 1999).

Beside this, glyphosate adsorption in soils also depends on pH. Increasing pH has been found to decrease the amount of glyphosate adsorbed by iron/aluminium oxides as well as in soils (Sheals *et al*., 2002; Barja *et al*., 2005). Again this in accordance to decreased adsorption of other phosphonates and phosphate with increasing pH.

In comparison to soils with high iron and aluminium content, soils dominated by permanent charged clay minerals such as illite, smectite and vermiculite adsorb less glyphosate (Vereecken, 2005; Gimsing *et al*., 2004, 2007; Gimsing and Borgaard, 2007). According to Borgaard and Gimsing (2008) discrepancy between the two mineral groups can be attributed to the number and distribution of sorption sites. However, release of cations from clay minerals and complexation of glyphosate by a cation-exchange reaction with solution protons has been proposed as an additional adsorption mechanism. In line with this, adsorption by the soils on three agricultural soils appeared to be related to the clay content and the cation-exchange capacity (CEC) of the soils (Glass *et al*., 1987). Also results of Miles and Moye (1988) indicated in the case of cation-saturated clays that the main mechanism of glyphosate adsorption is caused by hydrogen-bonding and ion-exchange mechanisms. Glyphosate adsorption varied inversely with the pH of the clay suspension in this study (Miles and Moye, 1988).
In an evaluation of the adsorption of glyphosate in three soils with illitic, kaolinitic and smectic clay minerals, before and after removal of organic matter, glyphosate adsorption could be related to the presence of clay minerals (Dion *et al*., 2001). In this study the presence of orthophosphate led to an increased competition with glyphosate for the sorption sites, thereby decreasing the adsorption of glyphosate again indicating that also glyphosate adsorption to clay minerals is related to adsorption of phosphate.

Soil organic matter (SOM) seems to play a controversial and dual role in soil adsorption of glyphosate. On the one hand, Piccolo *et al*. (1996) reported very high glyphosate adsorption by four different purified humus samples. Adsorption was explained by multiple hydrogen bondings which can occur among the various acidic and oxygen-containing groups in both molecules and was described by a Freundlich isotherm. The extent of adsorption was found to vary considerably between the humic substances and seemed to depend on the macromolecular structure and dimension. On the other hand, other investigations have shown that soil sorption of glyphosate is not, or is sometimes negatively, correlated with SOM content (Vereecken, 2005; Gimsing *et al*., 2004, 2007; Gerritse *et al*., 1996; McConnel *et al*., 1989). Experimental studies on the adsorption of glyphosate on sandy soils of Western Australia indicate the possibility that soluble soil organic matter competes for adsorption sites and thus inhibits adsorption of glyphosate (Gerritse *et al*., 1996). According to Borgaard and Gimsing (2008) soil organic matter may have indirect effects on glyphosate adsorption in two opposite ways. On the one hand soil organic matter might reduce glyphosate sorption by blocking sorption sites, but on the other hand soil organic matter might increase glyphosate adsorption because poorly ordered Al and Fe oxides with high sorption capacity are favoured at higher soil organic matter content (Borgaard and Gimsing, 2008).

Experimental studies on the ad-/desorption of AMPA (Barja *et al*., 2005) as the phytotoxic main metabolite of glyphosate in soils (Reddy *et al*., 2004; Laitinen *et al*., 2007, 2008) indicate high similarity to the ad-/desorption characteristics of glyphosate described above.

2.3.2 Degradation of glyphosate in soils

According to various authors soils can exhibit great variability in their ability to degrade glyphosate (Mamy *et al*. 2005; Sørensen *et al*., 2006; Carlisle and Trevors, 1988). Furthermore, degradation of glyphosate in soil has been found to be inversely correlated with the glyphosate adsorption capacity of the soil, (Sørensen *et al*., 2006; Moshier and Penner, 1978) i.e. if glyphosate adsorption to the soil matrix is strong, degradation of glyphosate is low, possibly because bioavailablity is low for microorganisms responsible for glyphosate degradation.
In line with this, several investigations indicate that glyphosate degradation is correlated with the general microbial activity (Franz *et al*., 1997; Rueppel *et al*., 1977; von Wirén-Lehr *et al*., 1997) e.g. no degradation occurred in sterile soil, whereas degradation took place under non-sterile soil conditions. Von Wirén-Lehr *et al*. (1997) reported that the soil microbial biomass, as measured by substrate-induced heat output and total adenylate content, was correlated with glyphosate degradation. Franz *et al*. (1997) found the glyphosate degradation rate to be correlated with the respiration rate (Strange-Hansen *et al*., 2004; Sprankle *et al*., 1975c; Rueppel *et al*., 1977).

Principally, glyphosate degradation by soil microorganisms can occur through two pathways (Jacob *et al*., 1985, 1988; Kishore *et al*., 1987; Lerbs *et al*., 1990; Liu *et al*., 1991; Dick and Quinn, 1995). Accordingly, one pathway leads to the intermediate formation of sarcosine and glycine, and the other leads to the formation of AMPA (Fig. 2.4). First step in the AMPA pathway appears to be the cleavage of the C–N bond by the enzyme glyphosate oxidoreductase producing AMPA and glyoxylate (Franz *et al*., 1997; Jacob *et al.*, 1988; Liu *et al.*, 1991; Barry *et al*., 1998; Balthazor and Hallas, 1986). In further steps AMPA is cleaved to produce inorganic phosphate and methylamine, which is ultimately mineralised to CO_2 and NH_3 (Franz *et al*., 1997; Balthazor and Hallas, 1986; Pipke and Amrhein, 1988). Cleavage of AMPA is achieved by the enzyme C–P lyase. Because cleavage of the C–P bond is a critical step in the degradation of glyphosate and other phosphonates in soils the C–P lyase enzyme has been extensively studied. However, so far the mechanism of degradation of AMPA is not fully understood. Glyoxylate is further metabolised via the glyoxylate cycle (Schnürer *et al*., 2006; Kryosko and Lupicka, 1997; Yakovleva *et al*., 1998; Obojska *et al*., 1999) (Fig. 2.4).

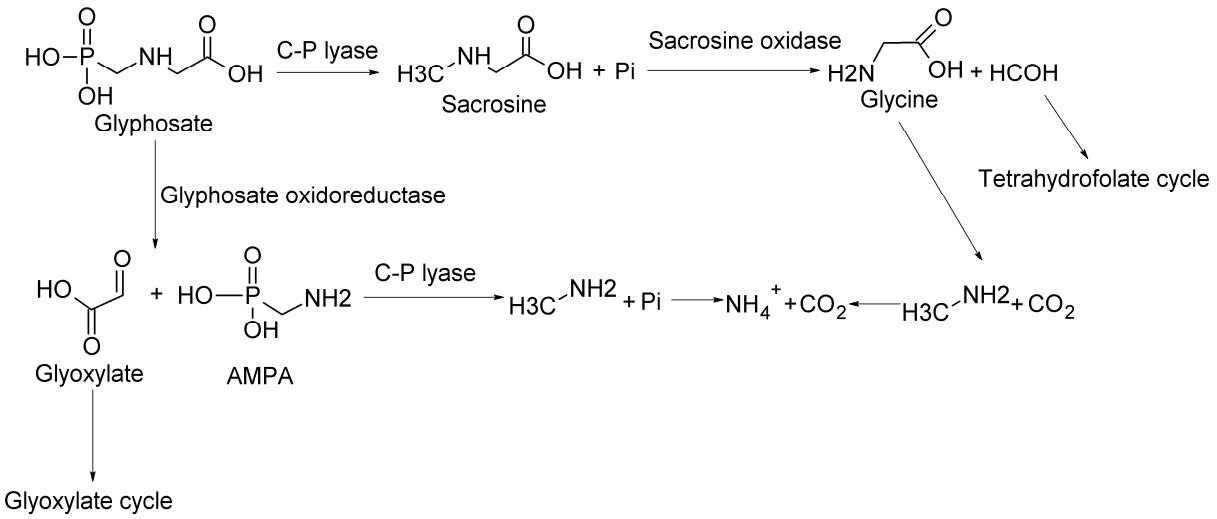

Fig. 2.4: Microbial degradation of glyphosate in soils via the sarcosine- or AMPA pathway

Beside microbial degradation, Barrett and McBride (2005) recently demonstrated abiotic degradation of glyphosate and AMPA by the manganese oxide birnesite.

In sum, due to the very low phytotoxic activity of glyphosate in soils caused on the one hand by rapid microbial degradation in soil solution (Giesy *et al*., 2000; Schnürer *et al*., 2006; Kryosko and Lupicka, 1997; Yakovleva *et al*., 1998; Obojska *et al*., 1999) and on the other hand by almost instantaneous inactivation by strong adsorption to the soil matrix (Piccolo *et al*., 1992, 1994; Piccolo and Celano, 1994; Nicholls and Evans, 1991; Hance, 1976; Hill, 2001; Borggaard and Gimsing, 2008) risks of glyphosate toxicity to non-target organisms in soils are generally considered as marginal. However, in contrast to this assessment of a relatively risk-free application of glyphosate there are increasing observations of negative side effects after glyphosate use under experimental and field conditions indicating risks for negative effects of glyphosate on plants and other organisms under certain conditions.

2.4 Risks of glyphosate application for plants

According to Fent (1998), effects of herbicides on organisms can be studied on several distinct levels of organisation ranging from the subcellular- and cellular levels to effects on single organisms, specific species and the community of organisms in a given ecosystem. This is indicating the multiplicity of possibilities to assess risks associated to applications of herbicides as xenobiotics and the unavoidable need to narrow the scope of research on particular aspects of effects of herbicides on organisms.
Thus, the focus of the present study was an assessment of risk associated to glyphosate application on crops in agro-ecosystems.

Accordingly, in this study risks of glyphosate application will be defined as (direct and/or indirect) negative effects on the physiology, development, growth and mineral nutrition status of crops as non-target plants that are not intended to be controlled, injured, killed or detrimentally affected in any way by glyphosate or its metabolites. During the study, the terms "non-target plants", "crops" and "crop plants" are used synonymously even so there is no single definition of the term "non-target plants". According to the Environmental Protection Agency of the United States, non-target plants are defined as "any plant species not considered to be pest at the location in which they are growing" and include desirable plants like crops and ornamentals within as well as outside the treatment area (USEPA, 1998). In contrast, according to the definition of the European and Mediterranean Plant Protection Organisation non-target plants are "plants outside the treatment area" (EPPO, 2003).

Thus, risks of glyphosate application for non-target plants are frequently separated according to these definitions into two groups: (a) risks for non-target plants outside the treatment area and (b) on-site risks for non-target plants inside the treatment area.
However, for the purpose of the present study, risks of glyphosate application for non-target plants are separated according to the potential pathway of exposure to phytotoxic glyphosate into: (a) risks associated to direct exposure of non-target plants during application of glyphosate and (b) risks associated to indirect exposure of non-target plants growing after an application of glyphosate. Potential risks associated to both pathways will be discussed in the following sections.

2.4.1 Potential risks for non-target plants associated to direct exposure to glyphosate

Similar to other pesticides, during a post-emergence application of glyphosate for weed control by commonly used application methods (hand sprayers, tractor-mounted sprayers, helicopters and aircrafts) unwanted events of glyphosate-drift cannot be completely avoided. Thus, drift of phytotoxic glyphosate during weed control and damage on crops outside the treatment area constitute a risk due to a direct (even so) unwanted exposure to glyphosate.

Extent of glyphosate drift contamination of non-target plants is strongly depending on factors including: formulation, susceptibility of the cultivars, growth stage, environmental conditions, nozzle size, height above the ground when the spray solution was released and the rate and timing of application (Atkinson, 1985). Off-target movement of herbicide during application can be somewhere between 1/10 and 1/100 of the applied rate (Wolf *et al*., 1992). However, according to the Mississippi Department of Agriculture, glyphosate application frequency has increased with the adoption of glyphosate-resistant crops (GR), and the application window has widened because of differences in planting dates among GR crops. Glyphosate drift complaints from ground or aerial applications are getting increasingly common in the Mississippi Delta but also other regions of the United States. In 2008, 56 cases of herbicide drift onto non-target crops were reported in Mississippi, an increase of 21 cases from 2007 (Reddy *et al*., 2010). Similarly, concerns of glyphosate-damage of non-resistant crop plants due to drift events are evident in publications and newsletters of extension services from Oregon, Louisiana, Wisconsin, Arkansas and other states of the United States indicate similar

increase of glyphosate drift events (Hensley *et al*., 2009; Griffin *et al*., 2003; Johnson *et al*., 2006; Boerboom, 2007; Skinkis, 2009; Scott, 2006; Thomas *et al*., 2005).

Effects of glyphosate drift on non-resistant crop plants have been also studied in considerable detail in scientific literature. Damage of crops after exposure to levels of phytotoxic glyphosate considered as realistic under drift conditions have been reported in model experiments and partly also under field conditions in wheat (*Triticum aestivum* L.), maize (*Zea maize* L.), rice (*Oryza sativa* L.), cotton (*Gossypium hirsutum* L.), soybean (*Glycine max* L.), tomato (*Lycopersicon esculentum* L.), pepper (*Capsicum annuum* L.), sunflower **(***Helianthus annuus* L.) and other crop plants (Roider *et al*., 2007; Brown *et al*., 2009; Cakmak *et al*., 2009; Ozturk *et al*., 2008; Eker *et al*., 2006; Miller *et al*., 2004; Henry *et al*., 2007; Lassiter *et al*., 2007; Norsworthy, 2004a; Reddy *et al*., 2010; Bellaloui *et al*., 2006, 2009; Gilreath *et al*., 2000a, 2000b).
In accordance to the primary mode of action of glyphosate in plants, damage of crops in these studies frequently included: stunted shoot growth, chlorosis and/or necrosis of young leaves, decline in density of crop plants, reduced biomass of shoots and roots, delayed ripening and significant yield loss. Additionally, accumulation of shikimate as specific indicator of glyphosate toxicity due to disruption of the shikimate pathway as primary mode of action of glyphosate was frequently detected and identified as reliable parameter for evaluation of glyphosate phytotoxicity and yield loss in model experiments and under field conditions (Roider *et al*., 2007; Brown *et al*., 2009; Miller *et al*., 2004; Henry *et al*., 2007; Lassiter *et al*., 2007; Norsworthy, 2004a; Reddy *et al*., 2010; Bellaloui *et al*. 2006, 2009; Gilreath *et al*., 2000a, 2000b). Damage of crop after simulated glyphosate drift generally increased in all crop species with increasing amounts of active ingredient applied again indicating glyphosate toxicity as primary cause of plant damage.
In contrast to these effects common to all crop species evaluated, results of these studies revealed also differential sensitivity of crops to simulated glyphosate drift depending on the plant species as well as the growth stage at exposure to phytotoxic glyphosate. Gramineous plant species like wheat, rice and maize responded to glyphosate application rates of 140 g ha^{-1} or 12.5% of the labelled use rate, realistic of what could be expected from herbicide drift (Wolf *et al*., 1992), with comparable yield reduction of 60-80% when applied in early growth stages and 30-50% when applied at late growth stages (Ellis *et al*., 2003; Roider *et al*., 2007). According to Ellis *et al*. (2003) based on yield reductions, rice and corn can be classified as equally sensitive to glyphosate. Height reduction and discoloration of foliage to both rice and corn associated with the lower glyphosate rates in some cases was minimal, but the negative effect on yield was significant (Ellis *et al*., 2003).
In contrast to this, particularly conventional soybean and in some extent also cotton appear to be more resistant to glyphosate toxicity after drift exposure. Al-Khatib and Peterson (1999) reported that glyphosate at 280 g ha^{-1} applied in juvenile growth stage (V3) caused transient injury to conventional soybean but did not reduce yields. Similarly, conventional soybean injury in vegetative growth stages caused by glyphosate application at rates up to 105g ha^{-1} was transient and did not affect yield (Ellis and Griffin, 2002). Transient glyphosate damage was also reported by Norsworthy (2004a) who additionally could show high vulnerability of soybean to glyphosate drift during (early) podfilling but not at later growth stages.

While these results very clearly indicate differential sensitivity of crops to glyphosate toxicity, the underlying causes are so far not studied in detail and poorly understood.
Beside effects on growth and yield of crops, physiological and metabolic disturbances induced by glyphosate drift were observed in conventional soybean (Reddy *et al*., 2003; Zablotowicz *et al*., 2007), maize (Buehring *et al*., 2007), rice (Koger *et al*., 2005), and sunflower (Eker *et al*., 2006; Ozturk *et al*., 2008). Recently a study under hydroponic conditions with glyphosate application rates between 1.25-6.0% of the recommended concentration, revealed that glyphosate is antagonistic to the uptake, translocation, and tissue concentration of Fe, Mn and Zn in sunflower plants (Eker *et al*., 2006). As mentioned earlier, based on the chemical properties glyphosate acts as potent chelator of di- and trivalent cations potentially causing negative effects on availability of mineral nutrients for crop plants. According to Eker *et al.* (2006) formation of poorly soluble glyphosate-metal complexes is possibly the main factor responsible for the antagonism between glyphosate and cationic micronutrients.

In line with this, Ozturk *et al*. (2008) showed that glyphosate application at 1, 3 and 6% of the recommended concentration reduced ferric reductase activity in sunflower roots. Under Fe deficiency 1.89mM glyphosate resulted in about 50% inhibition of ferric reductase activity within 6 h and complete inhibition within 24 h after the treatment (Ozturk *et al*., 2008). The observation that glyphosate decreased ferric reductase activity was attributed to impairment of soil Fe acquisition, resulting from the Fe-Gly complexes formed in soil (Ozturk *et al*., 2008; Sprankle *et al*., 1975c). Similar effects on ferric reductase activity were also observed by Bellaloui *et al*. (2009) in soybean.

Impaired activity after exposure to glyphosate has been also reported for nitrate reductase in soybean and maize plants (Bellaloui *et al*., 2006; Reddy *et al*., 2010). Due to the well-known rapid growth inhibition and yield loss caused by N-deficiency of plants, glyphosate induced impaired nitrate reductase activity highlight the potential for severe yield loss in crops exposed to glyphosate drift. Beside this effect on the nitrate reductase, as shown by de María *et al*. (2005) and Bellaloui *et al.* (2006) glyphosate might cause impaired N-supply in leguminous plants by inhibition of the enzyme nitrogenase in N_2-fixing symbionts. It has been shown that *Bradyrhizobium japonicum* possesses a glyphosate-sensitive EPSPS enzyme, thus and exposure to glyphosate may interfere with N_2 fixation due to direct toxicity.

Very recently Cakmak *et al.* (2009) showed that glyphosate rates between 0.06 and 1.2% of the recommended concentration for weed control affected not only micronutrients but also induced declined concentrations of Ca and Mg in young leaves and seeds of conventional soybean plants. According to Cakmak *et al.* (2009) due to their low transpiration capacity, young leaves, shoot tips and seeds are highly sensitive to small changes in Ca concentrations. Accumulation of glyphosate in such sink organs with low Ca concentration would induce physiological Ca deficiency by complexing Ca.
Interestingly, Gilreath *et al*. (2000a) observed in pepper that glyphosate induced incidence of blossom-end rot indicating a negative effect on Ca-status of plants. According to Cakmak *et al.* (2009) in contrast to Ca and Mg, the concentrations of phosphorus (P), potassium (K) and sulfur (S) were not affected by glyphosate indicating that the decline in tissue concentrations

of Mg and Ca by glyphosate is a specific phenomenon and cannot be generalised to all macronutrients.

2.4.2 Risks for glyphosate-resistant crops associated to direct exposure to glyphosate

Due to its non-selective mode of action glyphosate-based herbicides had to be applied before emergence of crop plants in the past. However, with the development of glyphosate-tolerant crops, application of glyphosate for weed control during the vegetation period of crops became possible.

In the United States, soybean was introduced in 1996 as the first glyphosate-resistant (GR) crops in agricultural plant production (Duke, 2005). Plants were developed by insertion of the cp4 epsps coding sequence derived from the common soil bacterium *Agrobacterium* sp. strain CP4 (Franz *et al*., 1997). This gene (*CP4 EPSPS*) directs the production of the 5-enolpyruvyl shikimate-3-phosphate synthase (EPSPS) that is less sensitive to inhibition by glyphosate compared to the endogenous EPSPS of non-transgenic soybean plants. Currently, glyphosate-resistant crops introduced in agricultural practice include soybean, maize, cotton, canola, sugar beet and alfalfa and wheat in the development process.
A new generation of glyphosate-resistant plants based on a new technique of *Agrobacterium*-mediated gene delivery to meristems of crop plants, where cells were directly induced to produce shoots and give rise to transgenic plants has been developed recently (Martinell *et al*., 2002). This technique, which has been so far only used in soybean, allowed direct transformation of the gene cassette into elite soybean germplasm such as Asgrow soybean variety A3244 (Paschal, 1997). Using elite germplasm as the base genetics, the superior agronomic characteristic of A3244 can be introgressed to other soybean varieties through crosses with the MON 89788 insertion event containing the *CP4 EPSPS* cassette (Taylor *et al.*, 2007). In 2008, these second generation of glyphosate-resistant (RR2) cultivars were commercially available for farmers and promoted higher yields relative to the previous RR cultivars. Arguably, the process of introduction of new GR-crops (most probably rice) and new RR-techniques will increase in the near future. In fact several companies continue to work on discovering such new GR mechanisms. For example, a recent patent application describes new bacterial enzymes that metabolically inactivate glyphosate. The enzymes referred as GDC-1 and GDC-2 have homology to known decarboxylases and use a pyrophosphate cofactor (Hammer *et al*., 2007).

Efficacy of glyphosate on a wide spectrum of weeds, simplicity and flexibility in application and lower herbicide cost, and the possibility to rotate GR-crops have encouraged farmers in the United States, Brazil, Argentina and other countries to plant more areas with glyphosate-resistant (GR) crops each year (Gianessi, 2008; Reddy *et al*., 2000). In 2007 the global area planted to all genetically modified crops exceeded 113 million ha on which GR soybean occupied 58.6 million ha (52% of the global area planted to biotech crops), followed by GR maize (35.2 million ha at 31% of global area), and GR cotton (13.4 million ha at 12% of global area) (James, 2007). Indeed, based on average application rate and frequency and area treated, the amount of glyphosate applied to GR soybean in the United States increased by 52 million kg from 1997 to 2004 (Benbrook, 2004). In 2009, 91% of the soybean, 71% of the

cotton, and 68% of the corn produced in the US was planted with GR-plants (U.S. Department of Agriculture, 2009).

However, in parallel to the increase in cultivation of GR-crops there is also increase in observations of damage of glyphosate-resistant crops after application of glyphosate by farmers, extension service members and scientists. Particularly in GR-soybean, two distinct symptoms of damage after direct exposure to glyphosate during application for weed control have been observed. On the one hand, visual symptoms noticed in the field after glyphosate application to GR soybean include "yellow flashing" or yellowing of the young trifoliar leafs resembling micronutrient (Fe, Mn and Zn) deficiency. On the other hand emergence of smaller, darker and morphological deformed (stretching, cupping, crinkling, rolling) trifoliar leafs and shoot apices resembling disturbance of plant hormonal status were observed in GR-soybean after glyphosate application. Similarly to damage of conventional soybean due to glyphosate drift, chlorosis on young leafs as well as leaf deformations appear to be transient in nature (Norsworthy, 2004b).
Field observations in Brazil and the United States indicate that applications of glyphosate may directly or indirectly induce Fe, Zn and Mn deficiencies in GR as well as non-GR plants (Huber, 2006; Jolley and Hansen, 2004; Huber and McCay-Buys, 1993). Under field conditions Gordon (2007) could show that GR soybean compared to their near-isogenic non-treated non-GR soybean required a higher Mn fertilisation to achieve maximum yields potentially indicating lower Mn use efficiency of GR-soybean due to insertion of genetically modified EPSP-synthase. In a recent study, Zobiole *et al.* (2010a, 2010b) found that glyphosate application, decreased macro- and micronutrients and shoot and root biomass in GR soybean as compared to their near-isogenic non-treated non-GR soybean or non-treated GR soybean. They also found that glyphosate application decreased stomatal conductance, chlorophyll concentration, and photosynthesis rate (Zobiole *et al*., 2010a, 2010c). In line with this, several studies reported growth depressions, chlorosis, leaf necrosis and micronutrient deficiencies after glyphosate applications with the recommended dosage (Duke *et al*., 2003; Jolley and Hansen, 2004; Reddy *et al*., 2004). Based on the well-documented ability of glyphosate to form stable complexes with metal cations such as Al, Fe, Zn, Mn and Ca (Sprankle *et al*., 1975c) hypothetically impaired micronutrient status of GR-crops might be related to the formation of metal-glyphosate complexes unavailable for plants.
However, the underlying mechanisms of impaired micronutritional status of GR-plants remain not well understood.

Emergence of morphological deformed trifoliar leafs and shoot apices are according to newsletters of extension services in United States frequently observed in GR-soybean after glyphosate application (Taylor, 2002; Bohner, 2002; Loux, 2007; Swoboda, 2004; Hager, 2005). Whether this damage is caused by glyphosate is unknown. Damage symptoms have been attributed to drift of the growth regulator type of herbicides like Clarity, Banvel or 2,4-D (Taylor, 2002; Bohner, 2002; Loux, 2007; Swoboda, 2004; Hager, 2005). In cases were drift contamination could be ruled out damage was attributed to the ability of glyphosate formulations to solubilise residues of previously used herbicides, if tank cleanout has been inadequate (Taylor, 2002). However, based on its mode of action glyphosate might interfere

with auxin-, cytokinin- and/or gibberellin-status of plants. Thus a direct effect glyphosate cannot be ruled out.

2.5 Potential risks for non-target plants associated to indirect exposure to glyphosate

In comparison to risks for conventional and genetically modified GR-crops associated to direct exposure to glyphosate, risk factors for non-target plants associated to indirect exposure to glyphosate have not been studied systematically so far.

In contrast to the obvious pathways of drift to/application on plants in case of a direct exposure, hypothetical pathways of indirect exposure to glyphosate are associated to transfer of phytotoxic glyphosate to non-target plants via the roots or rhizosphere of plants.

2.5.1 Risks for non-target plants associated to pre-sowing glyphosate application on weed plants

Glyphosate is often described as exhibiting little or no activity in soil due to nearly instantaneous adsorption on soil inorganic and organic particles (Piccolo *et al*., 1992, 1994; Piccolo and Celano, 1994; Gimsing *et al*., 2004; Zhou *et al*., 2004; Duke and Powles, 2008) and additionally because glyphosate in the soil solution is prone to rapid microbial and/or chemical degradation (Giesy *et al*., 2000; Gimsing *et al*., 2004). However, under field conditions considerable amounts of glyphosate can be intercepted and taken up by weed plants growing on the field before glyphosate is reaching the soil surface. Thus, an additional potential pool of glyphosate in soils is represented by the plant residues including roots of glyphosate-treated weeds and the fate of bound glyphosate in plant residues has not been widely considered in the past.
As systemic herbicide, glyphosate is first absorbed by foliage and then translocated throughout the plant. Plant organs with high metabolic activity and growth rates such as nodules, root tips and shoot apex represent a high sink activity for glyphosate. In many plant species, glyphosate is not readily metabolised and considerable amounts can accumulate particularly in young tissues (Reddy *et al*., 2004). Experimental evidences are available showing substantial accumulation of foliar-applied glyphosate in such sink tissues (Schulz *et al*., 1990; Hetherington *et al*., 1999; Feng *et al*., 2003). According to Feng *et al*. (2003) up to 80% of the glyphosate absorbed after foliar applications is translocated into shoot apex and root tips. Even at low foliar application rates, the sink tissues accumulate glyphosate at very high concentrations. A release of glyphosate by roots was first shown in a study of Coupland and Caseley (1979) who reported that intact quackgrass roots exuded significant amounts of ^{14}C-glyphosate into surrounding solution.

Neumann *et al*. (2006) investigated the potential transfer of foliar applied glyphosate, released from roots of weed plants to non-treated indicator plants (sunflower seedlings, *Helianthus annuus* L.) simultaneously cultivated in hydroponics and in soil culture systems. Results of this study clearly demonstrate a release of glyphosate via the roots of target plants, which can be subsequently taken up by simultaneous growing non-treated plants, exerting inhibitory effects on the shikimate pathway, uptake of micronutrients (Mn) and plant growth. According

to Neumann *et al*. (2006) the release of glyphosate may occur from damaged roots of dying target plants but can be also released as exudates from undamaged roots of weeds. In line with this, also Pline *et al*. (2002a, 2002b), Guldner *et al*. (2005) and Tesfamariam (2009) have shown that glyphosate can be exuded from roots to soil, and cause growth inhibition to adjacent plants and seedlings. Results of Pline *et al.* (2002a, 2002b) indicate that transfer of foliar applied glyphosate, released from roots of weed plants even affects GR cotton seedlings, potentially due to tissue-specific lower expression of glyphosate-tolerant EPSPS-synthesis in root tissue.

Interestingly, transfer of systemic herbicides through the rhizosphere has also been observed in case of mesotrione and imazapyr (Boydston *et al*., 2008; Silva *et al*., 2004; Kanampiu *et al.*, 2002). Similarly to glyphosate, the selective mesotrione is considered to be rapidly degraded in soils (Mitchell *et al*., 2001). Beside this, the major metabolites of mesotrione: 4-methylsulfonyl-2-nitrobenzoic acid 2 (MNBA) and 2-amino-4-methylsulfonyl benzoic acid 3 (AMBA) are not herbicidal (Armel *et al*., 2005). Under field conditions, selective application of mesotrione on single plants for control of volunteer potato in crops resulted in mesotrione damage of plants growing adjacent to treated volunteer potatoes. Apparently, mesotrione had not been rapidly degraded in the soil and moved from the treated plants to the adjacent non-treated plants (Boydston *et al*., 2008).

Beside damage of crop plants due to (immediate) rhizosphere transfer of accumulated glyphosate in roots of treated weed plants to roots of simultaneously growing crop plants, a few studies are indicating that root residues of glyphosate-treated weeds represent a potential pool of glyphosate in soils which is not readily degraded and thus potentially prolongs risks for non-target plants associated to phytotoxicity of glyphosate in the rhizosphere (Neumann *et al*., 2006; Tesfamariam, 2009).

In a field study of Laitinen and Rämö (2005) 40 days after the application glyphosate, root concentrations of glyphosate treated weeds reached up to 2.7 mg kg^{-1} of dry weight, while glyphosate concentrations detected in 0–5 cm and 5–35 cm soil layers adjacent to the roots were 0.17 and 0.07 mg kg^{-1} of dry weight. These results indicate strong effects of glyphosate plant application on local glyphosate concentrations in soils as well as delayed degradation and preservation of phytotoxic glyphosate in the root tissue of glyphosate treated weed plants. According to Doublet *et al.* (2009) the fate of pesticides in plant residues in soil is under-investigated and basically unknown. Studies with soybean and wheat suggested unspecific and non-covalent binding of glyphosate to starch and cell wall components (Komossa *et al*., 1992). Release and degradation of ^{14}C-labelled glyphosate in agricultural soils correlated with the soil-microbial activity but only after direct soil application. No such correlation was observed after soil incorporation of lyophilised soybean tissue cultures, contaminated with glyphosate. These findings suggest different mechanisms for degradation of glyphosate adsorbed to the soil matrix and bound in plant residues in the soils, respectively. In line with this Doublet *et al.* (2009) reported that absorption of glyphosate and sulcotrione in plants delays their subsequent soil-degradation, and particularly in case of glyphosate persistence in soil could increase two to six times.

Accordingly, results of model experiments (Neumann *et al*., 2006; Tesfamariam, 2009) and preliminary field trials in a no-tillage winter wheat cropping system (Römheld *et al*., 2008; Tesfamariam, 2009) gave indications of increased and prolonged phytotoxicity of glyphosate in case of plant application of glyphosate associated with impaired growth and nutritional status of crops (sunflower and wheat).
Interestingly, also Rodrigues *et al*. (1982) discovered that glyphosate application at levels of 2-12 L ha^{-1} on a low number of weed plants (5 plants pot^{-1}) had no effect or even a growth stimulating effect on simultaneously cultivated maize and soybean plants. However, when the number of weed plants was increased to 30 plants pot^{-1}, independent of the used soil type, significant damage was observed for both crops. Furthermore, the results of Rodrigues *et al*. (1982) suggest that exuded glyphosate was not adsorbed on soil constituents or biodegraded sufficiently rapid to exert growth effects on adjacent plants to occur. However, glyphosate damage of adjacent plants was most likely influenced by the density of treated weed plants.

According to Doublet *et al*. (2009), the modifications of herbicide degradation in soil due to interception by plants should be considered for environmental risks assessment. However, no attempt to study risk factors associated to a plant application of glyphosate has been conducted so far.

Beside of risks for non-target plants due to transfer of phytotoxic glyphosate from roots of glyphosate-treated weed plants, several studies indicate glyphosate-induced promotion of soil borne pathogens as cause for damage of non-target plants after pre-sowing glyphosate application.

Several studies reported increase of infection of wheat with fungal pathogens (*Fusarium*, *Phytium*, *Rhizoctonia*), and also “green bridge” effects, when total herbicides were used to control weeds shortly before seeding of cereals (Smiley *et al.*, 1992; Descalzo *et al*., 1998; Powell and Swanton, 2008). Particularly in cereals, the “green bridge” provided by volunteer cereals growing in summer is important for maintaining the life cycle of various pathogens (viruses, bacteria, fungi) and insect pests (Jiang *et al*., 2005). However, Bithell *et al*. (2009) observed that early or late timing of weed control with glyphosate before sowing of winter wheat caused similar infection rates of wheat plants by take all (*Gaeumannomyces graminis* var. *tritici*). Similarly, Fernandez *et al.* (2003, 2005, 2007, 2008, 2009) could show a long-term effect of glyphosate application on infection of cereal plants by soil-borne fungal pathogens, such as *Fusarium.*
Additionally, various studies reported increased susceptibility of glyphosate-resistant (GR) and non-resistant plants to a wide range of fungal pathogens (*Fusarium*, *Phytium*, *Rhizoctonia*) due to a direct effect of glyphosate by promotion of pathogens, or inhibition of plants own defence mechanisms (e.g. phytoalexins) caused by glyphosate toxicity on plants and/or antagonistic/ beneficial soil microorganisms or an impaired plant-nutritional status (Johal and Huber, 2009; Kremer *et al*., 2005; Kremer and Means, 2009; Yamada *et al*., 2009; Johal and Rahe, 1984, 1988, 1990; Neumann *et al*., 2006).

In contrast to reports indicating increased pathogen pressure associated with glyphosate application, recent studies on GR soybean and wheat have shown particularly in case of

infections with rust fungi, glyphosate exhibits anti-fungal activity , thereby providing disease control benefits (Anderson and Kolmer, 2005; Feng *et al*., 2005, 2008). Additionally, it has been reported that soil-applied glyphosate can protect plants also against other soil-borne fungi (Berner *et al*., 1992) and that glyphosate application in GR wheat may potentially cause yield increase on soils infested with *Rhizoctonia* or *Gaeumannomyces graminicola* (Baley *et al*., 2005).

2.5.2 Risks for non-target plants associated to re-mobilisation of phytotoxic glyphosate in the rhizosphere

As described earlier, half-life times of glyphosate in soils are reported to be variable ranging from 1 day up to several months depending on soil and environmental conditions. However, due to very low phytotoxic activity of glyphosate in soils caused by rapid inactivation by adsorption to the soil matrix (Gimsing and Borggaard, 2002a, 2002b; Piccolo *et al*., 1992; Morillo *et al*., 1999, 2002; Borggaard and Gimsing, 2008) and microbial degradation of glyphosate in soil solution (Giesy *et al*., 2000; Schnürer *et al*., 2006; Kryosko and Lupicka, 1997; Yakovleva *et al*., 1998; Obojska *et al*., 1999), risks of glyphosate toxicity to non-target organisms in soils are generally considered as marginal.

However, since glyphosate adsorption in soils resembles adsorption of inorganic phosphorus (P) (Borggaard and Gimsing, 2008), re-mobilisation from the soil matrix may cause phytotoxic effects on non-target plants. Re-mobilisation of glyphosate from the soil matrix might be caused by (a) plant own strategies for mobilisation of P and Fe (e.g. under limited P- and Fe supply), (b) by P-mobilising soil microorganisms or mycorrhizal fungi or (c) by application of fertilisers inducing pH changes in the rhizosphere (e.g. nitrate (NO_3^-) or ammonium (NH_4^+) or desorption of glyphosate (e.g. P fertilisers, chelators).
Very few if any studies have been conducted to assess risks for plants associated to a re-mobilisation of glyphosate from soils. Cornish (1992) reported detrimental effects of glyphosate pre-transplanting treatments on tomato in field and pot experiments on sandy loam soils, which were still detectable after waiting times of 3–4 weeks. However, this study used young tomato plants and no seeds, thereby increasing the risk of plant damage by glyphosate application. In his PhD research at the Institute for Plant Nutrition, Tesfamariam (2009) tried to assess the potential for re-mobilisation of glyphosate by application of synthetic P-mobilising root exudates (citric acid, sodium- and potassium-citrate) simulating the response of P efficient plant species to P deficiency and re-mobilisation of glyphosate by pH-changes in the rhizosphere induced by application of nitrate (NO_3^-) or ammonium (NH_4^+). In this studies repeatedly growth inhibition of sunflowers (e.g. root growth) was however not associated with significant accumulation of shikimate as indicator for glyphosate toxicity.

Adsorption and desorption characteristics of glyphosate in soils has been studied extensively to evaluate the risk of glyphosate leaching and groundwater pollution (Piccolo *et al*., 1992, 1994; Piccolo and Celano, 1994; Nicholls and Evans, 1991; Hance, 1976; Hill, 2001; Gimsing and Borggaard, 2002a, 2002b, 2007; Gimsing *et al*., 2004, 2007; Borggaard and Gimsing, 2008). Numerous of these studies showed that, depending on the soil conditions, phosphate

concentration is the most important factor in determining the amount of glyphosate adsorbed and that phosphate in some cases is able to completely desorb glyphosate fixed to the soil (Gimsing and Borggaard, 2002a; Nicholls and Evans, 1991). Thus, phosphate most likely plays an important role in determining the potential bioavailability of glyphosate (Cornish, 1992; Laitinen *et al*., 2008).

Surprisingly, the potential consequences of these interactions between glyphosate and phosphate for plants e.g. a remobilisation of phytotoxic glyphosate in soil by application of inorganic P fertilisers have not been systematically evaluated so far.

2.6 Aims of the study

As shown in the previous sections, numerous potential risks for crop plants due to direct or indirect exposure to glyphosate might exist in agro-ecosystems.
Mechanisms, pathways and consequences of glyphosate air drift for plants have been studied in considerable detail. By contrast, potential risk factors for crop plants associated with indirect exposure to glyphosate are presently poorly understood and not systematically evaluated so far. Also potential negative effects of glyphosate application on glyphosate-resistant plants are not fully understood. This is surprising, since glyphosate treatments for weed control before sowing of conventional crops or during vegetation period of GR crops are arguably the most important applications of glyphosate in agricultural and horticultural production systems.

Therefore, the aim of present study was a systematic evaluation of potential risks for crop plants by indirect exposure to phytotoxic glyphosate via the rhizosphere and risks for GR crops associated with application on the plants.
In the first part, a series of hydroponic-, soil (model experiments) and field studies were conducted to identify factors influencing the risk for crop plants associated to glyphosate phytotoxicity in the rhizosphere after application on weed plants.
In the second part, in a series of model experiments under greenhouse conditions, the potential for glyphosate remobilisation was evaluated in a bioassay with crop plants cultivated on contrasting soils.
Finally, in the third part of the study, a series of model experiments under hydroponic and soil conditions was conducted to identify factors influencing the risk for GR crop plants associated to application of glyphosate.

3 Comparison of glyphosate application methods for investigations of the pathway of glyphosate transfer in the rhizosphere

[Weed Science (2010) submitted as Research Note]

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Own contribution: set-up of experiments, plant cultivation, harvest and sample preparation (support of students in 1 of 2 experiments) ,analysis of shikimate by HPLC, manuscript preparation

3.1 Abstract

Since fate and release of potentially phytotoxic glyphosate stored in root residues of weed plants and damage of subsequently grown crops is most likely influenced by a number of factors hardly controllable under field conditions, for a better understanding of the underlying mechanisms model experiments under controlled conditions are needed. However, simulating pre-crop glyphosate application in small scale experiments is a challenging task.

Therefore, objective of this study was to compare suitable methods for pre-crop application of glyphosate on weeds in greenhouse experiments. For this purpose effects of different application methods by hand-held sprayer were compared to application with a track sprayer simulating application under field conditions with respect to their effect on treated weed- and subsequently crop plants.

Dilution of spray solution to achieve appropriate volumes for hand-sprayer application of correct amounts of glyphosate per surface area of pots as recommended by Monsanto for small scale experiments proved unsuitable due to reduced herbicidal efficiency. Because of the self-limited translocation of glyphosate *in planta*, glyphosate application with a hand-held sprayer based on the estimated leaf surface area of weed plants induced in comparison to a track sprayer highly similar damage of crop plants and accumulation of shikimate as physiological indicator of glyphosate toxicity.

Therefore, application of glyphosate with a hand-held sprayer based on the estimated leaf surface area of weed plants is potentially a suitable alternative glyphosate application method for experiments investigating risk factors for crop plants associated to glyphosate stored in roots and root residues of treated weed plants when track spraying devices are not available.

Nomenclature: Glyphosate, N-(phosphonomethyl)glycine; wheat, *Triticum aestivum* (L.); rye grass, *Lolium perenne* (L.); sugar beet, *Beta vulgaris* (L.); soybean, *Glycine max* (L.)

Keywords: Glyphosate; application methods; small scale experiments; weed plants; self-limited translocation; rhizosphere transfer

3.2 Introduction

Risks of glyphosate (N-(phosphonomethyl)glycine) toxicity to non-target organisms in soils are generally considered as marginal, since glyphosate in the soil solution is prone to rapid microbial degradation or almost instantaneous inactivation by sorption to the soil matrix (Giesy *et al.*, 2000).

However, an additional potential pool of phloem-mobile herbicides (e.g. glyphosate) in soils, which has not been widely considered so far, might be present in plant residues of treated weeds. Recently, Doublet *et al.* (2009) reported that absorption of herbicides in plant delays their subsequent soil-degradation, and particularly in case of glyphosate persistence in soil could be increased two to six times and should be considered for environmental risk assessments. In line with this, field trials in no- or reduced tillage systems revealed strong damage of winter wheat (*Triticum aestivum* L.) in case of short waiting time between glyphosate application to different weed plant species and sowing of plants (Römheld *et al.*, 2008). Accordingly, glyphosate residues in target weeds potentially act as a transient storage pool of active glyphosate in soils associated with a risk of contact contamination of crops.

To achieve results relevant under real farming conditions, assessments of risks associated to glyphosate stored in tissue of glyphosate treated weed plants need to be done under field conditions. However, fate and release of potentially phytotoxic glyphosate stored in weed plants and damage of subsequently grown crops is most likely influenced by factors such as weed densities, soil moisture levels and temperature at time of application on weed plants as well as at sowing of crop plants which are hardly controllable under field conditions. Therefore, for a better understanding of the underlying mechanisms model experiments under controlled conditions are needed.

However, translation of field application rates of glyphosate to small scale model experiments and correct implementation of glyphosate application are challenging tasks. Thus, the present study was initiated to compare potential methods for glyphosate application in small scale experiments investigating factors influencing the transfer of glyphosate from weeds to crops via the rhizosphere.

3.3 Material and methods

In greenhouse experiments under soil conditions glyphosate was applied in its commercial Roundup Ultramax® formulation in three different concentrations (28.4 mM, 56.8 mM and 113.6 mM) with a track-spraying device (hereafter TSA) or a hand-held sprayer to "self-sown wheat" pre-cultured for 10 days at a density of 4 g seed pot^{-1} as weed plants. Application of glyphosate by hand-held sprayer was performed in two ways: application based on the surface area of weed plants (Tesfamariam, 2009) (with approx. 1000 cm^2 pot^{-1}/ application of 1.86 ml pot^{-1}) (hereafter LSA) or as recommended by Monsanto based on the pot surface area (hereafter DPA). As already indicated in the recommendation of Monsanto for glyphosate application in small scale experiments, in case of application based on pot surface area spray solution had to be 1:10 diluted with double deionised water (final concentrations: 2.84 mM, 5.68 mM and 11.36 mM) to achieve manageable volumes of solution (Monsanto person. communication). To avoid the possibility for above ground contamination of indicator plants with glyphosate, shoots of treated weed plants were cut close to soil level and removed from the pots before indicator plants emerged (4-5 days after sowing).
In control treatments without glyphosate application, shoots of weed plants were removed manually by cutting at the soil level.

As bio-indicator of potential glyphosate damage, 10 seeds of winter wheat (cv. Isengrain-B) were sown subsequently into the same pots. In all experiments, visual plant damage, biomass production and intracellular shikimate accumulation in roots and shoots as physiological indicator of glyphosate toxicity were recorded.

3.4 Results and Discussion

Glyphosate application on weed plants 2 days before seeding of winter wheat as non-target plants conducted by TSA caused in case of application of 4-, 8- or 16 L glyphosate ha^{-1} effective desiccation of pre-cultured weed plants (Fig. 3.1, Fig. 3.2). Similarly, glyphosate application conducted by LSA caused in all glyphosate concentrations applied satisfying desiccation of weed plants. In contrast to this, application of 4 L glyphosate ha^{-1} conducted by DPA completely failed to desiccate weed plants within the vegetation period of 14 days (Fig. 3.1).
Some regrowth of weed plants was also observed in case of 8 L glyphosate ha^{-1} as DPA application (data not shown). However, during the ongoing vegetation period the treated weed plants died off as well at both higher application rates. However, 8- or 16 L glyphosate ha^{-1} do not represent realistic or recommended application rates at field conditions.
Herbicide uptake by leaves is according to Leaper and Holloway (2000) dependent on surface activity, hygroscopicity and intrinsic uptake behaviour and strongly influenced by the physicochemical properties of the adjuvant. Addition of surfactants/adjuvants to glyphosate spray solution most likely significantly improves spray retention, affects spreading of spray droplets, increases the rate of penetration or enhances its biological activity by suppression of inactivation of glyphosate by crystallisation on the leaf surface (Leaper and Holloway, 2000). Weed control efficiency of glyphosate has been reported to be reduced due to dilution of surfactants caused by decreased penetration of barriers of glyphosate uptake, decreased

surface activity and potentially increased run-off (Holloway, 1994; Briggs and Bromilow, 1994; Leaper and Holloway, 2000).
Hence, most likely limited desiccation of weed plants in case of DPA application of 4L glyphosate ha^{-1} as recommended by Monsanto for small scale experiments is explainable by dilution of surfactants causing limited uptake of glyphosate by leaves of weed plants. Therefore, results indicated that the DPA application method based on the pot surface area in diluted spray solution as recommended by Monsanto for small scale experiments is not suitable for experiments.

Dilution of herbicide spray solution is also frequently used to simulate events of glyphosate drift in model experiments and field trials. Therefore, decrease in efficiency of herbicide uptake by plants caused by dilution of adjuvants in glyphosate spray solution might be also of relevance in these studies.

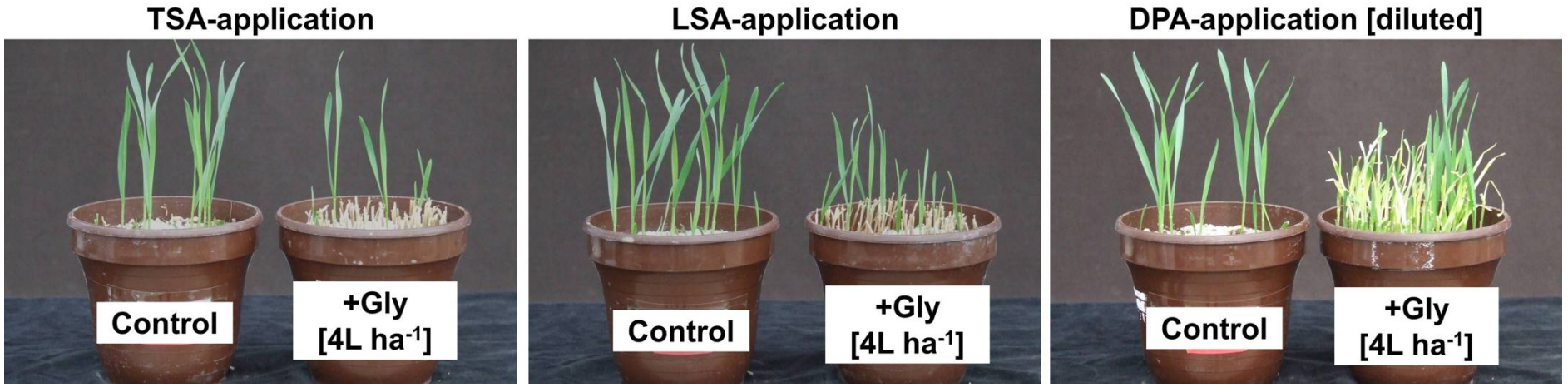

Fig. 3.1: Visual effects of different methods for glyphosate application on winter wheat

Visual effects were compared between different methods for application of Roundup Ultramax® (glyphosate) on "self-sown" wheat as pre-cultured weed plants and winter wheat (*Triticum aestivum* L.) sown as subsequently crop plants at a waiting time of two days after application. Glyphosate application with a track sprayer simulating application technique used under field conditions (TSA) (left) or application with a hand-held sprayer based on the estimated leaf surface area of weed plants (LSA) (middle) showed complete desiccation of weed plants and damage of subsequently grown indicator plants in case of glyphosate application levels of 4 L glyphosate ha^{-1}. Glyphosate application with a hand-held sprayer based on the pot surface area with diluted spray solution (DPA) as recommended by Monsanto for small scale experiments (right) failed to desiccate weed plants causing substantial regrowth of weeds.

Pre-crop glyphosate application 2 days before seeding of winter wheat caused a significant decline in shoot- and root biomass in comparison to untreated control in case of all three methods for glyphosate application evaluated. Surprisingly, different concentrations or different amounts of glyphosate applied did not cause different expression of damage in terms of lower shoot- or root biomass (Fig. 3.2).

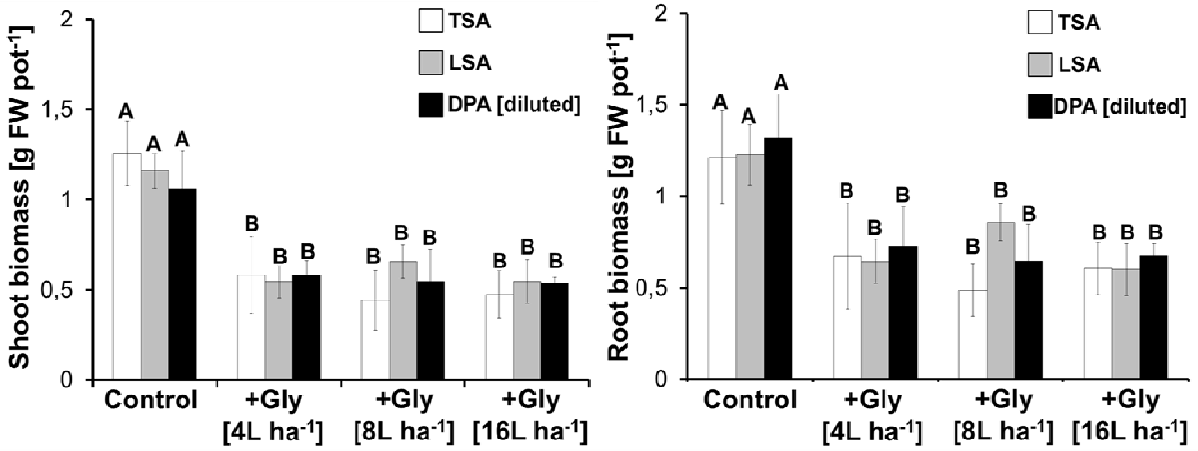

Fig. 3.2: Shoot and root biomass of winter wheat plants depending on glyphosate application method

Shoot and root biomass of winter wheat (*Triticum aestivum* L.) plants were measured at final harvest (14 days after germination). Winter wheat seeds were sown 2 days after different concentrations of glyphosate (4-,8- or 16 L glyphosate ha-1) were applied as Roundup Ultramax® on pre-cultured weed plants (wheat) with a track sprayer simulating application technique used under field conditions (TSA), with a hand-held sprayer based on the estimated leaf surface area of weed plants (LSA) or with a hand-held sprayer based on the pot surface area in diluted spray solution (DPA) as recommended by Monsanto for small scale experiments. Shoots of treated weed plants were cut close to soil level and removed from the pots before indicator plants emerged (4-5 days after sowing). In control treatments (Control) without glyphosate application, shoots of target-plants were removed by cutting at the soil level with a sharp knife. Data represent means and standard deviations of 4 independent replicates. Significant differences ($p<0.05$) are indicated by different characters.

Analysis of shikimate in root tissue of winter wheat plants as specific physiological indicator of glyphosate toxicity revealed a significant increase of accumulation in case of glyphosate TSA- and LSA-application of glyphosate on pre-cultured weed which was not observed in control treatment and in case of DPA-application at 4 L ha^{-1} rate (Fig. 3.3).

Later might indicate that the observed decline in shoot- and root biomass of indicator wheat plants was not caused by a transfer of phytotoxic glyphosate in the rhizosphere from treated weeds but by a high competition between not desiccated weed plants and emerging wheat crop plants.

In line with the results of other studies, damage of crop plants in case of short waiting time between glyphosate application on weeds and subsequent sowing of crops is most likely

caused by transfer of glyphosate from roots of treated target weeds through the rhizosphere to subsequently grown crop plants (Rodrigues *et al.*, 1982; Neumann *et al.*, 2006; Römheld *et al.*, 2008).

Similarly, in addition to equivalent expression of damage and decline in shoot- and root biomass, no significant differences in shikimate accumulation were observed between different concentrations and amounts of glyphosate applied with TSA- and LSA-application (Fig. 3.3).

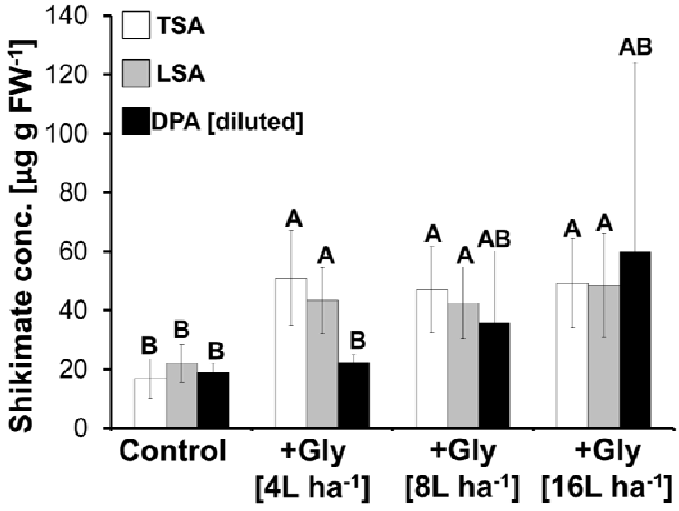

Fig. 3.3: Intracellular shikimate concentrations in root tissue of winter wheat depending on glyphosate application method

Shikimate concentrations as physiological indicator for glyphosate phytotoxicity were measured at final harvest (14 days after germination). Indicator winter wheat (*Triticum aestivum* L.) plants were sown 2 days after different concentrations of glyphosate (4-, 8- or 16 L glyphosate ha^{-1}) were applied as Roundup Ultramax® on pre-cultured weed plants (wheat) with a track sprayer simulating application technique used under field conditions (TSA), with a hand-held sprayer based on the estimated leaf surface area of weed plants (LSA) or with a hand-held sprayer based on the pot surface area in diluted spray solution (DPA) as recommended by Monsanto for small scale experiments. Shoots of treated weed plants were cut close to soil level and removed from the pots before indicator plants emerged (4-5 days after sowing). In control treatments (Control) without glyphosate application, shoots of target-plants were removed by cutting at the soil level with a sharp knife. Data represent means and standard deviations of 4 independent replicates. Significant differences ($p<0.05$) are indicated by different characters.

Most likely these results can be explained by self-limited translocation of glyphosate from leaves of treated weed plants to roots leading to comparable amounts of glyphosate in root tissue which are subsequently released into the rhizosphere and potentially transferred to crop plants. In line with this, Geiger *et al.* (1999) observed in case of foliar application of glyphosate on sugar beet (*Beta vulgaris* L.) clear indications for self-limited translocation of glyphosate. In this study export of glyphosate from treated leaves essentially stopped 10 hours after application in glyphosate-sensitive (GS) plants. According to various reports, after uptake of glyphosate by leaves of treated plants, glyphosate is not readily metabolised in most

plant species. Glyphosate is mobile in phloem and long distance transport through the plant following the same source-to-sink pattern that occurs for photoassimilates (Sprankle *et al.*, 1975c; Gougler and Geiger 1981). In a study by Geiger *et al.* (1999), inhibition of photoassimilate translocation and glyphosate translocation coincided, confirming that glyphosate export from treated leaves was inhibited by disruption of a process that drives carbon export. Similarly, Hess (1999) reported self-limitation of glyphosate translocation from treated leaves caused by effects on carbon metabolism in source leaves with various disturbances such as disruption of aromatic amino acid biosynthesis, increased diversion of carbon to shikimate pathway, reduction of carbon pool available for Calvin cycle, decreased starch synthesis, decreased export of triose to cytoplasm and decreased phloem transport of assimilates.

Because of the potential for self-limited translocation of glyphosate in treated weed plants results of studies like Rodrigues *et al.* (1982), Neumann *et al.* (2006) and Tesfamariam (2009) indicating a rhizosphere transfer of glyphosate from treated weeds to subsequently grown crops have practical relevance despite the amounts of glyphosate applied were higher compared to farmers practice/ legislations.

In additional experiments repeating the experimental set-up of Tesfamariam (2009), a comparison of the TSA- and LSA-application method revealed after glyphosate application on pre-cultured rye grass (*Lolium perenne* L.) significant damage of crops induced by rhizosphere transfer of glyphosate. However, again no significant differences between the application methods in terms of decline in biomass production (Fig. 3.4) or accumulation of shikimate as indicator of glyphosate toxicity was observed (data not shown).

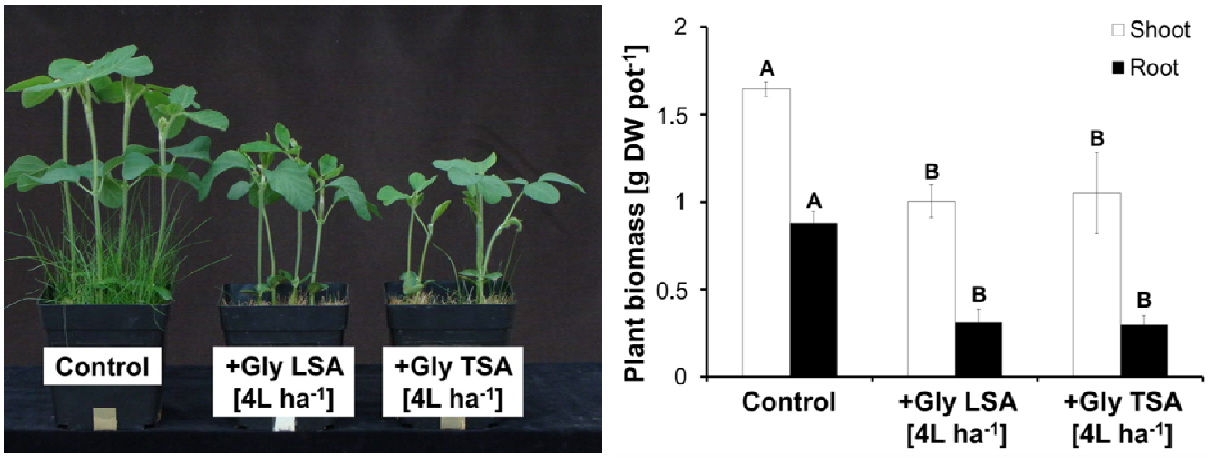

Fig. 3.4: Visual effects and plant biomass of soybean depending on glyphosate application method

Soybean (*Glycine max* L.) as indicator plants were sown 4 days after glyphosate (4 L ha^{-1}) was applied as Roundup Ultramax® on pre-cultured rye grass (*Lolium perenne* L.) plants with a track sprayer simulating application technique used under field conditions (TSA) or with a hand-held sprayer based on the estimated leaf surface area of weed plants (LSA). Shoots of treated weed plants were cut close to soil level and removed from the pots before indicator plants emerged (4-5 days after sowing). In control treatments (Control) without glyphosate application, shoots of target-plants were removed by cutting at the soil level with a sharp knife. Measurements were done at final harvest (20 days after sowing). Data represent means and standard deviations of 4 independent replicates. Significant differences ($p<0.05$) are indicated by different characters.

In conclusion, the achieved results indicated that glyphosate LSA-application with a hand-held sprayer based on estimation of leaf surface area of weed plants can be used as a realistic application method for studies investigating factors influencing rhizosphere transfer of glyphosate from weed- to subsequently grown crop plants in cases where preferable equipment for glyphosate application in small scale experiments (e.g. various track spraying devices) is not available.

4 Glyphosate in the rhizosphere - Role of waiting times and different glyphosate binding forms in soils for phytotoxicity to non-target plants

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Own contribution: set-up of experiments, plant cultivation, harvest and sample preparation, analysis of mineral nutrients, manuscript preparation in cooperation with Tsehaye Tesfamariam

4.1 Abstract

Glyphosate is the most widely used non-selective, systemic herbicide. It is easily translocated from shoot to roots and released into the rhizosphere, where it is immobilised at the soil matrix or microbially degraded. However, contradictory results are reported in the literature concerning the bio availability of glyphosate residues in soils and the potential risks for intoxication of non-target organisms. This study addresses the question whether plant residues of glyphosate-treated weeds (model plant perennial rye grass, *Lolium perenne* L.) or direct soil application of glyphosate bears an intoxication risk for subsequently cultivated sunflower (*Helianthus annuus* L.) seedlings. The experiments were conducted as greenhouse studies on two soils with contrasting properties (acidic, sandy Arenosol, calcareous loess subsoil). Also the potential role of different waiting times between glyphosate application and sunflower cultivation was considered.

On both soils, sunflower seedling growth and biomass production was strongly impaired by glyphosate pre-sowing treatments in the variants with 0 day waiting time and recovered within a waiting time of 7–21 days. Generally, the detrimental effects were more pronounced after glyphosate weed application (90% biomass reduction) compared with direct soil application (55-70 % biomass reduction) at waiting time 0 day. The inhibitory effects on seedling growth were associated with a corresponding increase in shikimate accumulation in the root tissue as physiological indicator for glyphosate toxicity. Glyphosate intoxication of sunflower seedlings was also associated with an impairment of the manganese-nutritional status, which was still detectable after a waiting time of up to 21 days, particularly on the Arenosol in the variants with glyphosate weed application. These findings indicate an important and yet un-investigated role of glyphosate in plant residues in determining the risk of non-target plant intoxication.

Keywords: Glyphosate, Manganese, Micronutrient, Rye grass, Shikimate, Sunflower

4.2 Introduction

Glyphosate (*N*-phosphonomethylglycine) is the most widely used broad-spectrum herbicide on global scale. After foliar application, it is absorbed by the foliage and translocated throughout stems, leaves and roots of the entire plant, finally accumulating preferentially in young growing tissues (Franz *et al*., 1997). The herbicidal effect is based on inhibition of the shikimate pathway enzyme 5-enolpyruvylshikimic acid-3-phosphate synthase (EPSPS), involved in the biosynthesis of aromatic amino acids and phenolic compounds (Della-Cioppa *et al.*, 1986; Franz *et al.*, 1997). Therefore, glyphosate application frequently induces intracellular accumulation of shikimate, which can be used as a sensitive physiological indicator for glyphosate toxicity (Henry *et al.*, 2007).

Glyphosate can reach the soil via foliar wash-off and undirected spray drift contamination (Al-Kathib and Peterson, 1999; Ellis and Griffin, 2002) and by exudation from roots or death and decomposition of treated plant residues (von Wirén-Lehr *et al.*, 1997; Neumann *et al.*, 2006; Laitinen *et al.*, 2007). However, risks of glyphosate toxicity to non-target organisms in soils are generally considered as marginal, since glyphosate is almost instantaneously inactivated by adsorption to clay minerals and cationic binding sites of the soil matrix (Piccolo *et al.*, 1992; Dong-Mei *et al.*, 2004), while glyphosate in the soil solution is prone to rapid microbial degradation (Giesy *et al.*, 2000).

An additional potential pool of glyphosate accumulation and stabilisation in soils is represented by the plant residues of glyphosate-treated weeds. Since in many plant species, glyphosate is not readily metabolised, considerable amounts can accumulate particularly in young tissues (Reddy *et al.*, 2004). However, the fate of bound glyphosate in plant residues has not been widely considered in the past. Studies with soybean and wheat suggested unspecific and non-covalent binding of glyphosate to starch and cell wall components (Komossa *et al.*, 1992). The release and degradation of ^{14}C-labelled glyphosate in various agricultural soils correlated with the soil-microbial activity but only after direct soil application. No such correlation was observed after soil incorporation of lyophilised soybean tissue cultures, contaminated with glyphosate. These findings suggest different mechanisms for degradation of glyphosate adsorbed to the soil matrix and bound in plant residues in the soils, respectively. No information exists on factors determining the stabilisation and release of glyphosate bound in plant residues and the potential risks for non-target organisms getting in contact with these residues.

An increasing number of yet unexplained observations of negative side effects after glyphosate application has been reported in the literature (Smiley *et al.*, 1992; King *et al.*, 2001; Kremer *et al.*, 2001; Charlson *et al.*, 2004; Fernandez *et al.*, 2005; Huber *et al.*, 2005; Yamada, 2006; Neumann *et al.*, 2006), which have been related to direct toxicity of glyphosate, impairment of the micro nutritional status and increased susceptibility to plant diseases. This study was initiated to investigate the influence of glyphosate residues in the root tissue of glyphosate-treated weeds

on plant biomass production, intracellular shikimate accumulation as indicator for glyphosate toxicity and the micronutrient status of subsequently cultivated non-target plants in comparison with direct glyphosate soil application. The study was conducted using rye grass (*Lolium perenne* L. cv. Kelvin) as target weed and sunflower (*Helianthus annuus* L. cv. Frankasol) seedlings as non-target plants, considering also the impact of different waiting times after glyphosate application for the subsequent culture, as well as two contrasting soils with different binding properties for glyphosate. In addition, the findings of these model pot experiments were compared with observations of field experiments of local farmers.

4.3 Materials and methods

Conditions for plant growth

Experiments were conducted under greenhouse conditions, using two contrasting soils with different cationic binding sites for glyphosate: a sandy acidic Ap horizon of an Arenosol with low buffering capacity (pH (CaCl2) 4.5; C_{org} 0.16 %; water-extractable Ca^{2+} and Mg^{2+} (Beck *et al.*, 2000) [mg kg^{-1} soil]: 0.4 and 0.4), and with a well-buffered calcareous loess subsoil (pH ($CaCl_2$) 7.6; C_{org} < 0.3%; $CaCO_3$ 23.3%; water-extractable Ca^{2+} and Mg^{2+} [mg kg^{-1} soil]: 59.9 and 11.3). Calcium chloride–diethylenetriamine penta acetic acid (CAT)-extractable micronutrient concentrations [mg kg^{-1} soil]: Mn= 7.4, Fe = 369.0, Zn = 0.8, B = 0.9, and Cu 0.5 (VDLUFA, 2004), exchangeable Al^{3+} (McLean, 1982) = 0.04 cmol kg^{-1} soil for the Arenosol and Mn= 15.0, Fe = 7.8, Zn = 0.6, B = 0.2 and Cu = 0.7 (VDLUFA, 2004). Soils were sieved by passing through a 2mm mesh size and fertilised with N as $Ca(NO_3)_2$ (100 mg N kg^{-1} soil), K as K_2SO_4 (150 mg K kg^{-1} soil), Mg as $MgSO_4$ (50 mg Mg kg^{-1} soil) and P as $Ca(H_2PO_4)_2$ (80 mg P kg^{-1} soil). In addition, the calcareous subsoil was supplied with Fe as FeEDTA (20 µmol kg^{-1} soil). Plant culture was performed in pots containing 500 g of fertilised soil and soil moisture was adjusted to 70% of the soil water-holding capacity (15 %, w/w for the Arenosol and 18 %, w/w for the calcareous loess subsoil). Water losses were determined gravimetrically and replaced by daily applications of deionised water.

Glyphosate plant application

To investigate the effects of glyphosate residues in the root tissue of target weeds on subsequently cultivated non-target plants, rye grass (*L. perenne* L. cv. Kelvin) was pre-cultivated as model-weed in 500 g pots filled with the fertilised soils. A sowing density of 2.2 g rye grass seeds (germination rate 70 %) per pot with a surface area of 100 cm^2 was used to simulate high weed coverage of the soil with intense root development (Fig. 1). At 10 days after sowing (DAS), the young rye grass seedlings were sprayed with the recommended dilution of Roundup Ultramax® glyphosate formulation (Monsanto Agrar, Düsseldorf, Germany), containing a glyphosate concentration of 28.4 mM in the spray solution using a hand-held sprayer. Each pot received 6.7 mL of glyphosate spray solution on the leaves, based on determination of the rye grass leaf area coverage (approximately 3300 cm^2 per pot) and the plants died within 7 days, a

typical time period usually observed also under field conditions (Pilot experiments with lower doses of glyphosate failed to desiccate the rye grass plants completely even within 3–4 weeks). Subsequently, sunflower seeds (*H. annuus* L. cv. Frankasol) were sown into the same pots (7 seeds per pot) at 0, 7, 14 and 21 days after rye grass glyphosate application. After desiccation, rye grass residues were removed and no disturbance of the soil in the pots was under taken. This time period was defined as "waiting time" (Fig. 4.1).

In control treatments without glyphosate application, rye grass shoots were removed by cutting at the soil level with a sharp knife. A time schedule with sequential sowing dates for the rye grass pre-culture was employed to ensure the same sowing day and thus the same external growth conditions for all sunflower seedlings, irrespective of the waiting time. All treatments were performed in 4 replicates (Fig. 4.1).

Glyphosate soil application

To assess the effects of glyphosate in the soil on non-target plants, the same amount of glyphosate as applied to the target weeds (6.7 mL of a Roundup Ultramax® solution containing a glyphosate concentration of 28.4 mM) was mixed directly with 500 g of the fertilised soils. Controls received only mineral nutrients and water. After a waiting time of 0, 7, 14 and 21 days, sunflower seeds were sown (7 seeds per pot) at the same day as in the treatments with rye grass weed pre-culture (Fig. 4.1).

Plant harvest

At 12 days after sowing (DAS), a first set of sunflower seedlings was removed from the pots. Roots and shoots were separated, frozen in liquid nitrogen and stored at −20 ◦C for shikimate analysis. In each pot, two seedlings were kept and further cultivated until 25 DAS. At final harvest, the root systems were washed out from the soil, and shoot and root parts were separated for biomass determination. The youngest fully expanded leaves were selected for analysis of micronutrients.

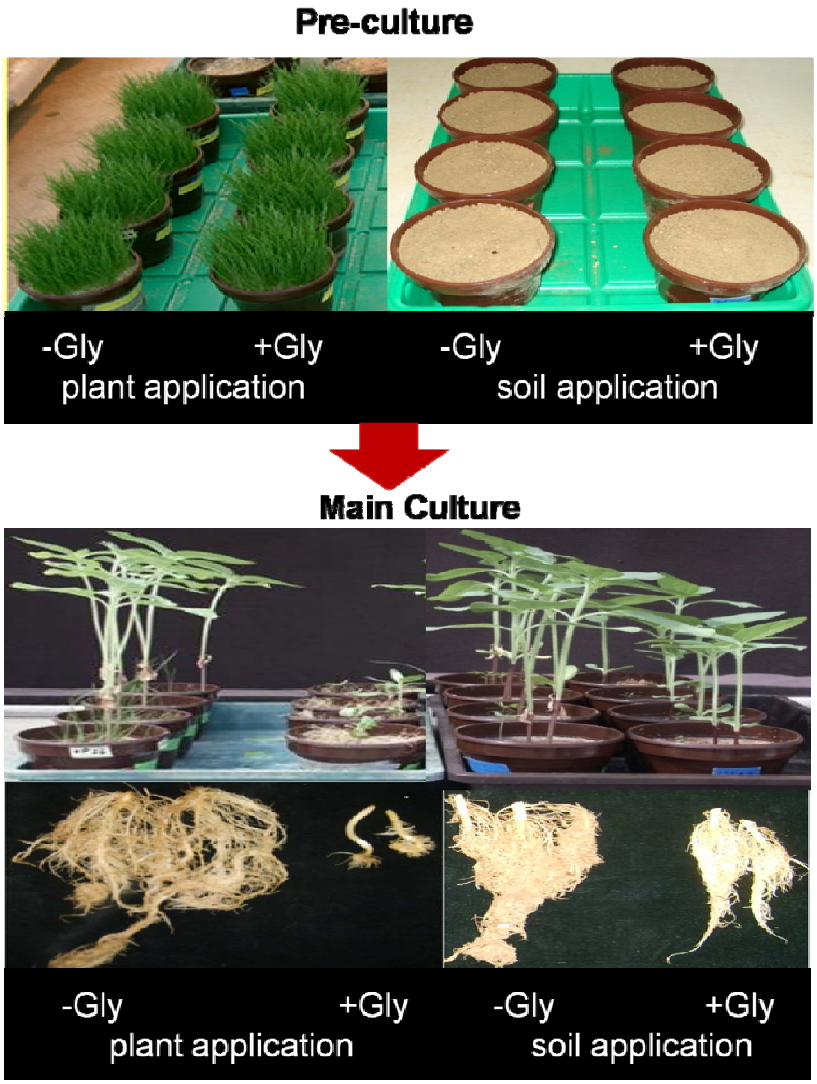


Fig. 4.1: Shoot and root development of sunflower depending on glyphosate application method

Sunflower seedlings were grown for 25 days after sowing on an acidic Arenosol with (+Gly) or without (−Gly) pre-sowing glyphosate treatments on a pre-culture with *Lolium perenne* or direct glyphosate soil application.

Shikimate analysis

Shikimate in acidic tissue extracts was analysed with modifications of the methods described by Singh and Shaner (1998) and Neumann (2006). The frozen plant tissue was homogenised with 5% orthophosphoric acid (1 mL 100 mg^{-1} fresh weight) using mortar and pestle. Insoluble material was removed by centrifugation (5 min at 20,000 × g) and the supernatant was used for HPLC analysis after appropriate dilution with the HPLC mobile phase. HPLC separation was performed by ion exclusion chromatography using an Aminex 87H column (Bio-Rad, Richmond, CA, USA) designed for organic acid analysis. A sample volume of 20µL was injected into the

isocratic flow (0.5 mL min^{-1}) of the eluent (2.5 mM H_2SO_4, 40°C) and organic acids were detected spectrophotometrically at 210 nm. Identification and quantification of shikimate was conducted by comparing the retention times, absorption spectra and peak areas with a known standard.

Analysis of micronutrients

Shoot mineral nutrients were determined according to Gericke and Kurmies (1952). Dried leaves (70 °C) were ground and ashed in a muffle furnace at 500 °C for 5 h. After cooling, the samples were extracted twice with 2mL of 3.4 M HNO_3 (v/v) and subsequently evaporated to dryness. The ash was dissolved in 2mL of 4M HCl, subsequently diluted 10-fold with hot deionised 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).

Statistics

All treatments comprised 4 replicates and pots were arranged in the greenhouse in a completely randomised block design. Analysis of variance was performed with SPSS statistics software package (SPSS Inc., IL, USA).

4.4 Results

Biomass production of sunflower seedlings was not influenced by the two contrasting soils (acidic Arenosol, calcareous loess subsoil) used for plant culture. However, glyphosate pre-sowing treatments substantially reduced seedling dry matter, particularly in the variant with a waiting time of 0 day after glyphosate application for sowing of sunflower (Tab. 4.1, 4.2). The inhibitory effect was more strongly expressed when glyphosate was applied on a pre-culture of rye grass, associated with a reduction of root and shoot biomass by approximately 90%, compared with direct soil application, leading to a reduction of shoot biomass by 55–57 % and of root biomass by 67–73 % (Tab. 4.1, 4.2; Fig. 4.1). The inhibitory effects declined with increasing waiting times, but still remained detectable even at 21 days after glyphosate application, although the differences were not significant in all cases.

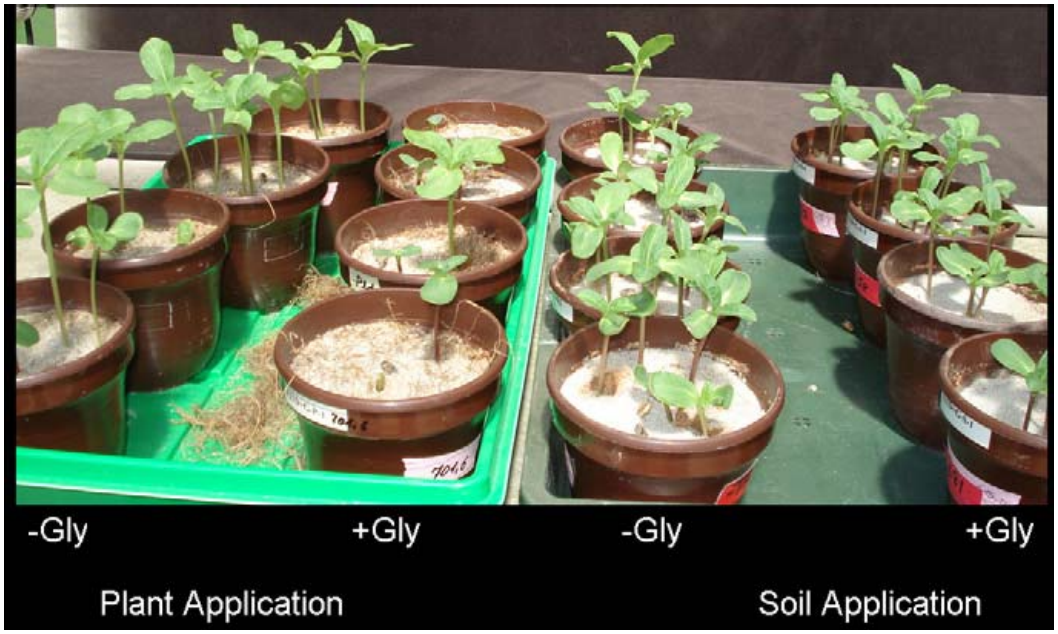


Fig. 4.2: Germination and seedling development of sunflower plants depending on glyphosate application method

Sunflower plants were grown on an acidic Arenosol at 21 days after desiccation of a ryegrass pre-culture by foliar glyphosate application (plant application) and after direct soil application of the same glyphosate dose (soil application).

Tab. 4.1 Shoot and root dry matter of sunflower plants depending on glyphosate application method and waiting time

Sunflower plants were grown 25 days after sowing on an acidic Arenosol with glyphosate application at 0, 7, 14 and 21 days before sowing to a pre-culture of rye grass or directly incorporated into the soil, respectively. Data represent means and standard deviations (±SD) of 4 independent replicates. Significant differences between treatments within a column are indicated by different characters.

Treatment	Shoot biomass (g)		Root biomass (g)	
	Plant application	Soil application	Plant application	Soil application
0d –Gly	0.59±0.05^{ab}	0.58±0.03^{ab}	0.27±0.03^{ab}	0.27±0.03^{ab}
0d +Gly	0.07±0.03^{c}	0.26±0.06^{bc}	0.04±0.02^{c}	0.09±0.02^{bc}
7d –Gly	0.32±0.04^{bc}	0.56±0.02^{ab}	0.32±0.07^{a}	0.27±0.02^{ab}
7d +Gly	0.40±0.3^{abc}	0.52±0.03^{ab}	0.27±0.19^{ab}	0.26±0.01^{ab}
14d –Gly	0.37±0.06^{bc}	0.56±0.07^{ab}	0.35±0.02^{a}	0.35±0.05^{a}
14d +Gly	0.57±0.06^{ab}	0.55±0.02^{ab}	0.33±0.06^{a}	0.28±0.01^{ab}
21d –Gly	0.75±0.11^{a}	0.54±0.05^{ab}	0.41±0.03^{a}	0.32±0.04^{a}
21d +Gly	0.46±0.46^{ab}	0.56±0.05^{ab}	0.24±0.24^{abc}	0.31±0.03^{a}

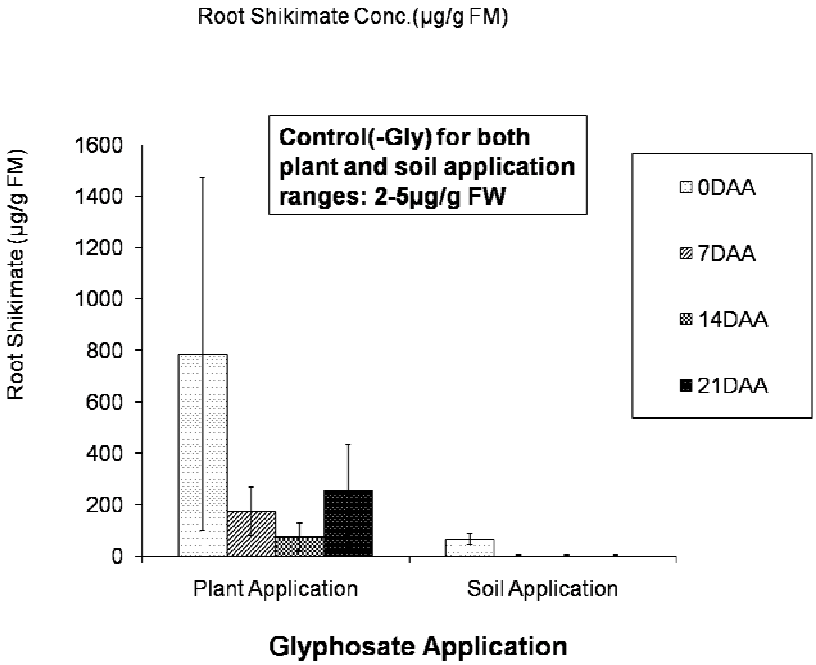
Tab. 4.2 Shoot and root dry matter of sunflower plants depending on glyphosate application method and waiting time

Sunflower plants were grown 25 days after sowing on a calcareous loess subsoil with glyphosate application at 0, 7, 14 and 21 days before sowing to a pre-culture of rye grass or directly incorporated into the soil, respectively. Data represent means and standard deviations (±SD) of 4 independent replicates. Significant differences between treatments within a column are indicated by different characters.

Treatment	Shoot biomass (g)		Root biomass (g)	
	Plant application	Soil application	Plant application	Soil application
0d –Gly	0.53±0.04^{abc}	0.59±0.06^{ab}	0.29±0.02^{abc}	0.26±0.01^{abc}
0d +Gly	0.05±0.02^{e}	0.23±0.09^{de}	0.03±0.02^{e}	0.07±0.03^{de}
7d –Gly	0.35±0.04^{bcd}	0.54±0.03^{abc}	0.28±0.03^{abc}	0.26±0.02^{abc}
7d +Gly	0.38±0.19^{bcd}	0.48±0.11^{abc}	0.17±0.12^{cd}	0.22±0.05^{bc}
14d –Gly	0.32±0.04^{cd}	0.45±0.03^{abcd}	0.33±0.05^{ab}	0.26±0.03^{abc}
14d +Gly	0.31±0.19^{cd}	0.42±0.07^{abcd}	0.22±0.07^{bc}	0.22±0.06^{bc}
21d –Gly	0.65±0.11^{a}	0.47±0.16^{abcd}	0.38±0.07^{a}	0.30±0.06^{abc}
21d +Gly	0.57±0.02^{ab}	0.53±0.02^{abc}	0.30±0.03^{abc}	0.30±0.05^{abc}

The detrimental effects of glyphosate pre-sowing treatments on plant growth were reflected in a corresponding increase in shikimate concentrations in the root tissue as a physiological indicator for glyphosate toxicity (Fig. 4.3, 4.4). In this case, the differences between the two glyphosate application modes already observed for inhibition of seedling growth (Tab. 4.1, 4.2) were even more expressed, and intracellular shikimate accumulation was increased by 10–100-fold in the treatment with glyphosate applied to pre-cultured rye grass seedlings, compared with direct soil application (Fig. 4.3, 4.4).

In contrast to direct soil application of glyphosate, the treatments with glyphosate application to the *Lolium* pre-culture were characterised by non-homogeneous germination and large differences in seedling development of sunflower (Fig. 4.2). This was reflected in a high variability of biomass data (Tab. 4.1, 4.2) and intracellular shikimate accumulation in the respective treatments (Fig. 4.3, 4.4).



Tab. 4.3 Intracellular shikimate accumulation in the root tissue of sunflower seedlings grown on an acidic Arenosol, depending on glyphosate application method and waiting time

Sunflower seedlings were grown 12 days after sowing on an acidic Arenosol with glyphosate application at 0, 7, 14 and 21 days before sowing to a pre-culture of rye grass or directly incorporated into the soil, respectively. Data represent means and standard deviations of 4 independent replicates. The background levels of shikimate concentrations are shown as numeric values.

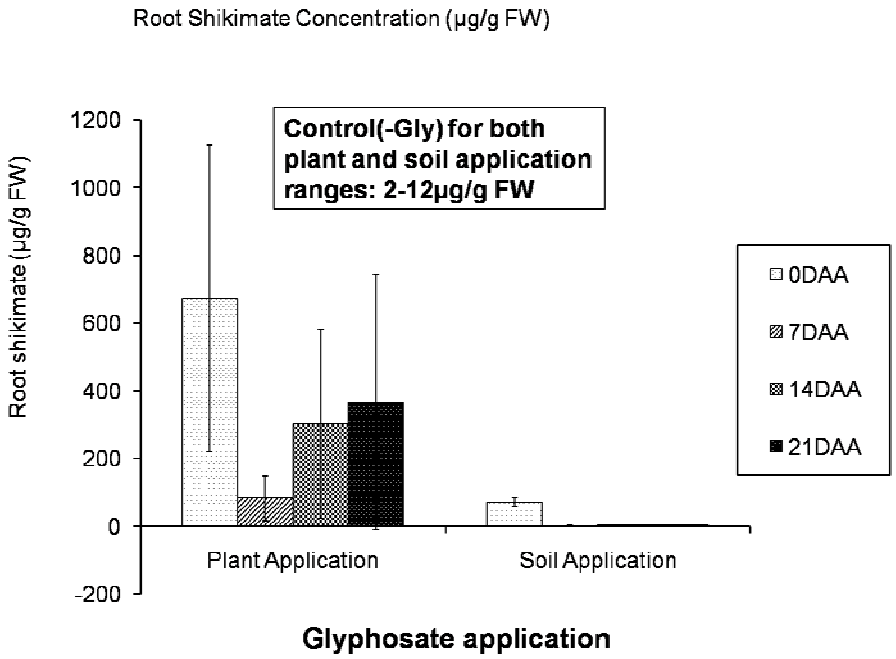


Fig. 4.3: Intracellular shikimate accumulation in the root tissue of sunflower seedlings grown on a calcareous loess subsoil, depending on glyphosate application method and waiting time

Sunflower seedlings were grown 12 days after sowing on a calcareous loess subsoil with glyphosate application at 0, 7, 14 and 21 days before sowing to a pre-culture of rye grass or directly incorporated into the soil, respectively. Data represent means and standard deviations of 4 independent replicates. The background levels of shikimate concentrations are shown as numeric values.

The pre-culture of rye grass without glyphosate application obviously increased Mn acquisition of sunflower on the Arenosol but not on the calcareous loess subsoil (Fig. 4.5). On both soils, glyphosate pre-sowing treatments affected Mn concentrations in the youngest fully expanded leaves in treatments with 0 day waiting time (Fig. 4.5, 4.6). Manganese concentrations recovered with increasing waiting times in all variants with exception of the rye grass glyphosate pre-sowing treatment on the Arenosol. In this case, glyphosate application induced a decline of Mn leaf concentrations even after a waiting time of three weeks and in some cases Mn concentrations dropped close to the critical level of Mn deficiency (Bergmann, 1992) (Fig. 4.5).

In contrast to the Mn-nutritional status, Fe and Zn nutrition of the sunflower seedlings were not affected by glyphosate pre-sowing treatments and Fe and Zn concentrations even increased in the glyphosate-treated variants with rye grass pre-culture and 0 day waiting time (data not shown). As a general feature of all measured parameters, data obtained from the treatments with glyphosate application to the rye grass pre-culture exhibited a much higher variation compared with those from the treatments with direct soil application of glyphosate (Tab. 4.1, 4.2; Fig. 4.3–4.6).

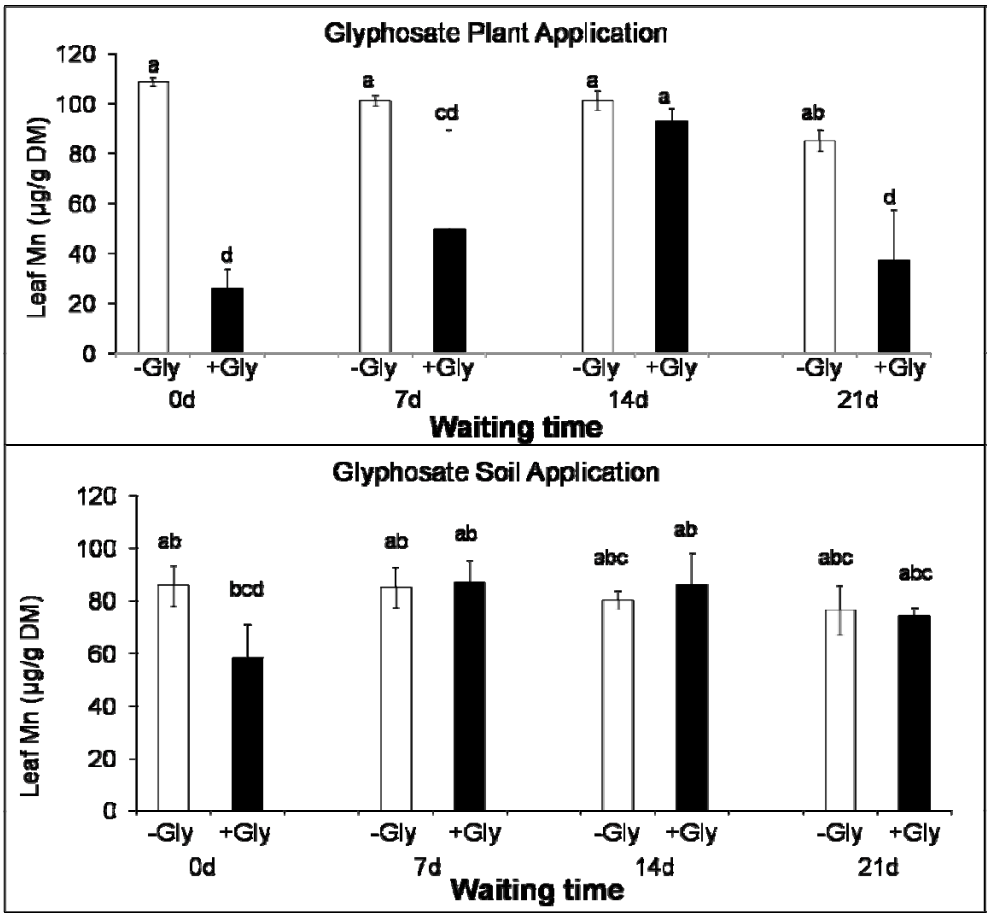


Fig. 4.4: Manganese concentration in the youngest fully expanded leaves of sunflower plants grown on an acidic Arenosol depending on glyphosate application method and waiting time

Sunflower plants were grown 25 days after sowing on an acidic Arenosol with glyphosate application at 0, 7, 14 and 21 days before sowing to a pre-culture of rye grass or directly incorporated into the soil, respectively. Data represent means and standard deviations of 4 independent replicates. Significant differences between treatments are indicated by different characters.

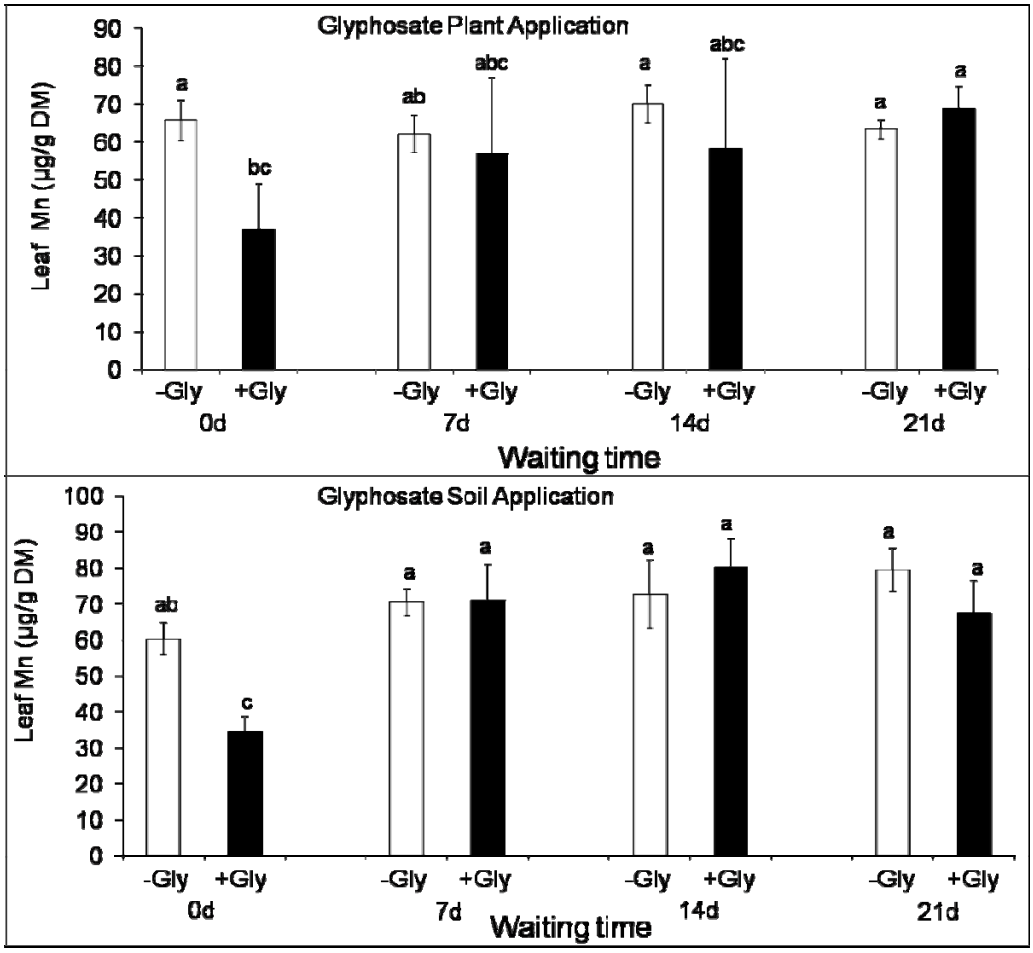


Fig. 4.5: Manganese concentration in the youngest fully expanded leaves of sunflower plants grown on a calcareous loess subsoil depending on glyphosate application method and waiting time

Sunflower plants were grown 25 days after sowing on a calcareous loess subsoil with glyphosate application at 0, 7, 14 and 21 days before sowing to a pre-culture of rye grass or directly incorporated into the soil, respectively. Data represent means and standard deviations of 4 independent replicates. Significant differences between treatments are indicated by different characters.

4.5 Discussion

In contrast to the common and recommended practice of glyphosate pre-sowing treatments, which frequently allows herbicide application even until the first days after sowing (Monsanto, Roundup Ultramax® product information), the results of this study underline the importance of waiting times, to avoid or at least minimise detrimental effects on the following culture. The analysis of physiological parameters, such as intracellular shikimate accumulation as metabolic indicator for glyphosate toxicity or the micronutrient status revealed, that the risk of toxic effects, induced by glyphosate pre-sowing treatments, increases with declining waiting time and can persist up to three weeks (Fig. 4.5), even when clearly visible effects on seedling growth and development are no more detectable by the first view (Tab. 4.1; Fig. 4.1). Similarly, Cornish

(1992) reported detrimental effects of glyphosate pre-transplanting treatments on tomato in field and pot experiments on sandy loam soils, which were still detectable after waiting times of 3–4 weeks. However, this study used young tomato plants and no seeds which increases the risk of plant damage by glyphosate application.

Glyphosate-induced impairment of Mn nutrition was more strongly expressed on the sandy Arenosol with low buffering capacity compared with the well-buffered calcareous subsoil (Fig. 4.5 and 4.6), indicating a role of different soil types in determining the expression of glyphosate toxicity. This was not associated with corresponding differences of intracellular shikimate accumulation or plant biomass production (Tab. 4.1, 4.2; Fig. 4.3, 4.4), suggesting rather soil-specific differences in Mn availability than differential expression of glyphosate toxicity on the two investigated soils as possible causes. Accordingly, soil analysis by CAT extraction (VDLUFA, 2004) revealed lower levels of available Mn in the Arenosol [7.4 mg kg^{-1} soil] as compared with the calcareous loess subsoil [15.0 mg kg^{-1} soil]. Glyphosate can form poorly soluble complexes with Mn (Sprankle *et al.*, 1975c) and may thereby reduce the already low level of available Mn in the Arenosol. Also glyphosate-induced inhibition of root growth (Tab. 4.1, 4.2; Fig. 4.1) may counteract Mn acquisition with the strongest consequences for Mn uptake on the Arenosol with low levels of plant-available Mn. Detrimental effects of glyphosate applications on the micronutrient status and particularly on Mn nutrition have been previously reported when glyphosate reached non-target plants as drift contamination in sub-lethal dosage (Eker *et al.*, 2006), via rhizosphere transfer from target weeds (Neumann *et al.*, 2006), or even in glyphosate resistant soybean (Jolley and Hansen, 2004). Since micronutrients, such as Mn and Zn are important physiological cofactors for mechanisms of plant disease resistance (Cakmak, 2000; Thompson and Huber, 2007), glyphosate-induced impairment of the micronutrient status may be linked with the observations of a higher susceptibility to plant diseases (e.g. *Fusarium, Corynespora, Rhizoctonia, Gaeumannomyces* and pathogenic nematodes) in response to glyphosate treatments (Smiley *et al.*, 1992; King *et al.*, 2001; Kremer *et al.*, 2001; Charlson *et al.*, 2004; Jolley and Hansen, 2004; Fernandez *et al.*, 2005; Huber *et al.*, 2005).

In contrast to the Mn-nutritional status in this study, Fe and Zn concentrations in the youngest fully developed leaves were not affected by glyphosate application, except of the treatments with rye grass pre-culture and 0 day waiting time. In these cases, Fe and Zn concentrations even increased in the leaves of glyphosate-treated variants (data not shown). Most probably, this represents a concentration effect of Fe and Zn seed reserves due to the extreme growth depression of the seedlings in these treatments.

Also calcium and magnesium are discussed as potential ligands, mediating glyphosate immobilisation and inactivation in soils (Sprankle *et al.*, 1975c) and in plants (Duke *et al.*, 1985). However, despite of much higher levels of $CaCO_3$ and of free water extractable $Ca2^+$ [59.9 mg kg^{-1} soil] and Mg^{2+} [11.3 mg kg^{-1} soil] in the calcareous subsoil compared with the Arenosol [Ca^{2+}: 0.4 mg kg^{-1} soil; Mg^{2+}: 0.4 mg kg^{-1} soil], glyphosate-induced inhibition of plant growth (Tab. 4.1, 4.2) and intracellular shikimate accumulation (Fig. 4.3, 4.4) were similarly expressed

on both soils. This finding suggests that on both soils, the plants were exposed to similar levels of free glyphosate, which induced similar effects of toxicity. The lack of Ca^{2+} and Mg^{2+} in the Arenosol may be compensated by much higher concentrations of available Fe^{3+} [369 mg kg^{-1} soil] and exchangeable Al^{3+} [0.04 cmol kg^{-1}] compared with the calcareous loess subsoil Fe^{3+} [7.8 mg kg^{-1} soil] and negligible exchangeable Al^{3+} as ligands for binding and complexation of glyphosate.

Toxicity of glyphosate pre-sowing treatments on sunflower seedlings was also strongly dependent on the mode of glyphosate application: When glyphosate was sprayed on pre-cultured rye grass seedlings, detrimental effects on plant growth and the Mn nutritional status, as well as increased intracellular shikimate accumulation in the root tissue were more strongly expressed than after direct soil application of the same amount of glyphosate. The lower expression of glyphosate toxicity after soil application is in line with the concept of rapid inactivation and detoxification of glyphosate in soils by adsorption to phosphate binding sites, such as Fe/Al-oxides and hydroxides, precipitation as calcium salts, and rapid microbial degradation of free glyphosate in the soil solution (Sprankle *et al.*, 1975c; Giesy, 2000; Monsanto, 2005a; Yamada, 2006). Accordingly, Cornish (1992) reported increased toxicity of glyphosate soil pre-treatments on tomato after simultaneous application of P fertilisers, which obviously increased the solubility and thus the bio-availability of glyphosate by competition for soil binding sites. It remains to be established, whether also the intense expression of root-induced mechanisms for phosphorus or iron mobilisation in the rhizosphere, reported for various plant species and cultivars (Neumann and Römheld, 2002), can similarly induce toxic effects by co-mobilisation of glyphosate adsorbed to P sorption sites. However, in the present short-term study, no relevance of these adaptive responses to nutrient limitation is expected, since only young seedlings were investigated, relying mainly on P and Fe seed reserves in this early developmental stage.

The increased expression of toxicity effects after glyphosate pre-sowing application to the rye grass pre-culture compared with direct soil application suggests, that also the root tissue of glyphosate-treated weeds represents a storage pool for glyphosate in the investigated soils. In this experiment, the bio-availability of glyphosate in plant residues to subsequently cultivated sunflower seedlings was obviously much higher than the bio-availability of glyphosate bound at the soil matrix. In most plant species, glyphosate is not readily metabolised and is preferentially translocated to young growing tissues of roots and shoots, where it can accumulate in millimolar concentrations (Reddy *et al.*, 2004; Monsanto, personal communication). In soil-grown target plants, this non-homogeneous distribution of glyphosate within the root tissues may lead to the formation of hot spots of root residues in soils, containing high levels of glyphosate, which is subsequently released during microbial degradation of the plant material. Without a fast immobilisation of glyphosate by adsorption on the soil matrix, glyphosate toxicity to non-target plants may be induced by root contact with these hot spots. The non-homogeneous distribution of glyphosate-contaminated plant material in the soil could also explain the much higher variation of the data on sunflower biomass production, shikimate accumulation and Mn-nutritional status after glyphosate application to the rye grass pre-culture as compared to direct soil application

(Fig. 4.2 but also Tab. 4.1, 4.2; Fig. 4.3–4.5). Since toxic effects can be expected only after direct root contact of the non-target plants with one of the hot spots of glyphosate-contaminated plant residues, sunflower seedlings without contact to the hot spots remained unaffected. In contrast, direct soil application of glyphosate resulted in a homogeneous distribution and lower bio-availability due to adsorption of the herbicide over the investigated soil profile.

The potential role of plant residues as a pool for glyphosate stabilisation in soils has not been widely considered in the past. Most of the available information originates from studies of glyphosate residues in foliage (Newton *et al.*, 1984; Feng and Thompson, 1990; Thompson *et al.*, 1994; Reddy *et al.*, 2004) and not in roots. In a model study with different agricultural soils, von Wirén-Lehr *et al.* (1997) investigated the degradation of bound ^{14}C-glyphosate residues in lyophilised cell cultures of soybean but only the water insoluble fraction was taken into account. Komossa *et al.* (1992) characterised the binding forms of glyphosate in wheat and soybean. However, in contrast to the fate of the herbicide applied to soils in a free state, systematic investigations on the bio-availability of glyphosate in real plant residues incorporated into soils are rare. The present study suggests a considerable contribution of this glyphosate pool in determining the risk of phytotoxicity to non-target organisms. The findings of this study are in line with recent field observations of plant damage in winter wheat after glyphosate pre-crop applications and waiting times shorter than two weeks in no-tillage systems (Römheld *et al.*, 2008). To improve bio-safety in face of the global increase in agricultural use of glyphosate, open questions to be considered for the future comprise the expression of these effects under a range of different field conditions, the impact of external factors, such as soil properties, soil moisture levels, temperature, period of season, soil-organic matter and biological activity and thus speed of microbial degradation of glyphosate containing crop residues, as well as the role of plant species, rooting densities and fertilisation management. The variability of these factors in agricultural practice may contribute to the explanation of contradictory results frequently reported in the literature and in field observations concerning the risks of negative side effects of glyphosate application on non-target organisms (for reviews see Monsanto, 2005a,b and Yamada, 2006 and references cited therein).

5 Rhizosphere transfer of glyphosate after pre-crop herbicide application

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Own contribution: set-up of model experiments, plant cultivation in model experiments, planning of field experiments (in cooperation with LTZ Augustenberg) harvests and sample preparation (model and field experiments), analysis of nutritional status of plants, analysis of shikimate by HPLC, manuscript preparation. Support of students: in 4 field and 3 model experiments

5.1 Abstract

Observations of crop damage and yield losses by farmers as well as in two preliminary field trials in South Germany indicate the possibility of a rhizosphere transfer of glyphosate from treated weeds to crops after pre-crop glyphosate application.
This study evaluated the potential for damage of wheat after pre-crop application of glyphosate on weeds in four field trials with different waiting times between glyphosate application and sowing of crops. To identify factors contributing to risks for crops associated to glyphosate in the rhizosphere, additional model experiments were conducted under soil and hydroponic conditions.

Results of field and model experiments consistently revealed a correlation of damage of winter wheat to waiting time after application of glyphosate on weed plants. Intensity and time window for crop damage induced by glyphosate and/or its metabolite aminomethylphosphonic acid (AMPA) in the rhizosphere of crops were correlated with the density of treated weeds and influenced by the developmental stage of plants.

There is substantial evidence that under certain conditions root residues of treated weeds represent a pool for prolonged glyphosate phytotoxicity in the rhizosphere of crops. Risks for crop damage can be limited by observance of waiting times of 14-21 days after application.

Keywords: glyphosate, pre-crop application, risk factors, winter wheat (*Triticum aestivum* L.), micronutrients

Abbreviations:

AI active ingredient

AMPA aminomethylphosphonic acid

cv. cultivar

DBS days before sowing

WT waiting time

5.2 Introduction

Due to low production costs and high efficiency, glyphosate, N-(phosphonomethyl)glycine, is the most extensively used herbicide in agricultural practice (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 the impairment of general metabolic processes, such as protein synthesis and photosynthesis (de María *et al.*, 2005; Geiger *et al.*, 1986). Glyphosate application frequently induces intracellular accumulation of shikimate, which can be used as a sensitive physiological indicator for glyphosate toxicity (Henry *et al.*, 2007).

As non-selective total herbicide glyphosate is particularly used in cropping systems with genetically modified glyphosate-resistant plants, but also as pre-crop application before sowing of plants in cropping systems with high weed pressure. Particularly in no-till and minimal tillage systems, control of weeds by application of effective herbicides (e.g. glyphosate) shortly before sowing is considered essential to minimise crop production losses caused by high weed pressure competing with the emerging crops (Lyon *et al.*, 1996; Calado *et al.*, 2010).
With the exception of drift contamination, glyphosate toxicity to crop plants and other non-target organisms is generally considered as marginal, since glyphosate is almost instantaneously inactivated by adsorption to the soil matrix (Piccolo *et al.*, 1992; Gimsing *et al.,* 2004). Glyphosate residues in the soil solution are prone to rapid microbial/chemical degradation (Giesy *et al.*, 2000; Gimsing *et al.*, 2004). Under agricultural soil conditions, depending on soil and environmental conditions half-life times of glyphosate in soils range from 1-197 days but are often less than 60 days (Giesy *et al.*, 2000).

Due to the very limited plant availability of glyphosate residues in soils glyphosate can be used in agricultural cropping systems without waiting times between application and sowing of crops.
However, poor establishment and growth of succeeding crops has been repeatedly reported by farmers and scientists when glyphosate or other non-selective herbicides have been used to kill weeds before sowing of crops in no tillage or conservation tillage systems in the United States, Australia and Germany (Smiley *et al.*, 1992; Descalzo *et al.*, 1998 and references cited therein; Römheld *et al.*, 2008). A stimulation of root pathogens such as *Rhizoctonia* and *Phytium* attracted by the decaying weed residues (Smiley *et al.*, 1992; Descalzo *et al.*, 1998; Powell and Swanton, 2008), the release of allelopathic compounds (Dudai *et al.*, 2009) and rhizosphere transfer of the herbicide during degradation of the weed residues to germinating seeds and seedlings of the subsequent crop (Neumann *et al.*, 2006; Römheld *et al.*, 2008; Tesfamariam, 2009) have been discussed as possible reasons, but the underlying mechanisms are still not clear.
Therefore the aim of the present study was

(i) a systematic investigation of the phenomenon in field experiments,
(ii) to simulate the effects in parallel model studies under controlled environmental conditions to identify the underlying mechanisms,

(iii) to characterise risk factors favouring the induction crop damage which should be excluded as far as possible in agricultural practice.

5.3 Material and Methods

Evaluation of pre-crop glyphosate application under field conditions

Set-up of field trials

Four field trials with winter wheat (*Triticum aestivum* L.) cropping systems were established together with local farmers between September and October 2008 in South West Germany near Tauberbischofsheim (TB), Dusslingen (DU), Bad Rappennau (BR) and Starzach (ST). The experiments were arranged in a randomised block design with three replicates for each treatment.

To evaluate waiting time effects between pre-crop herbicide applications and sowing date, time intervals of 18-20, 10 and 1-2 days between pre-crop herbicide application and sowing of winter wheat were investigated at the field sites DU, ST and TB. Due to unfavourable weather conditions waiting times at BR comprised 22, 14 and 9 days in a minimal tillage system.

The additional influence of different cropping systems (minimal tillage vs no-tillage) was investigated at the field sites TB and DU. At the field site ST, a potential impact of weed population density was evaluated by comparing herbicide applications to the natural weed population and to plots with additional sowing of 100 kg wheat ha^{-1} as target plants.

Herbicide application

Glyphosate was applied in two commercial formulations (Roundup Ultra Max®, 2 L ha^{-1} and Clinic, 2.4 L ha^{-1} diluted in 200 L water). Controls included the application of a mixture of the herbicides Basta® and Agil-S® approx. 20 days before sowing (DBS) and of Basta® at 1-2 DBS. The dominant herbicide-treated weed populations comprised a weakly developed barley (*Hordeum vulgare* L.) stand at TB, hail-damaged oat (*Avena sativa* L.) and slender meadow foxtail (*Alopecurus myosuroides* L.) at DZ, and self-sown wheat in BR and ST.

Plant sampling

During the two month growth period before the onset of winter in early December 2008 seedling development, crop density, expression of chlorosis (SPAD-value) and biomass production as indicators for crop damage were recorded. To achieve comparability in scoring of plant damage between the field sites under different climatic and soil conditions decline in seedling development, crop density SPAD-value and biomass were calculated in % of optimal treatment (e.g. long waiting time Basta®/Agil-S® and/or Roundup® treatments). The means of the calculated damage in % of optimal treatments was used as common damage index for all field trials.
Leaf and root samples for analysis of accumulation of shikimate as physiological indicator of glyphosate-toxicity were taken from all plots at growth stage BBCH 11-13 (approx. 4 weeks

after emergence) and at BBCH 16-17 (approx. 8 weeks after germination for TB. DU and BR) (Lancashire *et al.*, 1991).
Additionally, shoots sampled at growth stage BBCH 16-17 in TB, DU and BR and at BBCH 11-13 in ST were used for determination of the plant nutritional status.

Parts of the leaf samples taken for shikimate analysis during growth stage BBCH 11-13 were pooled and submitted to SGS Institute Fresenius GmbH (Taunusstein, Germany) for determination of glyphosate residues in shoots by HPLC (DFG, 1996).

Evaluation of pre-crop glyphosate application in model experiments

Model experiments with winter wheat (*Triticum aestivum* L.) cv. Isengrain-B were conducted on soils sampled from no-tillage field sites in South West Germany near Hirrlingen (pH ($CaCl_2$) 5.8; sand [%] 5.7; silt [%] 56.2; clay [%] 38.1; Corg [%] 1.96) and Tübingen (pH ($CaCl_2$) 7.1; sand [%] 8.4; silt [%] 67.8; clay [%] 23.8; Corg [%] 1.90).

Field soil was air-dried and sieved through 2 mm mesh size. Fertilisation was conducted 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 $Ca(H_2PO_4)_2$. After fertilisation, the soil was sieved again and soil moisture was adjusted to 70% of the soil water-holding capacity. During vegetation period water loss was determined gravimetrically and replaced by daily applications of deionised water.

Cultivation of target plants

To investigate the effects of glyphosate residues in root tissue of target weed on subsequently cultivated non-target plants in all experiments winter wheat *(Triticum aestivum* L. cv. Isengrain-B) was pre-cultivated as target weed in 550 g pots filled with fertilised field soils. In experiments with field soil from Hirrlingen studying the effect of short waiting time between glyphosate application on weeds and sowing of crops, a sowing density of 4 g target weed seeds (*Triticum aestivum* L.) (germination rate approx. 90 %) per pot with a surface area of 100 cm^2 was used to simulate high weed coverage of the soil with intense root development. In experiments with field soil from Tübingen studying effects of different waiting times and densities of glyphosate treated weeds on non-target plants, 1-, 2- and 4.5 g of target weed seeds pot^{-1} (*Triticum aestivum* L.) were sown in a sequential way to achieve an identical sowing date for crops of 21, 14, 7 and 0 days after pre-crop glyphosate application.

To ensure comparable environmental conditions for growth of target and subsequently sown non-target plants (winter wheat cv. Isengrain-B), as well as degradation of root residues of glyphosate-treated target plants, model experiments were conducted in a growth chamber under controlled environmental conditions with a light/dark regime of 14/10 h at 18-16 °C, light intensity of 200 µmol m^{-2} s^{-1} at canopy height, provided by fluorescent lamps (Osram HQL-R 400, Osram, Munich, Germany) and 60% relative humidity.

Glyphosate application

To simulate the application technique and applied amounts realistic under field conditions a track-spraying device (Wöhrle WST 144, Germany) (application volume 400 L ha^{-1}, application speed 800 mm s^{-1}, application pressure 6 bar, application height 50 cm) was used. Ten days after sowing (DAS) glyphosate was applied to target weed seedlings (wheat) in concentrations of 28.4 mM, representing an application amount of 4 L ha^{-1} which is realistic and recommended for most field conditions.

Two days (Hirrlingen) or 4 days (Tübingen) after application of glyphosate shoots of treated target-wheat plants were cut approx. ½ cm above soil surface and removed from the pots. Similarly in control treatments without glyphosate application, target-wheat shoots were removed by cutting at the soil surface with a sharp knife.

Cultivation of non-target plants

Subsequently, after a waiting time of 2 days (soil from Hirrlingen) or 21, 14, 7 and 0 days (soil from Tübingen) 10 seeds of winter wheat (*Triticum aestivum* L., cv. Isengrain-B) were sown into the same pots. During the whole growth period of both experiments parameters such as seedling development, leaf development, leaf morphology and leaf surface area, plant growth and expression of chlorosis (SPAD-value) were recorded and scored as visual indicators of glyphosate toxicity. Two weeks after sowing plants were harvested and fresh weights of all plant parts (roots and shoot) were separately determined. Roots and shoots were separated, frozen in liquid nitrogen and stored at -20 °C for analysis of accumulation of shikimate in plant tissue as physiological indicator of glyphosate toxicity. The frozen shoot tissue was subsequently homogenised under liquid nitrogen using mortar and pestle and separated into two parts used for shikimate analysis and for the determination of nutritional status of plants. Fresh shoot homogenate used for mineral analysis were weighted into crucibles and carefully dried at 60 °C in a dry-oven.

Root and seed exposure of winter wheat to glyphosate and AMPA

Hydroponic experiments were performed in a growth chamber under controlled environmental conditions with a light/dark regime of 14/10 h at 24/20 °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.

Preculture of wheat seedlings and application of glyphosate and AMPA

Winter wheat seeds (*Triticum aestivum* L.) cv. Isengrain-B were sterilised 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 $CaSO_4$. Thereafter seedlings were cultivated for 24 hours in continuously aerated falcon tubes containing double deionised water (control), glyphosate as Roundup UltraMax®, glyphosate as N-phosphono-methylglycine or AMPA in concentrations of 10 µM. Thereafter, plants were transferred to pots (6 plants per 2.8 L plastic pot) containing

continuously aerated nutrient solution (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, 1 µM H_3BO_3, 0.5 µM $ZnSO_4$, 0.5 µM $MnSO_4$. 0.2 µM $CuSO_4$ and 0.01 µM $(NH_4)_6Mo_7O_{24}$).

Plant sampling

During the whole growth period of 21 days, parameters of shoot growth and development were recorded for indications of potential glyphosate and AMPA toxicity. In addition, 72 h after the end of herbicide treatment pattern of root growth and root morphology of plants were analysed using the WinRhizo Pro® digital imaging software (Regent Instruments, Quebec, Canada). At day 7 after transfer, a first set of 3 plants was removed from the pots. Roots and shoots were separated, frozen in liquid nitrogen and stored at -20°C for analysis of accumulation of shikimate in plant tissue as physiological indicator of glyphosate toxicity. In each pot, three plants were kept and further cultivated until final harvest 21 days after transfer. At final harvest, fresh weights of all plant parts (roots and shoot) were determined. Dry weights of roots and shoots were determined after oven-drying at 60 °C. Subsequently dried shoots were grinded for analysis of nutritional status of plants.

To evaluate the effect of glyphosate and AMPA on germination, 140 seeds of winter wheat (*Triticum aestivum* L.) cv. Isengrain-B were put for 5h into 200ml double deionised water, or 200ml solution containing 10 µM glyphosate (Roundup®) or AMPA. Solutions were continuously carefully shaken. Afterwards, seeds were placed on filter paper for germination. For each treatment 4 filter papers each with 35 seeds were put in an upright position in germination boxes containing approx. 150 ml of 2 mM $CaSO_4$ solution. For initial germination plants were caped for 80h in controlled conditions (no light, 22 °C). To avoid effects of fungal infection or potential stress of $CaSO_4$ after initial germination filter papers were opened seeds/seedlings were checked for fungal infection and placed on new filter paper soaked with 2 mM $CaSO_2$. Subsequently, wheat seedlings were cultivated on filter paper for additional 48 h at a light/dark regime of 14/10 h at 22 °C. Afterwards germination rate and shoot and root fresh weight of germinated seedlings were determined.

Shikimate analysis

Shikimate in acidic tissue extracts was analysed with modifications of the methods described by Singh and Shaner (1998) and Neumann (2006). The frozen plant tissue was homogenised 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 20.000 x g) and the supernatant was used for HPLC analysis after appropriate dilution with the HPLC mobile phase. HPLC separation was performed by ion exclusion chromatography using an Aminex 87H column (Bio-Rad, Richmond, CA, USA) designed for organic acid analysis. A sample volume of 20 µL was injected into the isocratic flow (0.5 mL min^{-1}) of the eluent (2.5 mM H_2SO_4, 40 °C) and organic acids were detected spectrophotometrically at 210 nm. Identification and quantification of shikimate was conducted by comparing the retention times, absorption spectra and peak areas with a known standard.

Glyphosate analysis

Glyphosate determination in 10 g FW samples of shoot tissue of wheat seedlings sampled at the field sites was conducted at the SGS Institute Fresenius GmbH, Taunusstein, Germany. By HLPC with detection of fluorescence after post column derivatisation according to method 405 developed by the Deutsche Forschungsgemeinschaft (DFG) for determination of glyphosate and AMPA in water, soil or plant samples (DFG, 1996).

Analysis of mineral nutrients

One hundred milligram of dried shoot material was ashed in a muffle furnace at 500°C for 5 h. After cooling, the samples were extracted twice with 1 mL of 3.4 M HNO_3 and evaporated until dryness to precipitate SiO_2. The ash was dissolved in 1 mL of 4 M HCl, subsequently diluted ten times with hot deionised water, and boiled for 2 min to convert meta- and pyro-phosphates to orthophosphate. 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). Spectrophotometrical determination of orthophosphate was conducted after addition of molybdate-vanadate colour reagent according to the method of Gericke and Kurmis (1952). Determination of Mg was conducted by atomic absorption spectrometry, while K and Ca were measured by flam photometry.

Statistics

Field trials were conducted in a randomised block design with three replicates for each herbicide treatment. Soil experiments were conducted in a completely randomised block design with four replicates per treatment. Nutrient solution experiments were conducted in a completely randomised block design with three 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).

5.4 Results

Role of waiting times after pre-crop application of glyphosate

In three out of four field experiments (DU, TB, ST) short waiting times (1-2 days) after pre-crop glyphosate application resulted in a significant damage of subsequently sown winter wheat. Damage symptoms were first detectable at BBCH 16-17 (approx. 8 weeks after germination) and comprised reduced plant density, stunted shoot growth, chlorosis and necrosis of older leaves and needle-shaped deformations of young leaves (Fig. 5.1). Crop damage was induced by application of two commercial glyphosate formulations (Roundup Ultramax® 2 L ha^{-1}; Clinic® 2.5 L ha^{-1}) but was not detectable after waiting times of 18-20 days. At the field site BR with a waiting time of 9 days as shortest time span between glyphosate application and sowing of winter wheat, no damage symptoms were observed.

A certain degree of plant damage also appeared in the glufosinate (Basta®) variants after short waiting times of 1-2 days but significant effects were detected only at the field site DU (Tab. 5.1).

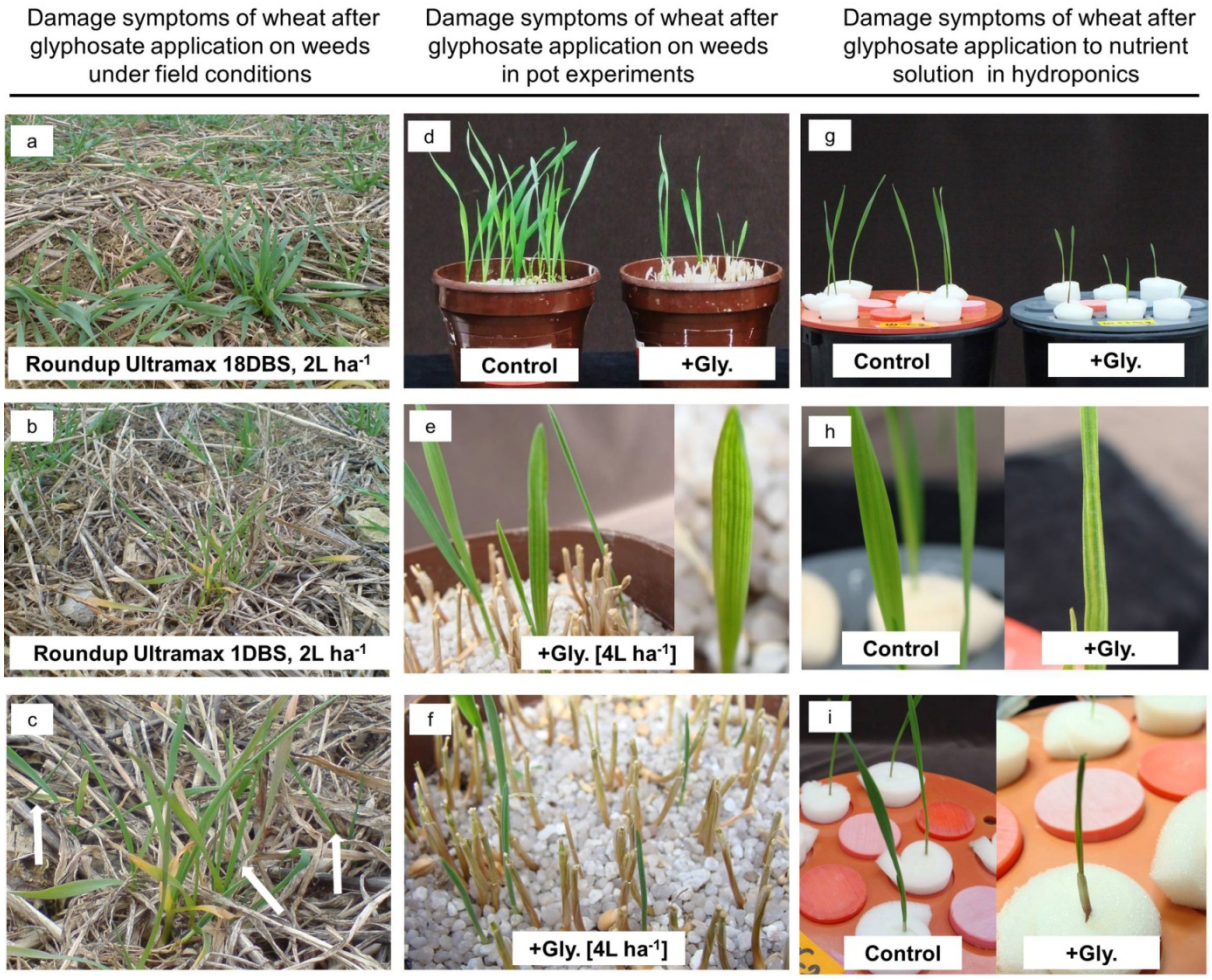

Fig. 5.1: Plant growth and symptoms of glyphosate-induced damage of winter wheat in the field, in pot experiments and in hydroponics

Plants were grown under no tillage conditions on a field site in TB in case of long- (18 days) and short waiting time (1 day) after glyphosate application on a dense population of weed plants (a, b, c), in model experiments under soil conditions with short waiting time (2 days) after pre-crop glyphosate application with track-sprayer (d, e, f) and under hydroponic conditions after short term (24h) root exposure to 10µM glyphosate (Roundup UltraMax®) (g, h, i). Comparable damage symptoms comprised of delayed and weak development (b, c, d, g), chlorosis (b, c, e, h) and needle-shaped leaf defomations (c, f, i) was observed in case of phytotoxic glyphosate in the rhizosphere. Needle-shaped leaf deformations are indicated with arrows in the close-up of damaged wheat plants in case of short waiting after glyphosate under field conditions (c).

Tab. 5.1 Damage index of plants at the field sites of Dusslingen, Tauberbischofsheim and Bad Rappenau

Damage was observed after pre-crop application of glyphosate (Roundup UltraMax®, Clinic®), Basta®/Agil-S® mix (control) or Basta® (control) at different waiting times before sowing (DBS) of winter wheat (*Triticum aestivum* L.) under no-tillage (left) and or minimal tillage conditions (right). Data represent means and standard deviations of 3 independent replicates. Significant differences ($p<0.05$) are indicated with different characters.

		Damage Index [%]					
Days	**Waiting time/ herbicide treatment**	**no tillage**			**minimal tillage**		
				Dusslingen			
18 /	**Roundup®UltraMax**	**8**	±2	D	**4**	±3	C
18 /	**Clinic®**	**10**	±1	D	**8**	±2	C
18 /	**Agil+Basta®**	**5**	±2	D	**5**	±0	C
10 /	**Roundup®UltraMax**	**36**	±3	C	**25**	±3	AB
10 /	**Clinic®**	**43**	±2	BC	**24**	±4	AB
1 /	**Roundup®UltraMax**	**52**	±2	A	**32**	±3	A
1 /	**Clinic®**	**50**	±3	AB	**30**	±4	A
1 /	**Basta®**	**36**	±6	C	**20**	±3	B
				Tauberbischofsheim			
18 /	**Roundup®UltraMax**	**1**	±0	D	**8**	±1	BC
18 /	**Clinic®**	**2**	±2	D	**7**	±4	C
18 /	**Agil+Basta®**	**5**	±2	CD	**12**	±2	ABC
10 /	**Roundup®UltraMax**	**10**	±2	CB	**14**	±6	ABC
10 /	**Clinic®**	**8**	±1	CB	**17**	±1	AB
1 /	**Roundup®UltraMax**	**19**	±1	A	**19**	±1	A
1 /	**Clinic®**	**18**	±1	A	**19**	±5	A
1 /	**Basta®**	**11**	±3	B	**16**	±3	ABC
				Bad Rappenau			
22 /	**Roundup®UltraMax**	-	-	-	**13**	±3	n.s
22 /	**Clinic®**	-	-	-	**11**	±9	n.s
22 /	**Agil+Basta®**	-	-	-	**10**	±9	n.s
14 /	**Roundup®UltraMax**	-	-	-	**16**	±9	n.s
14 /	**Clinic®**	-	-	-	**20**	±11	n.s
9 /	**Roundup®UltraMax**	-	-	-	**22**	±3	n.s
9 /	**Clinic®**	-	-	-	**25**	±12	n.s
9 /	**Basta®**				**14**	±1	n.s

The same symptoms of plant damage as described for the field sites DU, TB, ST were observed in pot experiments under controlled environmental conditions on two soils of the field trial programme, when glyphosate (Roundup Ultramax® 4 L ha^{-1}) was applied with a track sprayer on a wheat pre-culture as target plants with subsequent sowing of winter wheat with waiting times of 0 -2 days. Germination was significantly reduced by up to 20 % (Tab. 5.3) Plant biomass reduction, stunted growth (Fig. 5.1d; Fig. 5.4a) chlorosis of older leaves and deformation of young leaves (Tab. 5.2; Fig. 5.1e,f) was similarly expressed as in the field experiments (Fig.5.1c). This pattern of damage symptoms was unexpected since glyphosate toxicity usually affects the youngest leaves first but associated to significant increase in intracellular accumulation of shikimate as physiological indicator for glyphosate toxicity in root tissue (Tab. 5.2).

Tab. 5.2 Parameters of plant damage of winter wheat plants

Development of needle-shaped leaf-deformations, chlorosis scoring, shoot and root biomass of winter wheat plants (*Triticum aestivum* L.) at final harvest and concentrations of shikimate as indicator for glyphosate toxicity in roots of plants were measured in case of short waiting time of 2 days after pre-crop application of glyphosate (+Gly) applied with a track-sprayer at an application level of 4 L ha^{-1}. In control treatments (Control) without glyphosate application, shoots of target-plants were removed by cutting at the soil level with a sharp knife. Data represent means and standard deviations of 4 independent replicates. Significant differences ($p<0.05$) are indicated with different characters.

	Parameters of plant damage [2 days waiting time]				
treatment	**leaf deformations**	**Green value of leaves**	**Shoot biomass**	**Root biomass**	**shikimate concentration in roots**
	[% of plant pot^{-1}]	[SPAD]	[g fresh weight]	[g fresh weight]	[µg g fresh weight]
Control	0±0 B	43±2 A	1.26±0.2 A	1.21±0.3 A	15±6 B
+ Gly	43±14 A	30±1 B	0.58±0.2 B	0.67±0.3 B	51±16 A

Comparable plant damage was detectable in pot experiments when the aboveground parts of the target plants were removed 2 days after glyphosate application prior to sowing of the subsequent winter wheat culture. In contrast, no crop damage occurred in control treatments without glyphosate application when the shoots of the target plants were removed by cutting at the shoot base. These findings suggest that root residues of glyphosate treated target plants are a source of toxicity for the subsequent crop (Tab. 5.2).

To investigate toxicity symptoms in a scenario when only roots and not the shoot tissues of winter wheat seedlings are exposed to glyphosate, (e.g by rhizosphere transfer from rot residues of glyphosate-treated target weeds), a series of nutrient solution experiments was conducted with different concentrations of glyphosate, applied to the growth medium. Under

soil conditions, the described rhizosphere transfer of glyphosate is supposed to be a transient process, since glyphosate is rapidly inactivated by adsorption to the soil matrix and by microbial degradation. Therefore, the roots of winter wheat seedlings in the nutrient solution experiments were only exposed to glyphosate (10 µM) during a time period of 24 h. Plant damage was already detectable after exposure to glyphosate concentrations of 10 µM with comparable symptoms of growth inhibition, chlorosis of older leaves, needle-shaped deformations of young leaves (Fig. 5.1h, g, i; Fig. 2a) as observed in the field studies (Fig. 5.1a,b,c) and in the pot experiments (Fig. 5.1d,e,f). In contrast, the same amounts of AMPA as the main metabolite of glyphosate in soil, applied to the nutrient solution, did not induce plant damage (Fig. 5.2a,b, c). However, exposure of wheat seeds to solutions of AMPA (10 µM) during a time period of 5 h significantly reduced the germination rate, while germination was not affected by the same amount of glyphosate (Fig. 5.2d).

Shikimate accumulation as physiological indicator for glyphosate toxicity was detected in the root tissue of the damaged plants in all pot experiments (Tab. 5.2; Fig.5.2c, 5.4c) while in the field studies, a significant increase of shikimate concentrations in the surviving plants of the treatments with short waiting times (1-2 days) after glyphosate application was only detectable at the field site TB (data not shown). Glyphosate and AMPA in the shoot tissue of the surviving plants in the field experiments remained below the detection limits of 0.5 µg g^{-1} fresh weight.

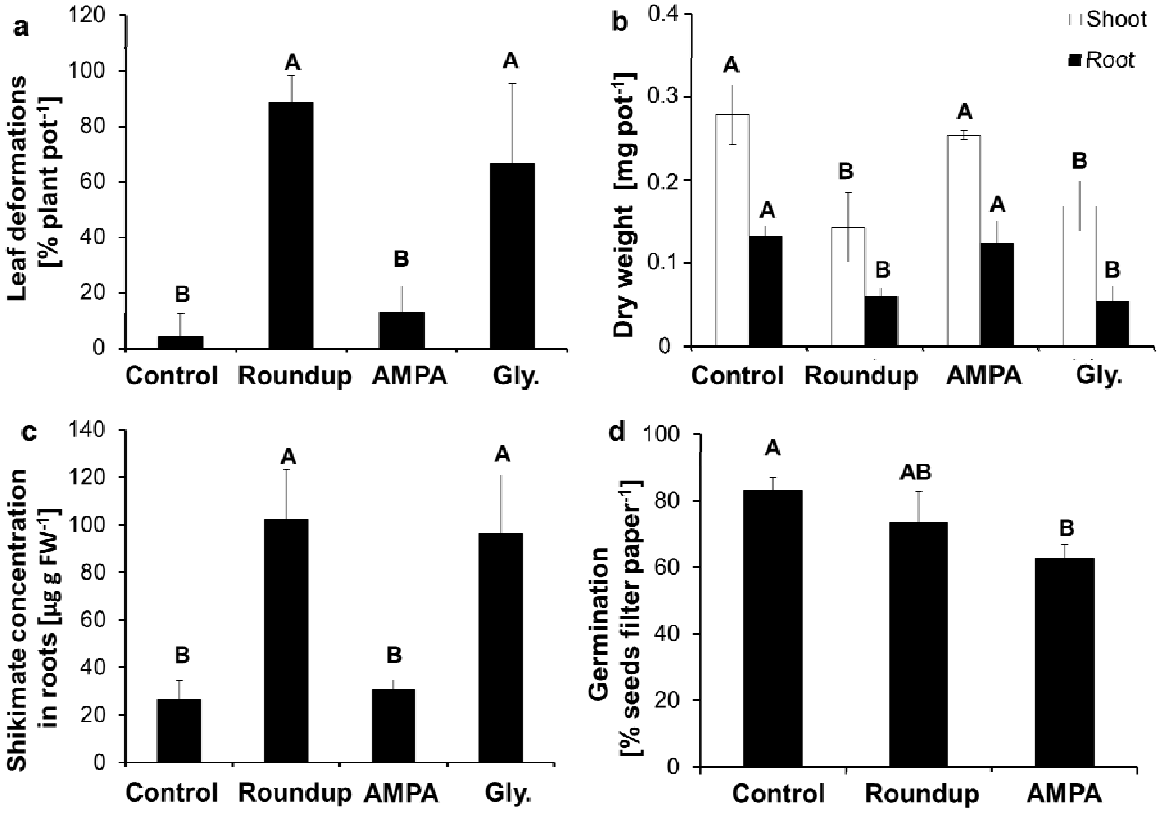

Fig. 5.2: Leaf deformation, biomass and shikimate concentrations of winter wheat depending on glyphosate and AMPA application

Leaf deformations (a), shoot and root dry weight at final harvest (b) and shikimate concentrations in roots (c) of wheat (*Triticum aestivum* L.) were measured in case of 24 h root exposure to concentrations of 10µM of glyphosate Roundup® UltraMax (glyphosate), AMPA (main metabolite) under hydroponic conditions and germination rate (% seeds^{-1}) after 5 h seed exposure to Roundup® UltraMax or AMPA before germination on filter papers (d). Data represent means and standard deviations of 3 independent replicates in case of hydroponic experiments (a,b,c) and 4 independent replicates for germination tests on filter paper (d). Significant differences ($p<0.05$) are indicated with different characters.

Role of the population density of target weeds

At the field sites TB and DU, dense populations of pre-damaged barley and oat were dessicated by the herbicide treatments prior to sowing of winter wheat. At the field site ST, two different densities of weed populations were tested including (i) the natural weed population and (ii) additional sowing of wheat (100 kg ha^{-1}). Fig 3 demonstrates that high densities of the weed population promote the expression of damage in the subsequent culture (here expressed as biomass of winter wheat seedlings) sown with a short waiting time after pre-crop glyphosate application.

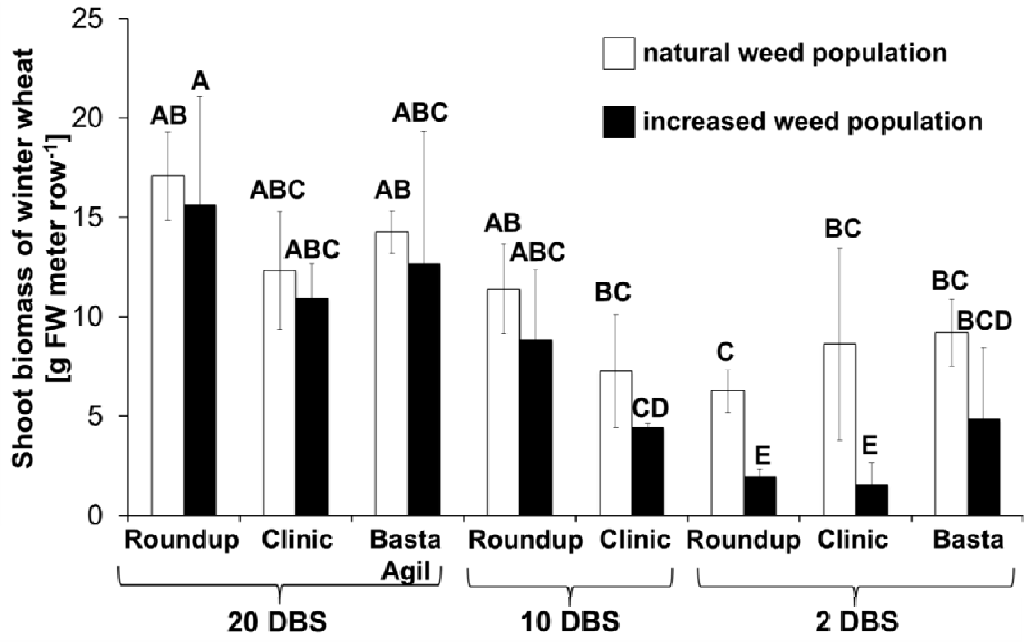

Fig. 5.3: Shoot biomass of winter wheat depending on herbicide application and weed population

Shoot biomass [g fresh weight m row^{-1}] of winter wheat (*Triticum aestivum* L.) grown under field conditions (BBCH 30-31) at the field site located in Starzach (Southwest Germany) were measured in a minimal-tillage system with natural and artificially increased density of weeds and different waiting times between herbicide application and sowing of winter wheat (days before sowing: DBS). Data represent means and standard deviations of 3 independent replicates. Significant differences ($p<0.05$) are indicated with different characters.

Similarly, in a pot experiment under controlled environmental conditions short waiting times after pre-crop glyphosate application on increasing densities of weed plants (1, 2 or 4.5 g wheat seeds pot^{-1}) caused in comparison to controls significantly impaired germination, declined shoot biomass production and increased intracellular shikimate concentrations. Intensity of glyphosate-induced plant damage of winter wheat plants was positively correlated to increasing density of glyphosate treated weed plants (Tab. 5.3; Fig 5.4a, b, c).

Tab. 5.3 Germination of winter wheat depending on waiting time and density of glyphosate-treated weeds

Emergence of winter wheat (*Triticum aestivum* L.) grown under climate chamber conditions on a soil of the field trial programme (Tübingen, Southwest Germany) were determined 4, 5, 6, 8, 9 and 11 days after sowing (DAS) in % of seeds pot^{-1} depending on the impact of waiting time until sowing after glyphosate pre-crop application and of sowing density (1, 2 or 4.5g seeds pot-1). In control treatments (Control) without glyphosate application, shoots of target-plants were removed by cutting at the soil level with a sharp knife. Data represent means and standard deviations of 4 independent replicates. Significant differences ($p<0.05$) are indicated with different characters.

	Germination of wheat [% seeds pot^{-1}]					
treatment	**4 DAS**	**5 DAS**	**6 DAS**	**8 DAS**	**9 DAS**	**11 DAS**
Control			**0 days waiting time**			
bare soil control	21±1 D	75±2 B	90±1 A	90±1 A	92±1 AB	94±1 A
1g weed pot-1	25±0 D	75±1 B	85±1 B	92±1 A	94±1 A	94±1 A
2g weed pot-1	50±1 A	81±2 A	85±2 B	90±2 A	90±2 AB	90±2 AB
4.5g weed pot-1	46±1 B	77±1 B	85±1 B	85±1 B	90±1 B	90±1 B
+ Gly			**0 days waiting time**			
1g weed pot-1	8±1 F	67±2 C	73±2 C	79±1 C	79±1 D	79±1 D
2g weed pot-1	6±1 F	40±1 D	65±1 D	73±1 D	75±1 E	75±1 E
4.5g weed pot-1	4±1 F	44±1 D	63±1 D	69±1 E	69±1 F	69±1 F
Control			**21 days waiting time**			
1g weed pot-1	17±1E	79±1 B	88±1 AB	94±1 A	94±1 A	94±1 A
2g weed pot-1	13±2 E	85±1 A	88±1 AB	92±1 A	94±1 A	94±1 A
4.5g weed pot-1	15±1 E	75±1 B	83±1 B	85±3 AB	88±1 C	88±1 C
+ Gly			**21 days waiting time**			
1g weed pot-1	44±3 B	81±3 A	83±3 B	85±3 AB	85±3 C	85±3 C
2g weed pot-1	35±1 C	79±2 B	81±2 B	85±5 AB	85±2 C	85±2 C
4.5g weed pot-1	29±1 C	71±2 C	83±2 B	90±2 A	90±2 AB	92±2 AB

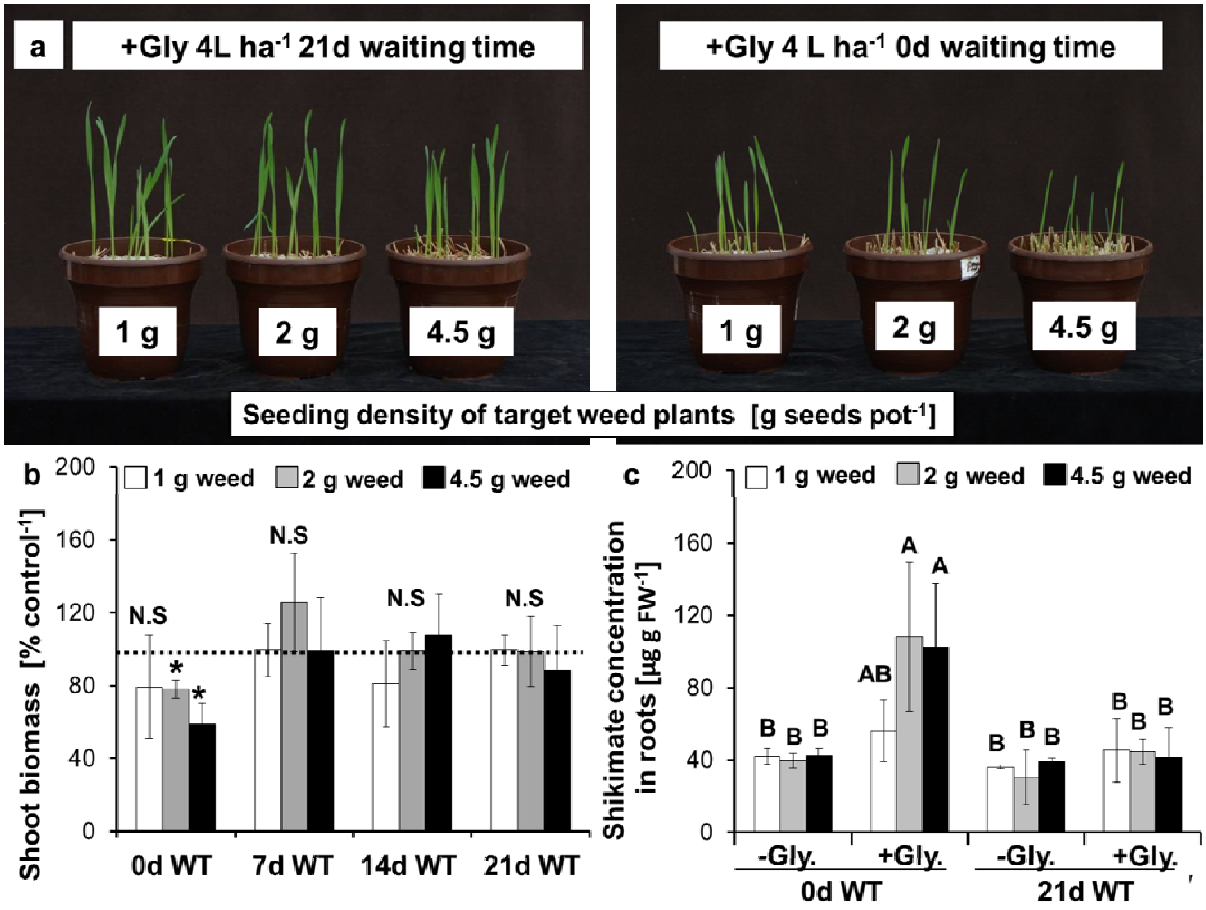

Fig. 5.4: Shoot biomass and shikimate concentration of winter wheat depending on waiting time and density of glyphosate-treated weeds

(a) Effects of pre-crop application of glyphosate and different weed densities in case of 21 days (21d) waiting time and 0 days (0d) waiting time until sowing of winter wheat (*Triticum aestivum* L.). (b) Relative shoot biomass of winter wheat (% control-1) 18 days after germination depending on a waiting time (WT) of 0, 7, 14 or 21 days between glyphosate application on different weed densities and sowing of crops. (c) Shikimate concentrations in roots of the respective wheat seedlings in case of 21 days waiting time and 0 days waiting time until sowing of winter wheat. Data represent means and standard deviations of 4 independent replicates. Significant differences ($p<0.05$) are indicated with different characters.

Role of tillage treatment

At the field sites TB and DU, effects of desiccation barley and oat as weed plants by herbicide treatments prior to sowing of winter wheat were studied in no- and minimal tillage conditions.

At the field site DU, damage of winter wheat in case of short waiting times (1-2 days) after pre-crop glyphosate application was substantially increased under no-tillage compared to minimal tillage conditions. No differences between the tillage treatments in (marginal) plant damage could be detected in case of long waiting time (Tab. 5.1). At the field site of TB development of winter wheat was generally weaker in case of minimal tillage- compared to no-tillage treatment. However, in comparison to long waiting time treatments damage of

winter wheat induced by short waiting time after pre-crop glyphosate application was increased under no-tillage conditions (Tab. 5.1).

Effects on the plant nutritional status

There was no consistent relationship between pre-crop herbicide applications, waiting time until sowing and nutritional status of the following crop. In model experiments with short waiting times (2days) after pre-crop glyphosate application in young wheat seedlings (10 DAS), particularly macronutrient concentrations (Ca, Mg, K) declined below the critical levels, while micro nutrients (Fe, Zn, Mn) remained unaffected. By contrast, in the field experiments shoot macronutrient concentrations were not influenced by herbicide treatments but on some field sites Zn and Mn concentrations declined close to/ below critical levels in case of short waiting time after pre-crop application of glyphosate (and Basta®).

Effects on final yield

Reduced crop densities in the short waiting time treatments after pre-crop glyphosate applications were still detectable in spring at BBCH 30-31. Thereafter, the remaining plants increasingly compensated these effects by improved plant development and tillering due to less competition as a consequence of lower plant densities. Particularly at the field site DU this was also reflected by delayed senescence probably caused by increased nitrogen availability to individual plants.

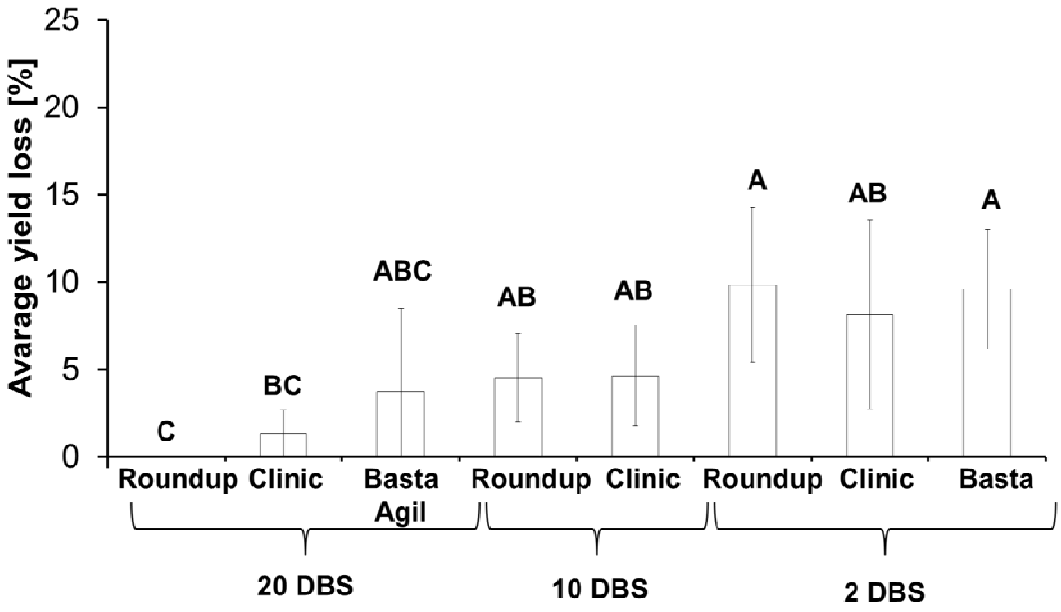

Fig. 5.5: Average yield loss of winter wheat at the field sites of Dusslingen, Tauberbischofsheim and Starzach depending on waiting time between glyphosate application and sowing

Winter wheat (*Triticum aestivum* L.) plants were grown under minimal or no-tillage conditions at the field sites Dusslingen, Tauberbischofsheim and Starzach (Southwest Germany), sown after different waitings times (days before sowing; DBS) between herbicide application of glyphosate (Roundup UltraMax®, Clinic®) or herbicide controls (Basta®/Agil-S® mix or Basta®). Data represent means and standard deviations of 3 independent replicates. Significant differences ($p<0.05$) are indicated with different characters.

5.5 Discussion

Similarly to results of preliminary experiments under field conditions (Römheld *et al.*, 2008) results of the field trials in TB, DU, BR and ST revealed consistently damage of winter wheat in juvenile growth stages in case of short waiting time between pre-crop application of glyphosate and sown of crop plants (Tab. 5.1). Moreover, plant damage comprising reduced plant density, stunted shoot growth, chlorosis and necrosis of older leaves and needle-shaped deformations of young leaves were correlated to glyphosate application on a dense population of weed plants (Tab. 5.1; Fig. 5.1a, b, c). In comparison between reduced tillage systems, damage of winter wheat induced by short waiting time after pre-crop glyphosate application was at the field sites of TB and DU more pronounced in no-tillage compared to minimal tillage conditions (Tab. 5.1).

Glyphosate transfer in the rhizosphere

Results of the model experiments under soil- and hydroponic conditions offered further evidence supporting the rhizosphere transfer of phytotoxic glyphosate from treated weed plants to subsequently wheat plants as causal explanation for damage observed under field conditions.

Experiments under hydroponic conditions gave clear indications that damage symptoms of wheat plants highly similar to those observed in field trials and model experiments under soil conditions were primarily caused by glyphosate (Fig. 5.2a,b,c), while its main metabolite aminomethylphosphonic acid (AMPA), considered also as phytotoxic (Reddy *et al.*, 2004) caused significantly declined germination (Fig. 5.2d). Recently, a Monsanto patent on production of AMPA-resistant crops reported an inhibitory effect of AMPA on wheat in an embryo germination test (Barry, 2009). Under field conditions heterogeneity of damage symptoms in crop plants may arise, depending on whether glyphosate or AMPA or both are present in a damaging amount in a sensitive developmental stage of plants.

In model experiments under soil condition shoots of glyphosate treated weed plants were removed from the pots prior to emergence of subsequently sown winter wheat plants. Because of this, damage of wheat plants in these experiments (Tab. 5.2; Fig. 5.4a, b, c) was plausibly caused by a transfer of phytotoxic glyphosate from treated weeds to subsequently sown crops through in the rhizosphere which is also in accordance with the knowledge on the behaviour of glyphosate *in planta*.

In most plant species, glyphosate is not readily metabolised and is preferentially translocated to young growing tissues of roots and shoots, where it can accumulate in millimolar concentrations (Reddy *et al.*, 2004). A subsequent release and transfer of glyphosate via the rhizosphere to causing damage to following crop plants have been reported by Neumann *et al.* (2006) and Tesfamariam (2009).

According to Reddy *et al.* (2004) distribution of glyphosate within the root tissues is inhomogeneous. Thus, according to Tesfamariam (2009) this potentially leads to the formation of hot spots of root residues in soils, containing high levels of glyphosate. Glyphosate toxicity to subsequently grown crop plants may be induced by root contact with these hot spots (Tesfamariam, 2009).

In contrast to model experiments under hydroponic and soil conditions (Tab. 5.2; Fig. 5.2c, 5.4c), significant accumulation of shikimate as physiological indicator of glyphosate toxicity was detectable under field conditions only at the field trial in TB. However, according to results of Bresnahan *et al.* (2003) a transient phenomenon which potentially peaks already 3-7 days after exposure to phytotoxic glyphosate. Thus, limited detectability of shikimate accumulation in shoot and root of wheat plants under field conditions might be related to a relatively late sampling date earliest approx. 4 weeks after emergence. Moreover, it is likely that plants strongly damaged by glyphosate died off before the first sampling while moderately damaged plants remained. This might have also limited detectability of glyphosate concentrations in shoot tissue of wheat, which remained below the detection limit of the HPLC-based method (0.5 µg g DW^{-1}) (DFG, 1996). However, according to Allister *et al.* (2005) and Wagner *et al.* (2003) in case of root supplied glyphosate up to 80% remain in roots, while impaired shoot growth is also ready induced by uptake of amounts of 1µg glyphosate $seedling^{-1}$. Accordingly, a re-growth of 1-2 g fresh weight during recovery from glyphosate toxicity potentially cause glyphosate concentrations close to or even below the

detection limit of the HPLC-based method (0.5 µg g DW^{-1}) used for determination of glyphosate.

Transfer of systemic herbicides through the rhizosphere has been also observed in case of mesotrione and imazapyr (Boydston *et al.*, 2008). Under field conditions, selective application of mesotrione on single plants for control of volunteer potato in crops resulted in mesotrione damage of plants growing adjacent to treated volunteer potatoes.

The mobility of glufosinate (Basta®) in planta employed in the present study as control is not entirely understood. Even so it is considered phloem-immobile, Steckel *et al.* (1997) showed that depending on the plant species up to 80 % of the translocated Basta® was found below the treated leaves (e.g. roots). Thus, it cannot be ruled out that damage of wheat in Basta®/control treatments was caused by a phytotoxic effect after transfer in the rhizosphere.

Role of the population density of target weeds

Results of the present study revealed both under field conditions (Fig. 5.3) as well as in model experiments (Fig. 5.4a, b, c) a significant correlation between the density of glyphosate treated weed plants and intensity- and duration of damage of subsequently cultivated winter wheat. Therefore it is plausible that glyphosate in root tissue of treated weed plants additionally acts as storage pool of glyphosate in the soil which is released after/during microbial degradation of treated root material affecting and prolonging the time-window of potential glyphosate phytotoxicity to subsequently sown crop plants.
In line with this, Doublet *et al.* (2009) reported that absorption of glyphosate in plants delays its subsequent soil-degradation causing two to six increased times persistence in soil. Results of von Wirén-Lehr *et al.* (1997) suggest different mechanisms for degradation of glyphosate adsorbed to the soil matrix and bound in plant residues in the soils, respectively.

Role of tillage treatment

As the results of the present study indicate, in comparison between different tillage treatments damage of crops induced by short waiting time after glyphosate application was at least in tendency stronger expressed in case of no-tillage- compared to minimal tillage treatments (Tab. 5.1). This effect might be explainable by the increased dispersion and soil mixing of glyphosate containing root material in the minimal tillage treatment potentially causing destruction of glyphosate spots with high phytotoxic activity and/or the destruction of root channels of glyphosate-treated weed roots as preferential pathway for root growth of subsequently sown crops (Chapter 4). Potentially minimal tillage might also increase in speed of decomposition of glyphosate treated root residues and subsequently inactivation of glyphosate by microbial degradation or adsorption to the soil (Alleto *et al.*, 2010; Giesy *et al.*, 2000).

Effects on the plant nutritional status

Because glyphosate is a potent chelator for divalent cations (e.g. Mn, Fe, Zn, Ca, Mg), competitive interactions limiting acquisition, uptake, translocation and intra-cellular utilisation of cationic nutrients have been discussed as putative causes for glyphosate-induced

nutrient limitation (Sprankle *et al.*, 1975c; Subramaniam and Hoggard, 1988; Eker *et al.*, 2006; Ozturk *et al.*, 2008; Cakmak *et al.*, 2009). However, in the present study there was no consistent relationship between pre-crop herbicide applications, waiting time until sowing and nutritional status of the following crop. Under field conditions glyphosate concentrations in shoots were generally below 0.5 µg g DW^{-1} while concentration of divalent cations like Mn or Zn ranged between 30-40 µg g DW^{-1}. Therefore, results suggest that competitive interaction of glyphosate with certain cationic nutrients was not the major limiting factor for nutrient acquisition. Since root growth determines the spatial acquisition particularly of sparingly soluble nutrients, impairment of root growth by glyphosate toxicity observed in the present study might explain the variability of glyphosate-induced nutrient limitations.

Pathogen infection of plants and allelopathic effects

Several studies reported increase of infection of wheat with fungal pathogen (*Fusarium*, *Phytium*, *Rhizoctonia*) via the "green bridge", when total herbicides were used to control weeds shortly before seeding of cereals (Smiley *et al.*, 1992; Descalzo *et al.*, 1998; Powell and Swanton, 2008). Similarly, it is known that residues of weed plants in soil can cause allelopathic effects on wheat (Dudai *et al.*, 2009).
In line with this alternative explanation for crop damage after pre-crop herbicide application, first results of the field trials at the field site of TB and DU indicate increased infection of wheat with soil-borne pathogens (*Fusarium*, *Phytium* and/or *Rhizoctonia*) in case of short waiting time after glyphosate or Basta® application.
However, in present study comparable crop damage of winter wheat was observed in case of pre-crop glyphosate application on a variety of different weed plants on different soils. Similarly crop damage after pre-crop application has been reported by several authors over a range of different weed/crop plant-systems (Rodrigues *et al.*, 1982; Neumann *et al.*, 2006; Römheld *et al.*, 2008; Tesfamariam, 2009). Therefore, in the present study potentially increased soil-borne pathogens infection was more likely a consequence of glyphosate-transfer from weeds to crops inducing weak plant development rather than the primary cause of crop damage. However, as the observation of complete crop failure induced by infection of glyphosate-damaged winter wheat plants by barley yellow dwarf virus (Römheld *et al.*, 2008) indicate, glyphosate-damaged crops are obviously more susceptible to soil-borne pathogens, toxicity of allelopathic compounds or other stress factors.

Effects on final yield

In contrast to results of Römheld *et al.* (2008) and results at juvenile growth stages revealing significant damage (Tab. 5.1; Fig. 5.1a, b, c, 5.3), at final harvest only a small yield loss of in average 10 % (Fig. 5.5) was detectable. This is indicating a recovery of plants in glyphosate treatments in later growth stages. The ability of plants to recover from various stress factors, among them initial damage after exposure to glyphosate is well known (Ellis and Griffin, 2002; Norsworthy, 2004c). An additional explanation based on the observation of lower plant density in case of short waiting times after pre-crop glyphosate application might be that lower plant density caused a higher N-availability per plant and thus enhanced plant recovery.

Increased yield losses observed in case of long waiting time between combined application of Agil-S® and Basta® as control potentially explainable by insufficient weed control and subsequently high competition between crops and weeds in this treatments. In contrast, no damage and yield loss crops in case of long waiting times after application of glyphosate-based herbicides highlighted, that even under conditions of high weed pressure the advantageous weed control ability of glyphosate is not lost by observation of waiting times.

5.6 Conclusions

Even so underlying mechanisms could not be entirely clarified, there is considerable evidence for glyphosate stored and released from root residues of treated weed plants as primary cause causes for plant damage induced by short waiting time after pre-crop glyphosate application under field conditions.

Therefore, short waiting times between pre-crop application of glyphosate and sowing of crops should be avoided under no- or minimal tillage conditions particularly in case of dense weed populations. Potentially good agricultural practice and optimised plant production could minimise pathogen pressure and thereby reduced additional risk for damaged plants associated soil-borne pathogens and other potential stress factors.

Climatic- (temperature, precipitation), soil physical and microbiological factors in soils potentially affecting the risk of crop damage after glyphosate application to weeds before sowing need to be evaluated in further studies.

6 Important factors for rhizosphere transfer of glyphosate: I. Role of weed density and soil type for phytotoxic effects in crop plants

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Own contribution: set-up of experiments, plant cultivation, harvest and sample preparation, analysis of nutritional status of plants (support of students in 1 of 2 experiments) ,analysis of shikimate by HPLC, manuscript preparation

6.1 Abstract

Damage of crops induced by the herbicide glyphosate at short waiting time between application for weed control and sowing repeatedly detected under field conditions suggest a transfer of phytotoxic glyphosate from roots of treated weeds to subsequently crops. Beside this, transfer of soil-borne pathogens via a green bridge or allelopathic effects of weed residues might be possible reasons but the underlying mechanisms are still not clear.

To test the hypothesis of a glyphosate rhizosphere transfer and to evaluate the influence of soil characteristics and different densities of glyphosate treated weed plants as possible risk factors for crop damage, a series of green-house experiments in pots with two contrasting soils were conducted. After an application of glyphosate directly into contrasting soils (Arenosol, Regosol) or on different densities of pre-cultured model weeds (*Lolium perenne* L.) glyphosate-sensitive (GS) soybean were sown after 4 days in these pots and evaluated for potential expression of glyphosate-induced damage. Near isogenic glyphosate-resistant (GR) soybean were cultivated as controls under the same conditions as GS soybean to evaluate whether potential damage is primarily caused by glyphosate transfer, allelopathic effects of weeds or soil-borne pathogens.

Visual symptoms of glyphosate toxicity, reduced plant biomass, intracellular shikimate accumulation as physiological indicator for glyphosate toxicity and a decreased nutritional status of plants were observed on both soils only in case of glyphosate plant application in GS soybean. Similar but significantly weaker expressed damage was observed in GR soybean plants. Significant differences in intensity of damage of GS and GR soybean plants and in accumulation of shikimate in root tissue of plants a indicate rhizosphere transfer of glyphosate as primary cause for plant damage. Glyphosate-induced damage of GS and GR soybean was strongly correlated to the speed of death and decay of glyphosate treated weed plants affected by the soil type and the weed density. The correlation between development, intensity and expression of damage symptoms of crop plants and death of glyphosate treated weed suggest a close connection between the glyphosate transfer from root residues of treated weeds to subsequently cultivated crops and the biotic and abiotic growth conditions of weeds and crop plants.

Key words: glyphosate, pre-crop application, risk factors, weed residues, soybean (*Glycine max* L.), micronutrients

Abbreviations:

AI active ingredient

AMPA aminomethylphosphonic acid

cv. cultivar

EPSP(S) 5-enolpyruvylshikimate-3-phosphate (synthase)

DAS days after sowing

DBS days before sowing

WT waiting time

6.2 Introduction

Since its introduction in 1974, glyphosate (N-(phosphonomethyl)glycine), the active ingredient (AI) of systemic, broad-spectrum, non-selective post-emergence herbicide like Roundup®, has become by any measure the most extensively used herbicide in agricultural practice and due to low production costs and high efficiency in weed control the world's bestselling agrochemical compound (Baylis, 2000). Glyphosate acts by inhibiting the biosynthesis of aromatic amino acids, thereby causing impairment of general metabolic processes, such as photosynthesis, protein synthesis and biosynthesis of secondary aromatic compounds (Geiger *et al*., 1986). As non-selective total herbicide glyphosate is particularly used in cropping systems with genetically modified glyphosate-resistant (GR) plants, but also as pre-crop application before sowing of glyphosate-sensitive (GS) plants in minimal/no tillage cropping systems were it is considered essential in order to minimise crop production losses caused by high weed infestations (Lyon *et al*., 1996; Pline *et al*., 2002).

Since glyphosate is strongly adsorbed to the soil matrix by one of its three polar functional groups and additionally prone to microbial degradation in soil solution, risks for phytotoxicity of glyphosate in soils are considered as marginal (Giesy *et al*., 2000). However, poor establishment and impaired growth of crop plants has been reported when glyphosate or other non-selective herbicides have been used for weed control before crop sowing in no tillage or conservation tillage systems in Australia, Germany and the United States (Smiley *et al*., 1992; Descalzo *et al*., 1998., Römheld *et al*., 2008). A stimulation of root pathogens attracted by the decaying weed residues (Smiley *et al*., 1992; Descalzo *et al*., 1998; Powell and Swanton, 2008) and the release of allelopathic compounds by decaying weeds have been discussed as possible reasons by Dudai *et al.* (2009).
In addition, glyphosate-induced damage of crops caused by rhizosphere transfer from weeds to crops was also frequently but not always observed in field trials (Römheld *et al*., 2008; Tab. 5.1; Fig.5.3). This suggests that risks associated to glyphosate toxicity in the rhizosphere might be influenced by abiotic and biotic factors at the field site such as the soil type and/ or the density of glyphosate treated weed plants.

Significant damage of crop plants potentially caused by a transfer of glyphosate from treated roots and/ or root residues of treated weeds to subsequently sown crops was repeatedly observed in model experiments under controlled conditions (Rodrigues *et al*., 1982; Neumann *et al*., 2006; Tesfamariam, 2009). However, the studies did not allow a differentiation between glyphosate transfer, allelopathic effects of decaying weed residues and/or soil-borne pathogens as primary cause of damage.

Therefore, to evaluate the hypothesis that damage of crop plants after pre-crop glyphosate application on weed plants is primarily induced by a rhizosphere transfer, but depending on abiotic factors such as soil type as well as biotic factors such as the density of glyphosate treated weed plants, a series of green-house experiments on two contrasting soils were conducted. After an application of glyphosate directly to contrasting soils (Arenosol, Regosol) or on different densities of rye grass (*Lolium perenne* L.) pre-cultured as model weed plants, glyphosate-sensitive (GS) soybean were sown after 4 days in these pots and evaluated for

potential expression of glyphosate-induced damage. Genetically modified GR cultivars are approx. 50 times less sensitive to phytotoxic glyphosate than their parental GS genotypes (Nandula *et al*., 2007) but not significantly different in their sensitivity to allelopathic compounds (Norsworthy, 2004c) or soil borne pathogens (Johal and Huber, 2009; Kremer *et al*., 2005). Therefore, near isogenic glyphosate-resistant (GR) soybean were cultivated under the same conditions as GS soybean as additional controls to evaluate potential damage caused by allelopathic effects of weeds, soil-borne pathogens or glyphosate transfer.

6.3 Material and Methods

Plant material and growth conditions

Soybean (*Glycine max* L.) seeds of the GR cv. BSR Valiosa RR and/or 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). To evaluate the effect of glyphosate application two soil culture experiments were conducted.
Two contrasting soils were used: a sandy acidic Ap horizon of an Arenosol (pH ($CaCl_2$) 4.5; Corg [%] 0.16); calcium chloride-diethylenetriamine pentaacetic acid (CAT)-extractable micronutrient concentrations [mg kg^{-1} soil]: Mn=7.4, Fe=369, Zn=0.8, B=0.9 and Cu=0.5) and a clay-rich Regosol soil (pH ($CaCl_2$) 7.1; Corg [%] 3.8%); calcium chloride-diethylenetriamine pentaacetic acid (CAT)-extractable micronutrient concentrations [mg kg^{-1} soil]: Mn=7.6, Fe=34.5, Zn=5.2, B=0.54 and Cu=1.5 (VDLUFA, 2004).

Soils were sieved through a 2 mm mesh and then fertilised 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 $Ca(H_2PO_4)_2$. After fertilisation, the soil was sieved again to guarantee homogeneous distribution of the fertilisers. Previous measurements showed no profound changes in soil pH after identical fertiliser application to both soil types.

Glyphosate plant application

To investigate the effects of glyphosate residues in root tissue of different target weeds on subsequently cultivated crop plants, rye grass *(Lolium perenne* L. cv. Kelvin) was pre-cultivated as weed in 900 g plastic pots filled with the fertilised soils. In the first soil experiment a sowing density of 2.2 g rye grass seeds per pot with a surface area of 100 cm^2 was used.

To compare effects of different plant densities, in a second experiment sowing density of rye grass included a low sowing density of 2.2 g and a high sowing density 4.0 g rye grass seeds per pot.

In all soil experiments, at 10 days after sowing (DAS), the young rye grass seedlings were sprayed with glyphosate as Roundup Ultramax® formulation (Monsanto Agrar, Düsseldorf, Germany). In the first and the second experiment glyphosate was applied with a hand-held sprayer. Each pot received 4.3 mL of glyphosate spray solution on the leaves, based on determination of the rye grass leaf area coverage (approx. 2300 cm^2 per pot). Due to the known self-limited translocation of glyphosate, experiments with wheat and soybean revealed in terms of damage of crop plants no significant difference between glyphosate application based on the surface area of weed plants and application with a track spraying device simulating application technique under field conditions (Fig. 3.1-3.4).

Subsequently, 4 days after glyphosate application to rye grass soybean seeds were sown into pots (12 seeds per pot). In the first experiment seeds of glyphosate-resistant (cv. Valiosa) and non-resistant (cv. Conquista) soybean were used, while in the second experiment only non-resistant (cv. Conquista) soybean were sown. The time period between application of glyphosate to the rye grass pre-culture and sowing of soybean was defined as "waiting time". After desiccation rye grass residues were not removed and no disturbance of the soil in the pots was undertaken.
In control treatments without glyphosate application, rye grass shoots were removed by cutting at the soil level with a sharp knife.

Glyphosate soil application

To assess the effects of glyphosate in the soil on non-target plants, the same amount of glyphosate as applied to the target weeds (4.3 mL of a Roundup Ultramax® solution containing a glyphosate concentration of 28.4 mM) was mixed directly with 900 g of the fertilised soils. Controls received only mineral nutrients and water. After a waiting time of 4 days, soybean seeds were sown (12 seeds per pot) at the same day as in the treatments with rye grass weed pre-culture.

Evaluated parameters

During the whole growth period of both experiments parameters such as germination, seedling development, leaf morphology, plant growth and expression of chlorosis (SPAD-value) were recorded and scored as visual indicator of glyphosate toxicity. At 10 days after sowing (DAS), a first set of soybean seedlings was removed from the pots and fresh weights of all plant parts (roots and shoot) were determined. Roots and shoots were stored for analysis of accumulation of shikimate in plant tissue as physiological indicator of glyphosate toxicity as described below. In each pot, four soybean plants were further cultivated until final harvest 28 days after germination for the determination of the nutritional status of plants. At final harvest, plants were separated in young and old shoot parts and roots. Fresh weights of all plant parts (roots and shoot) were determined at harvest and dry weights after oven-drying at 60°C. Subsequently dried shoots were grinded for analysis of nutritional status of plants.

Shikimate analysis

Shikimate in acidic tissue extracts was analysed with modifications of the methods described by Singh and Shaner (1998) and Neumann *et al*. (2006). The frozen plant tissue was homogenised 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 20.000 x g) and the supernatant was used for HPLC analysis after appropriate dilution with the HPLC mobile phase. HPLC separation was performed by ion exclusion chromatography using an Aminex 87H column (Bio-Rad, Richmond, CA, USA) designed for organic acid analysis. A sample volume of 20 µL was injected into the isocratic flow (0.5 mL min^{-1}) of the eluent (2.5 mM H_2SO_4, 40 °C) and organic acids were detected spectrophotometrically at 210 nm. Identification and quantification of shikimate were conducted by comparing the retention times, absorption spectra and peak areas with a known standard.

Analysis of mineral nutrients

One hundred milligram of dried shoot material was ashed in a muffle furnace at 500°C for 5 h. After cooling, the samples were extracted twice with 1 mL of 3.4 M HNO_3 and evaporated until dryness to precipitate SiO_2. The ash was dissolved in 1 mL of 4 M HCl, subsequently diluted ten times with hot deionised water, and boiled for 2 min to convert meta- and pyro-phosphates to orthophosphate. 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). Spectrophotometrical determination of orthophosphate was conducted after addition of molybdate-vanadate colour reagent according to the method of Gericke and Kurmis (1952). Determination of Mg was conducted by atomic absorption spectrometry, while K and Ca were measured by flam photometry.

Statistics

All experiments were conducted in a completely randomised block design. Soil experiments were conducted with four replicates per treatment for GS soybean and eight for GR soybean. Analysis of variance and the Tukey test for detection of significant differences were performed using the SigmaStat-software (Jandel Scientific, Sausalito, CA, USA).

6.4 Results

Glyphosate efficiency on weed pre-culture

Determination of shoot fresh weight of weed plants after cutting in control treatments showed no significant difference in weed plant biomass between the two soils, but significantly differences in shoot biomass in comparison of low and high weed density pre-culture were found (low density: 8-10 g FW pot^{-1}; high density: 16-20 g FW pot^{-1}). However, soils differed significantly in speed of death of glyphosate treated weed plants. In both experiments, independent of the density of weed plants rye grass plants died off within the first 7-10 days after application of glyphosate on the Regosol. By contrast, on the Arenosol, complete death

of rye grass was delayed of approx. 5-7 days in case of glyphosate application on a low weed density, and of approx. 10 days in case of a high weed density (Fig. 6.1, 6.2).

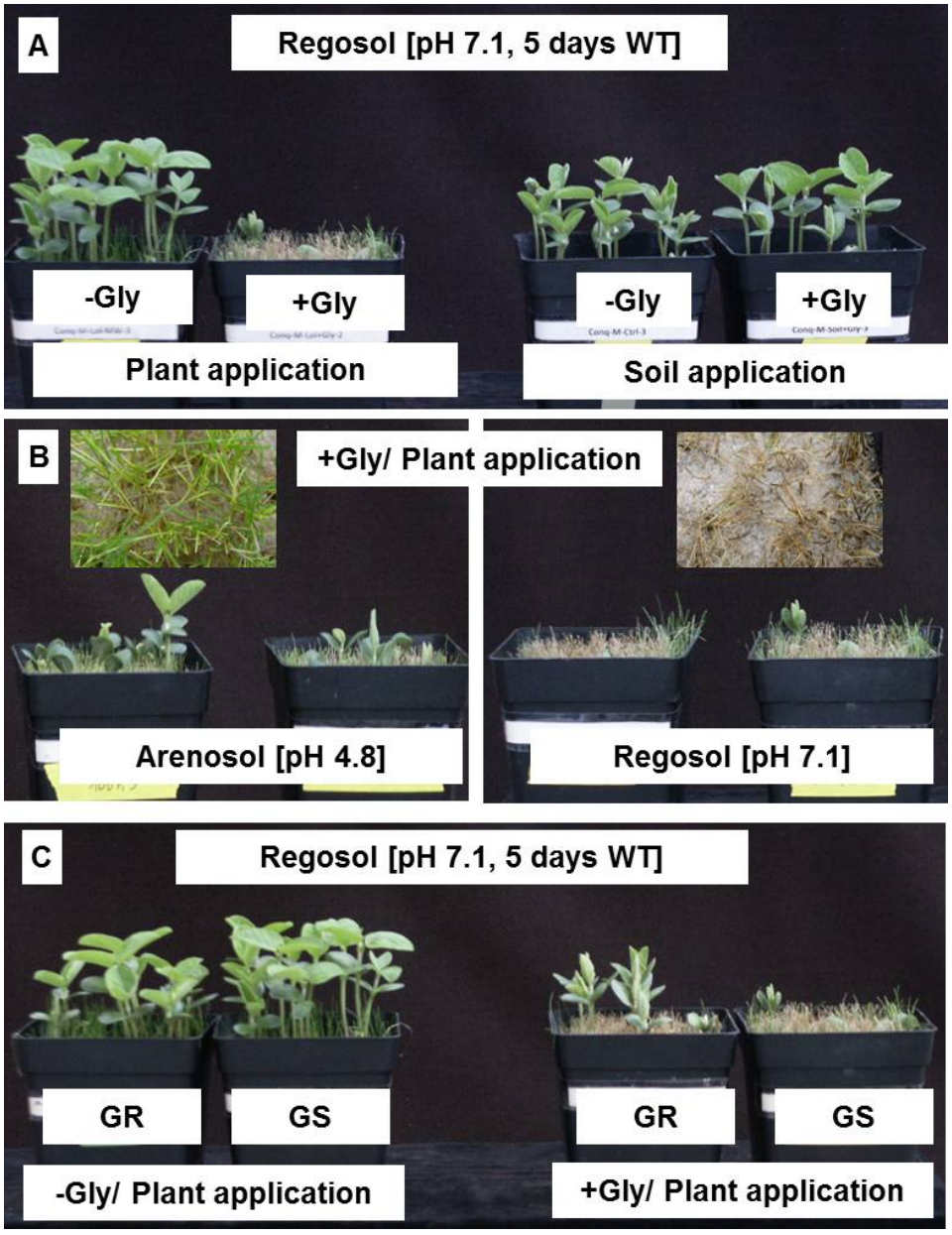

Fig. 6.1: Germination and seedling development of glyphosate-sensitive (GS) and glyphosate-resistant (GR) soybean depending on glyphosate application and soil type

(A) Germination and seedling development of GS soybean (*Glycine max* L.) plants grown on a clayly neutral Regosol soil sown after a waiting time of 5 days (5 days WT) after an application on pre-cultured rye grass plants (*Lolium perenne* L.) (Plant application) or after direct soil application of the same glyphosate dose (Soil application). (B) Comparison on effects of pre-crop plant application of glyphosate (Plant application) on pre-cultured rye grass plants of GS soybean cultivated on a sandy acidic Arenosol and a clayly neutral Regosol. (C) Comparison on effects of pre-crop plant application of glyphosate on pre-cultured rye grass plants (+Gly/Plant application) on GS soybean (GS) and glyphosate-resistant soybean (GR) cultivated a clayly neutral Regosol with control (-Gly/ Plant application).

Evaluation of plant damage in early vegetation period

Scoring of visual symptoms of glyphosate toxicity in soybean seedlings revealed in case of pre-crop plant-, but not soil application of glyphosate on both soils significantly lower germination, delayed seedling development and deformations of primary leaves in comparison to both controls. Correlated to the earlier death of glyphosate treated weed plants on the Regosol, scoring of damage of GS and GR soybean seedlings showed significantly stronger damage on the Regosol compared to the Arenosol. In comparison between GS and GR soybean on both soils delay in seedling development and deformations of emerging primary leaves were significantly stronger expressed in GS soybean (Tab. 6.1; Fig. 6.1).

A comparison of risks for damage of GS soybean depending on glyphosate application on low and high weed densities showed severe inhibited germination, delayed seedling development and deformations of primary leaves of GS soybean grown on the Regosol, but no significant difference in expression of crop damage depending on the densities of treated weeds. By contrast, on the Arensol damage of soybean was less expressed and there were indications for increased damage in case of glyphosate application to low density of weed plants compared to high weed density (Tab. 6.2; Fig. 6.2).

Tab. 6.1 Scoring of plant damage in glyphosate-sensitive (GS) and glyphosate-resistant (GR) soybean depending on glyphosate application and soil type in early growth stages

Quantitative and qualitative indicators of crop damage were determined in early growth stages (5-12 days after sowing, DAS) on GS and GR soybean (*Glycine max* L.) seedlings cultivated on a sandy acidic Arenosol and a clayly neutral Regosol soil with (+Gly) or without (−Gly) pre-sowing glyphosate treatments on a pre-culture with rye grass (Lolium perenne L.) (Plant) or direct glyphosate soil application (Soil). Time point of evaluations in days after sowing (DAS) and status of glyphosate treated weed plants are indicated. Values represent the average of 4 replicates for GS soybean and 8 replicates for GR soybean. Significant differences (P<0,05) are indicated by different characters.

Scoring of plant damage in early growth stages (5-12 DAS)

	Arenosol [pH 4.8]				Regosol [pH 7.1]			
Date (DAS)	**Application**				**Application**			
	Plant		**Soil**		**Plant**		**Soil**	
	- Gly	**+ Gly**	**- Gly**	**+ Gly**	**- Gly**	**+ Gly**	**- Gly**	**+ Gly**
5 DAS	**Status of weed plants [died off]**							
GS soybean		**<10%**				**>90%**		
GR soybean		**<10%**				**>90%**		
5 DAS	**Germination [% seeds pot^{-1}]**							
GS soybean	**98**±4^{A}	**83**±7^{B}	**96**±5^{A}	**96**±5^{A}	**92**±7^{AB}	**42**±7^{C}	**77**±21^{AB}	**73**±14^{AB}
GR soybean	**77**±6^{A}	**88**±9^{A}	**79**±14^{A}	**82**±14^{A}	**80**±9^{A}	**64**±5^{B}	**76**±5^{A}	**80**±7^{A}
8 DAS	**Delayed seedling development [% plants pot^{-1}]**							
GS soybean	**0**±0^{C}	**83**±0^{B}	**2**±4^{C}	**0**±0^{C}	**9**±7^{C}	**98**±5^{A}	**12**±10^{C}	**14**±12^{C}
GR soybean	**8**±11^{B}	**9**±7^{B}	**0**±0^{B}	**4**±5^{B}	**0**±0^{B}	**73**±6^{A}	**6**±8^{B}	**6**±7^{B}
11 DAS	**Deformation of emerging primary leaves [% plants pot^{-1}]**							
GS soybean	**0**±0^{B}	**93**±9^{A}	**0**±0^{B}	**0**±0^{B}	**0**±0^{B}	**100**±0^{A}	**0**±0^{B}	**0**±0^{B}
GR soybean	**0**±0^{B}	**0**±0^{B}	**0**±0^{B}	**0**±0^{B}	**0**±0^{B}	**35**±9^{A}	**0**±0^{B}	**0**±0^{B}
12 DAS	**Shikimate concentration in root tissue [µg g FW^{-1}]**							
GS soybean	**125**±118^{B}	**4257**±3524^{A}	**90**±33^{B}	**32**±10^{B}	**147**±65^{B}	**7480**±1559^{A}	**68**±8^{B}	**97**±39^{B}
GR soybean	**80**±41$^{N.S}$	**209**±165$^{N.S}$	**71**±11$^{N.S}$	**32**±10$^{N.S}$	**107**±32$^{N.S}$	**485**±421$^{N.S}$	**54**±5$^{N.S}$	**32**±10$^{N.S}$

Determination of intracellular shikimate concentrations in soybean root tissue as physiological indicator of glyphosate toxicity 15 days after glyphosate application on pre-cultured weed plants showed on both soils in GS and GR soybean elevated concentrations of shikimate only in case of pre-crop plant application of glyphosate. In comparison to control, significant differences in shikimate concentrations were detectable on both soils in GS soybean, while in GR soybean shikimate concentrations were only significantly increased in case of plants cultivated on the Regosol (Tab. 6.1).

Shikimate concentrations in roots of GS soybean cultivated on both soils after glyphosate application to low or high density of pre-cultured weed were in both cases significantly increased compared to controls. Shikimate concentrations in GS soybean roots were highest in case of glyphosate application on a high density of weed plants on the Regosol. In contrast, on the Arenosol highest shikimate concentrations were observed in case of glyphosate application on a low density of weeds (Tab. 6.2).

Tab. 6.2 Scoring of plant damage in glyphosate-sensitive (GS) soybean depending on soil type and density of glyphosate-treated weeds in early growth stages

Quantitative and qualitative indicators of crop damage were determined in early growth stages (5-12 days after sowing, DAS) on GS soybean (*Glycine max* L.) seedlings cultivated on a sandy acidic Arenosol and a clayly neutral Regosol soil with (+Gly) or without (−Gly) pre-sowing glyphosate treatments on a low (Low) and high (High) density of pre-cultured weed plants (*Lolium perenne*). Time point of evaluations in days after sowing (DAS) and status of glyphosate treated weed plants are indicated. Values represent the average of 4 replicates. Significant differences (P<0,05) are indicated by different characters.

Scoring of plant damage in early growth stages (5-12 DAS)								
	Arenosol [pH 4.8]				Regosol [pH 7.1]			
Date (DAS)	Weed densities				Weed densities			
	Low		High		Low		High	
	- Gly	+ Gly	- Gly	+ Gly	- Gly	+ Gly	- Gly	+ Gly
5 DAS	Status of weed plants [died off]							
GS soybean		<10%		<10%		>90%		>90%
5 DAS	Germination [% seeds pot^{-1}]							
GS soybean	98±4^{A}	83±12^{B}	96±5^{A}	81±8^{B}	95±8^{A}	35±10^{C}	93±8^{A}	45±10^{C}
10 DAS	Delayed seedling development [% plants pot^{-1}]							
GS soybean	0±0^{C}	57±20^{B}	0±0^{C}	43±10^{B}	0±0^{C}	90±12^{A}	0±0^{C}	87±6^{A}
10 DAS	Deformation of emerging primary leaves [% plants pot^{-1}]							
GS soybean	0±0^{C}	76±20^{AB}	0±0^{C}	48±18^{B}	0±0^{C}	89±12^{A}	0±0^{C}	87±6^{A}
11 DAS	Shikimate concentration in root tissue [µg g FW^{-1}]							
GS soybean	61±47^{B}	1488±621^{A}	94±26^{B}	996±654^{A}	101±54^{B}	2058±973^{A}	77±28^{B}	2631±1612^{A}

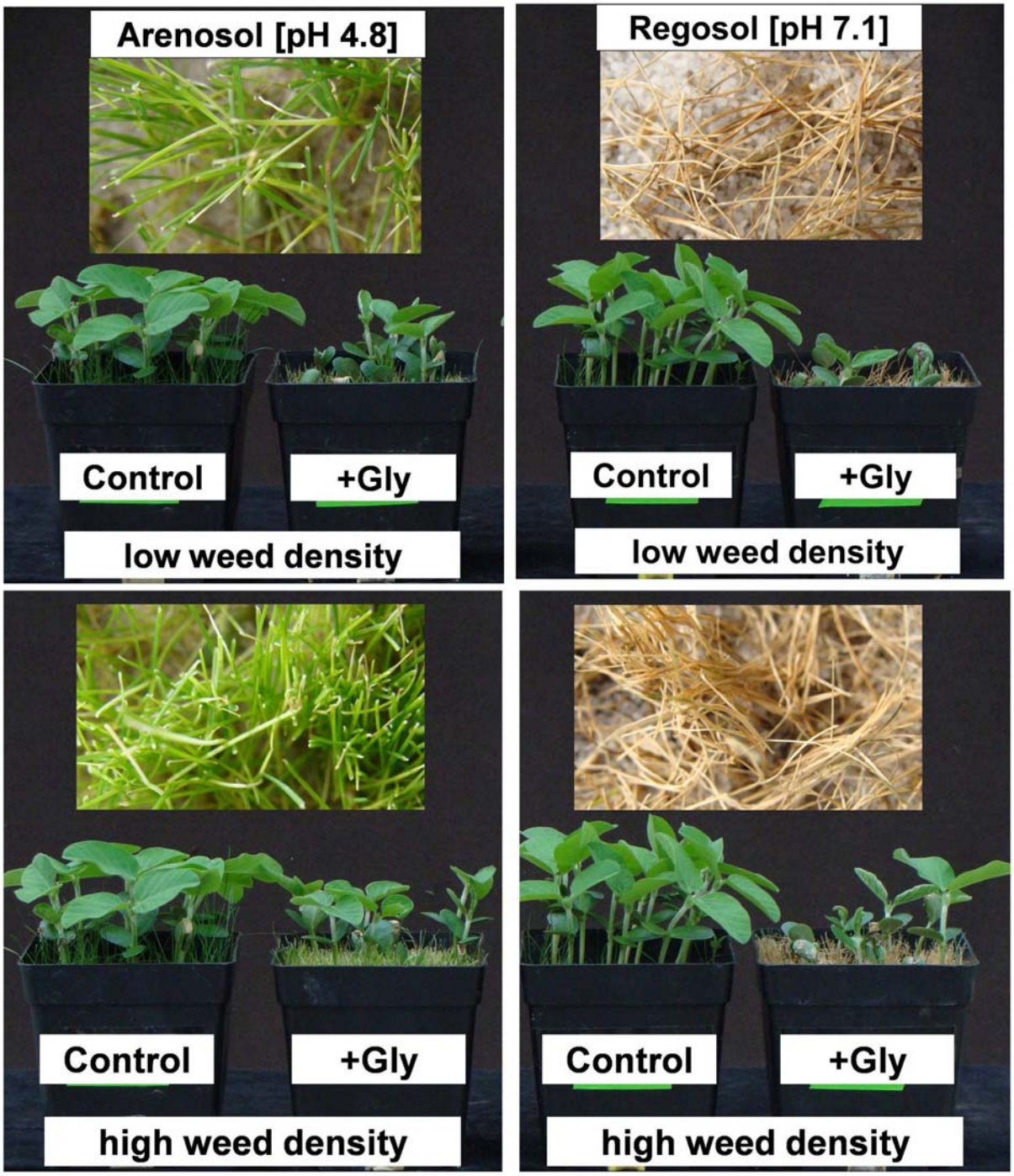

Fig. 6.2: Seedling development of glyphosate-sensitive (GS) soybean depending on density of glyphosate-treated weeds

GS soybean (*Glycine max* L.) plants were cultivated on a sandy acidic Arenosol (pH 4.8) (left) and a clayly neutral Regosol (pH 7.1) (right) with (+Gly) or without (Control) glyphosate application at 5 days before sowing to varied densities of weed plants (*Lolium perenne* L.) (above: Low weed density, below: High weed density) pre-cultured for 10 days before glyphosate application in the same pots.

Evaluation of plant damage in mid vegetation period

Evaluation of visual symptoms of glyphosate toxicity gave indications for damage of GS and GR soybean only in case of pre-crop application of glyphosate on weed plants. At this growth stage, damage of GS and GR soybean comprised impaired development of main shoots (e.g. no formation of trifoliar leaves on main shoots), decreased shoot height and significantly increased formation of deformed/ inversely cordated trifoliar leaves (Tab. 6.3; Fig. 6.3). In comparison between GS and GR soybean on both soils symptoms of damage were significantly stronger expressed in GS soybean. In contrast to damage in earlier growth stages, there were no significant differences in intensity of damage of GS and GR soybean between the Regosol (died off weed plant) and the Arenosol (died off weed plants) in this experiment (Tab. 6.3).

Tab. 6.3 Scoring of plant damage in glyphosate-sensitive (GS) and glyphosate-resistant (GR) soybean depending on glyphosate application and soil type in mid vegetation period

Quantitative and qualitative indicators of crop damage were determined on GS and GR soybean (*Glycine max* L.) plants in mid vegetation period (14-21 days after sowing, DAS) cultivated on a sandy acidic Arenosol and a clayly neutral Regosol soil with (+Gly) or without (−Gly) pre-sowing glyphosate treatments on a weed pre-culture with (*Lolium perenne* L.) (Plant) or direct glyphosate soil application (Soil). Time point of evaluations in days after sowing (DAS) and status of glyphosate treated weed plants are indicated. (*) In case of GS soybean trifoliar leaves were severely damaged/ did not fully develop in this treatment) Values represent the average of 4 replicates for GS soybean and 8 replicates for GR soybean. Significant differences (P<0,05) are indicated by different characters.

Scoring of plant damage in mid vegetation period (14-21 DAS)

	Arenosol [pH 4.8]				Regosol [pH 7.1]			
Date (DAS)	**Application**				**Application**			
	Plant		**Soil**		**Plant**		**Soil**	
	- Gly	**+ Gly**	**- Gly**	**+ Gly**	**- Gly**	**+ Gly**	**- Gly**	**+ Gly**
14 DAS	**Status of weed plants [died off]**							
GS soybean	regrowth	>90%			regrowth	>90%		
GR soybean	regrowth	>90%			regrowth	>90%		
18 DAS	**Impaired primary shoot development [% plant pot^{-1}]**							
GS soybean	**0**±0^{B}	**38**±25^{A}	**0**±0^{B}	**0**±0^{B}	**0**±0^{B}	**25**±0^{A}	**0**±0^{B}	**0**±0^{B}
GR soybean	**0**±0^{B}	**0**±0^{B}	**0**±0^{B}	**0**±0^{B}	**0**±0^{B}	**0**±0^{B}	**0**±0^{B}	**0**±0^{B}
20 DAS	**Shoot height [cm]**							
GS soybean	**20**±2^{A}	**9**±2^{B}	**23**±2^{A}	**23**±3^{A}	**22**±3^{A}	**7**±3^{B}	**21**±2^{A}	**23**±3^{A}
GR soybean	**21**±3^{A}	**17**±2^{B}	**23**±1^{A}	**23**±3^{A}	**23**±2^{A}	**16**±2^{B}	**22**±3^{A}	**23**±1^{A}
21 DAS	**Deformation of fully developed trifoliar leaves* [% plants pot^{-1}]**							
GS soybean	**0**±0^{B}	**97***±6^{A}	**0**±0^{B}	**0**±0^{B}	**0**±0^{B}	**92***±10^{A}	**0**±0^{B}	**0**±0^{B}
GR soybean	**6**±11^{B}	**72**±29^{A}	**16**±13^{B}	**9**±13^{B}	**9**±13^{B}	**59**±26^{A}	**6**±11^{B}	**6**±11^{B}

However, in experiments evaluating risks for crop damage of GS soybean depending on glyphosate application on low and high weed densities, plant damage was significantly stronger expressed on the Regosol in comparison to the Arenosol. While no significant differences in terms of plant damage were observed between both weed densities on the Regosol, on the Arenosol glyphosate application to low weed density induced significantly increased damage of soybean plants compared to high weed density (Tab. 6.4).

Tab. 6.4 Scoring of plant damage in glyphosate-sensitive (GS) soybean depending on soil type and density of glyphosate-treated weeds in mid vegetation period

Quantitative and qualitative indicators of crop damage were determined on GS soybean (*Glycine max* L.) in mid vegetation period (13-17 days after sowing, DAS) cultivated on a sandy acidic Arenosol and a clayly neutral Regosol soil with (+Gly) or without (−Gly) pre-sowing glyphosate treatments on a low (Low) and high (High) density of pre-cultured weed plants (*Lolium perenne* L.). Time point of evaluations in days after sowing (DAS) and status of glyphosate treated weed plants are indicated. Values represent the average of 4 replicates. Significant differences (P<0,05) are indicated by different characters.

Scoring of plant damage in mid vegetation period (13-17 DAS)

	Arenosol [pH 4.8]				Regosol [pH 7.1]			
Date (DAS)	**Weed densities**				**Weed densities**			
	Low		**High**		**Low**		**High**	
	- Gly	**+ Gly**	**- Gly**	**+ Gly**	**- Gly**	**+ Gly**	**- Gly**	**+ Gly**
13 DAS	**Status of weed plants [died off]**							
GS soybean	regrowth	>90%	regrowth	>50%	regrowth	>90%	regrowth	>90%
17 DAS	**Leaf surface area of primary leaves [cm^2]**							
GS soybean	**177**±11^{A}	**94**±7^{C}	**184**±14^{A}	**122**±10^{B}	**174**±6^{A}	**91**±10^{C}	**175**±9^{A}	**94**±7^{C}
17 DAS	**Chlorosis scoring of primary leaves [SPAD-value]**							
GS soybean	**45**±1^{A}	**38**±1^{BC}	**44**±1^{A}	**40**±1^{B}	**44**±1^{A}	**34**±1^{D}	**44**±1^{A}	**36**±1^{CD}
17 DAS	**Impaired main shoot development [% plant pot^{-1}]**							
GS soybean	**8**±10^{B}	**54**±15^{A}	**8**±10^{B}	**54**±15^{A}	**4**±8^{B}	**79**±15^{A}	**8**±10^{B}	**79**±15^{A}

Evaluation of plant damage in late vegetation period stage

Shortly before harvest, evaluation of glyphosate-toxicity symptoms in GS and GR soybean demonstrated significant increase in damage symptoms including increase in auxiliary shoot formation, cupping of young trifoliar leaves and chlorosis on youngest fully developed leaves only in case of pre-crop application of glyphosate on weed plants. In comparison between GS and GR soybean, delay in seedling development and deformations of emerging primary leaves were significantly stronger expressed in GS soybean on both soils (Tab. 6.5).

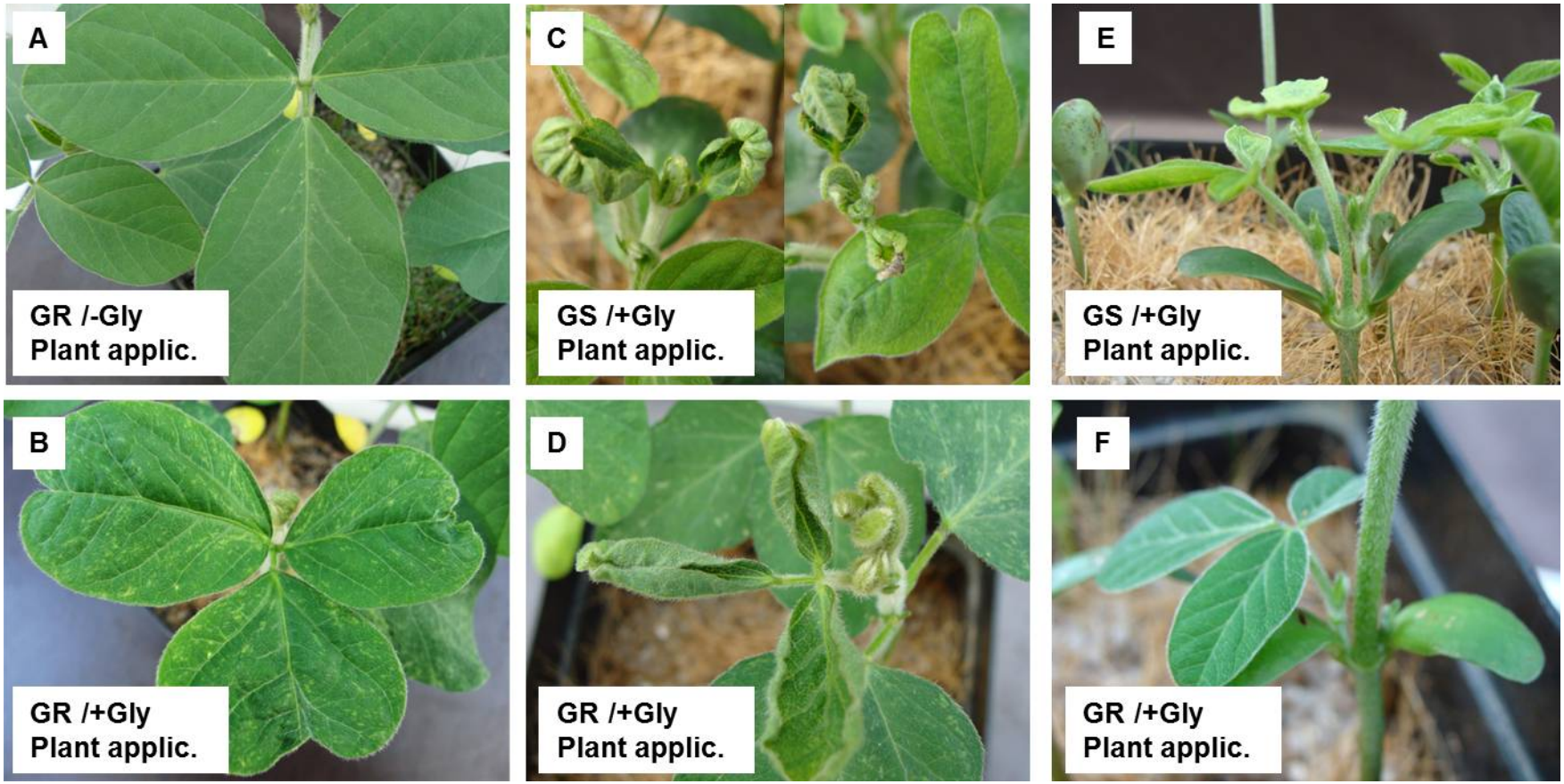

Fig. 6.3: Visual symptoms of glyphosate-sensitive (GS) and glyphosate-resistant (GR) soybean plants after glyphosate plant application

Visual symptoms of damage were observed on leaves and stems of GS (GS) and GR (GR) soybean (*Glycine max* L.) cultivated on a sandy acidic Arenosol and a clayly neutral Regosol with (+Gly) or without (-Gly) glyphosate application at 5 days before sowing to a pre-culture of rye grass (*Lolium perenne* L.) (Plant applic.). (A) Undamaged fully developed trifoliar leaves of GR soybean in control, (B) Deformed fully developed trifoliar leaves of GR soybean after glyphosate application on weed plants. Cupping of emerging trifoliar leaves and coiling of shoot apices of (C) GS soybean and (D) GR soybean after glyphosate application on weed plants. Increased formation of auxiliary shoots on main shoots of (E) GS soybean and (F) GR soybean after glyphosate application on weed plants are shown.

In contrast to early growth stages, symptoms of glyphosate damage were stronger expressed in GS and GR soybean on the Arenosol than on the Regosol (Tab. 6.5; Fig. 6.3). In line with this finding, also in the experiment with varied weed density GS soybean showed a higher glyphosate damage on the Arenosol (Tab. 6.6). Weed plants had died of completely on both soils at this time (Tab. 6.6).

Tab. 6.5 Scoring of plant damage in glyphosate-sensitive (GS) and glyphosate-resistant (GR) soybean depending on glyphosate application and soil type in late vegetation period

Quantitative and qualitative parameters of crop damage were determined on GS and GR soybean (*Glycine max* L.) plants in late vegetation period (26 days after sowing, DAS) cultivated on a sandy acidic Arenosol and a clayly neutral Regosol soil with (+Gly) or without (−Gly) pre-sowing glyphosate treatments on a weed pre-culture (*Lolium perenne* L.) (Plant) or direct glyphosate soil application (Soil). Time point of evaluations in days after sowing (DAS) and status of glyphosate treated weed plants are indicated. Values represent the average of 4 replicates for GS soybean and 8 replicates for GR soybean. Significant differences (P<0,05) are indicated by different characters.

Scoring of plant damage in late vegetation period (26 DAS)

	Arenosol [pH 4.8]				Regosol [pH 7.1]			
Date (DAS)	Application				Application			
	Plant		Soil		Plant		Soil	
	- Gly	+ Gly	- Gly	+ Gly	- Gly	+ Gly	- Gly	+ Gly
26 DAS	**Chlorosis scoring of trifoliar leaves [SPAD-value]**							
GS soybean	**27**±1^{B}	**19**±1^{C}	**26**±1^{B}	**26**±2^{B}	**33**±1^{A}	**18**±4^{C}	**33**±2^{A}	**32**±1^{A}
GR soybean	**26**±1^{B}	**27**±1^{B}	**27**±2^{B}	**26**±2^{B}	**31**±2^{A}	**31**±1^{A}	**32**±1^{A}	**31**±1^{A}
26 DAS	**Increased secondary shoot formation [% plants pot^{-1}]**							
GS soybean	**0**±0^{B}	**97**±6^{A}	**0**±0^{B}	**0**±0^{B}	**0**±0^{B}	**92**±10^{A}	**0**±0^{B}	**0**±0^{B}
GR soybean	**9**±13^{B}	**72**±29^{A}	**16**±13^{B}	**15**±13^{B}	**9**±13^{B}	**59**±26^{A}	**6**±13^{B}	**6**±13^{B}
26 DAS	**Cupping of young trifoliar leaves [% plants pot^{-1}]**							
GS soybean	**6**±13^{C}	**100**±0^{A}	**6**±13^{B}	**0**±0^{B}	**0**±0^{B}	**100**±0^{A}	**0**±0^{B}	**0**±0^{B}
GR soybean	**6**±13^{C}	**94**±13^{A}	**0**±0^{C}	**0**±0^{C}	**6**±13^{C}	**69**±13^{B}	**6**±13^{C}	**0**±0^{C}

Tab. 6.6 Scoring of plant damage in glyphosate-sensitive (GS) soybean depending on soil type and density of glyphosate-treated weeds in late vegetation period

Quantitative and qualitative parameters of crop damage were determined on GS soybean (*Glycine max* L.) in late vegetation period (20-27 days after sowing, DAS) cultivated on a sandy acidic Arenosol and a clayly neutral Regosol soil with (+Gly) or without (−Gly) pre-sowing glyphosate treatments on a low (Low) and high (High) density of pre-cultured weed plants (*Lolium perenne*). Time point of evaluations in days after sowing (DAS) and status of glyphosate treated weed plants are indicated. Values represent the average of 4 replicates. Significant differences (P<0,05) are indicated by different characters.

Scoring of plant damage in late growth stages (20-27 DAS)

	Arenosol [pH 4.8]				Regosol [pH 7.1]			
Date (DAS)	**Weed densities**				**Weed densities**			
	Low		**High**		**Low**		**High**	
	- Gly	**+ Gly**	**- Gly**	**+ Gly**	**- Gly**	**+ Gly**	**- Gly**	**+ Gly**
20 DAS	**Status of weed plants [died off]**							
GS soybean	regrowth	>90%	regrowth	>90%	regrowth	>90%	regrowth	>90%
23 DAS	**Deformation of trifoliar leaves* [% plants pot^{-1}]**							
GS soybean	**0**±0^{C}	**100**±0^{A}	**0**±0^{C}	**100**±0^{A}	**0**±0^{C}	**79**±15^{B}	**0**±0^{C}	**79**±15^{B}
27 DAS	**Increased secondary shoot formation [% plants pot^{-1}]**							
GS soybean	**0**±0^{C}	**100**±0^{A}	**0**±0^{C}	**94**±13^{AB}	**0**±0^{C}	**69**±24^{B}	**0**±0^{C}	**88**±14^{AB}
27 DAS	**Leaf surface area of trifoliar leaves [cm^{2}]**							
GS soybean	**515**±58^{A}	**162**±44^{B}	**495**±35^{A}	**193**±32^{B}	**507**±11^{A}	**173**±13^{B}	**528**±35^{A}	**201**±26^{B}
27 DAS	**Chlorosis scoring of trifoliar leaves [SPAD-value]**							
GS soybean	**32**±1^{A}	**27**±0^{B}	**31**±0^{A}	**27**±0^{B}	**32**±1^{A}	**33**±1^{A}	**32**±1^{A}	**28**±2^{B}
27 DAS	**Cupping/coiling of emerging trifoliar leaves [% plants pot^{-1}]**							
GS soybean	**6**±13^{C}	**100**±0^{A}	**0**±0^{C}	**100**±0^{A}	**0**±0^{C}	**25**±0^{B}	**6**±13^{C}	**25**±0^{B}

Determination of shoot and root fresh- and dry weights at final harvest revealed on both contrasting soils decline in plant biomass of GS and GR soybean only in case of pre-crop glyphosate application on weed plants which was however only significantly declined in GS soybean. Comparing shoot- and root biomass of GS and GR soybean there was no significant difference between the two soils, while initially damage was significantly greater on the Regosol (Fig 6.4).

Determination of shoot and root dry weights of GS soybean cultivated on the two soils after glyphosate application on low or high weed densities showed in case of glyphosate application on weeds significantly impaired shoot- and root biomass production. However,

there were significant differences in terms of impaired shoot- and root biomass of GS soybean neither between the two soils nor between the different weed densities (Fig. 6.5).

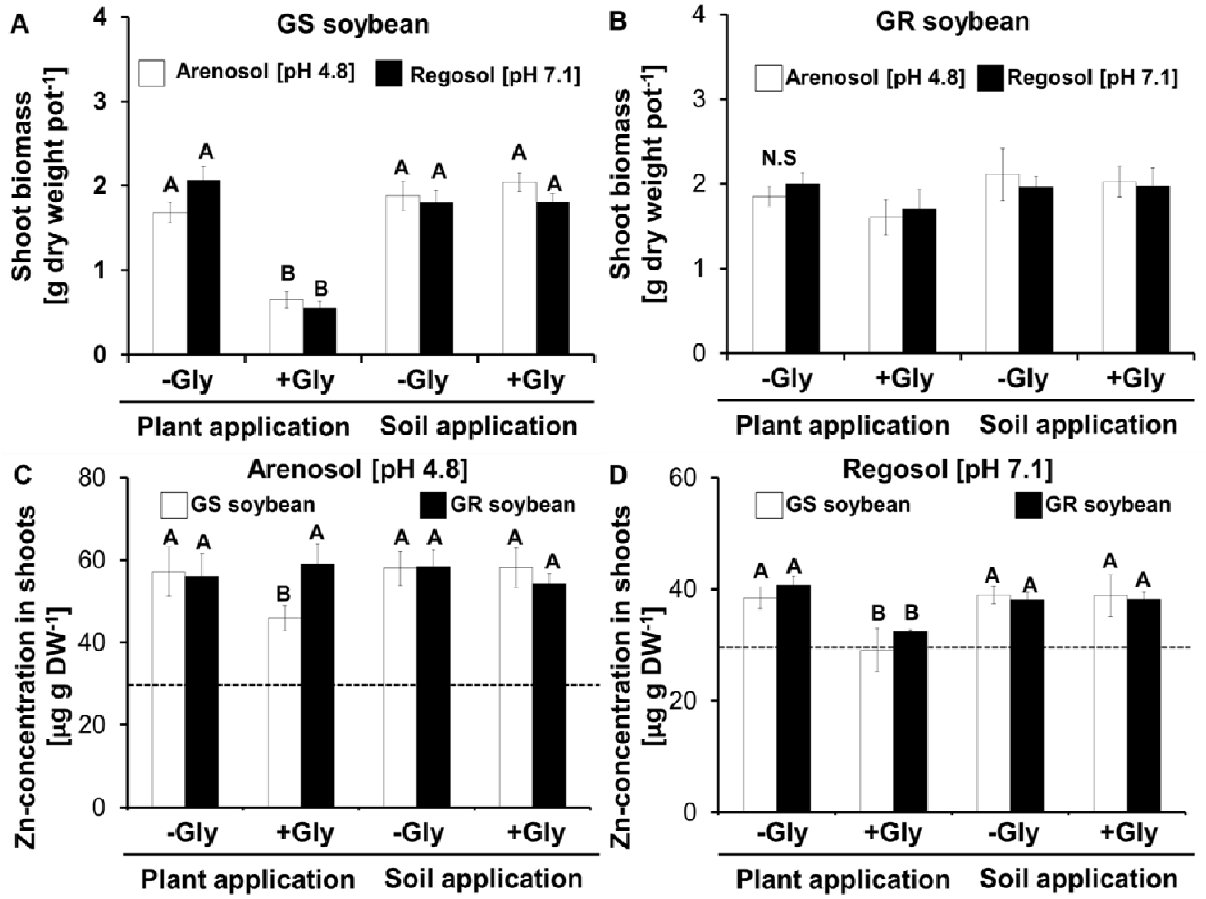

Fig. 6.4: Biomass and Zinc concentration in shoots of glyphosate-sensitive (GS) and glyphosate-resistant (GR) soybean depending on soil type and glyphosate application

Shoot dry matter (A, B) and Zn concentration (C,D) in the shoots of GS and GR soybean (*Glycine max* L.) plants were measured 28 days after sowing (DAS) on a sandy acidic Arenosol (pH 4.8) and a clayly neutral Regosol (pH 7.1) with (+Gly) or without (-Gly) glyphosate application at 5 days before sowing to a pre-culture of rye grass (*Lolium perenne* L.) (Plant application) or directly incorporated into the soil (Soil application), respectively. Data represent means and standard deviations of 4 independent replicates for GS soybean and 8 replicates for GR soybean. Significant differences between treatments are indicated by different characters.

Evaluation of nutritional status of plants

Determination of nutrient concentrations in shoots of GS and GR soybean showed for plants grown on the Arenosol only in GS soybean significantly lower concentrations of Zn (Fig. 6.4), Mn, Ca and Mg (data not shown) in case of glyphosate application on weeds in comparison to control and soil application of glyphosate. On this soil, significant decline in Mn and Zn concentrations in shoots of GS soybean were also induced by application of

glyphosate to varied densities of pre-cultured weeds. However, concentrations were for all mineral nutrients still above the deficiency threshold (Fig. 6.5).

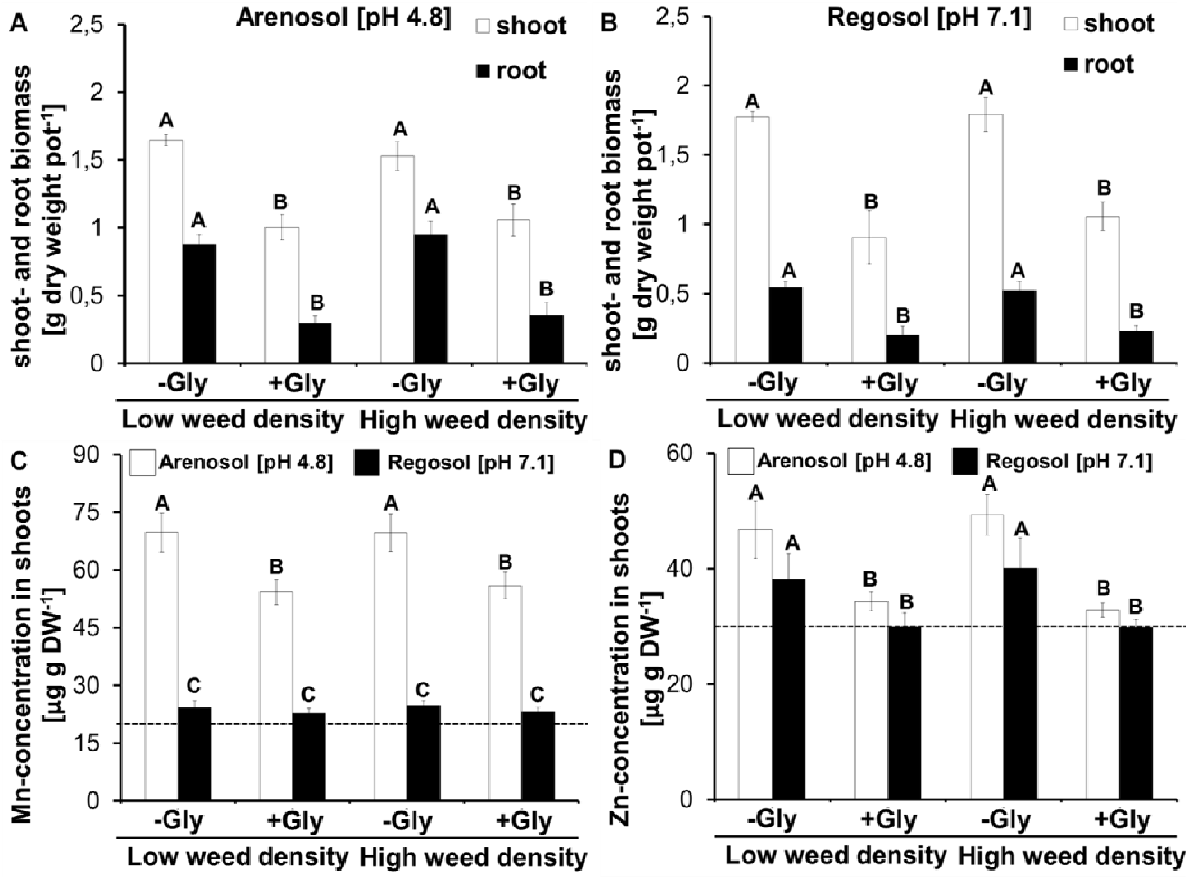

Fig. 6.5: Shoot, root biomass and micronutrients concentrations in shoots of glyphosate-sensitive (GS) soybean depending on soil type and density of glyphosate-treated weeds

Shoot and root dry matter (A, B) and (C) Mn- or (D) Zn concentration in shoots of GS soybean (*Glycine max* L.) plants were measured 28 days after sowing (DAS) on a sandy acidic Arenosol (pH 4.8) and a clayly neutral Regosol (pH 7.1) with (+Gly) or without (-Gly) glyphosate application at 5 days before sowing to a low (Low) – or a high (High) density of weed plants (*Lolium perenne* L.) pre-cultured for 10 days before glyphosate application in the same pots. Data represent means and standard deviations of 4 independent replicates. Significant differences between treatments are indicated by different characters.

By contrast, Zn concentrations in shoots of GS and GR soybean grown on the Regosol were significantly declined and close to/below the Zn deficiency threshold in case of glyphosate application on weed plants (Fig. 6.4). Similarly, a significant decline in Zn concentrations in shoots of GS soybean below the Zn deficiency threshold was also induced by application of glyphosate to varied densities of pre-cultured weeds on this soil (Fig. 6.5).

Mn, Fe, Cu, Ca, Mg, K and P concentrations in shoots of GS and GR soybean grown on the Regosol showed no significant differences between glyphosate- and control treatments (data not shown).

6.5 Discussion

Glyphosate transfer in the rhizosphere

In the present study, an application of glyphosate directly to the soils caused no glyphosate-induced damage of soybean such as accumulation of shikimate, decline of plant biomass and impaired nutrient status of GS and GR soybean (Tab.6.1, 6.3, 6.5; Fig. 6.4). These findings support the concept of low soil toxicity of glyphosate caused by rapid glyphosate detoxification by adsorption and/ or microbial degradation (Giesy *et al*., 2000; Borggaard and Gimsing, 2008).

However, application of an identical amount of glyphosate on pre-cultured rye grass as target weed induced in GS and GR soybean significant damage in terms of declined germination, delayed seedling development, deformations of leaves, impaired plant growth, accumulation of shikimate as physiological indicator of glyphosate toxicity and impaired nutrient status of GS and GR soybean (Tab.6.1, 6.3, 6.5; Fig. 6.3, 6.4). Significantly damage of GS- and GR soybean plants after glyphosate application on pre-cultured weeds (Tab. 6.1-6.6; Fig. 6.4, 6.5) are most likely caused by a rhizosphere transfer of phytotoxic glyphosate from roots of glyphosate treated weed plants to subsequently grown crop plants, which is also in accordance with the knowledge on behaviour of glyphosate *in planta*. After uptake by leaves glyphosate is rapidly translocated throughout the plant, but preferentially to young plant tissue with high metabolic activity and growth rates such root tips and shoot apices where potentially up to up to 80 % of the absorbed glyphosate accumulates (Hetherington *et al*., 1999; Reddy *et al*., 2004). Recently, Doublet *et al*. (2009) reported that absorption of herbicides in plant delays their subsequent soil-degradation, and particularly in case of glyphosate persistence in soil could increase two to six times.

In experiments conducted in Chapter 4, glyphosate application to weeds resulted in much stronger and longer lasting damage of sunflowers in case of short waiting times before sowing of the subsequent crop compared to application of the same amount of glyphosate directly mixed with the soil.
There are several reports in literature on comparable crop damage induced by short waiting time between glyphosate application on weeds and sowing of crops on a number of soils with contrasting soil characteristics (Rodrigues *et al*., 1982; Römheld *et al*., 2008; Tesfamariam, 2009). According to Tesfamariam (2009) risk of crop damage associated to rhizosphere transfer of glyphosate from weeds to crops due to a contact contamination by roots in the soil and therefore largely soil independent. However, in other studies the soil type considerably influenced the risk for crop damage induced by glyphosate toxicity in the rhizosphere (Neumann *et al*., 2006; Tesfamariam *et al*., 2009)

In the present study, significant differences between both used soil types in terms of speed, intensity and expression of symptoms indicating glyphosate toxicity in GS and GR soybean were detectable during the vegetation period (Tab. 6.1-6.6). However, no significant difference between the two soils in terms of glyphosate-induced impaired plant biomass was observed at final harvest (Fig. 6.4, 6.5). Therefore, results support the conclusion that risk for crop damage associated to rhizosphere transfer of glyphosate is largely soil independent. But

results also indicate that the soil type might have high importance for risk of crop damage induced by glyphosate transfer from weeds to crops due to effects on the growth conditions of glyphosate treated weeds as well as factor influencing the decay of root residues of treated weed plants.

Results of Rodrigues *et al*. (1982) and of Chapter 5 (Fig. 5.4) indicate that strength and duration of damage of maize and wheat induced by phytotoxic glyphosate was directly correlated to the density of glyphosate-treated weed plants acting as storage pool of glyphosate in soils.
In contrast, in both experiments of the present study expression, strength and development of damage symptoms of GS (and GR) soybean were highly correlated to the speed of death of weed plants (Tab. 6.1-6.6; Fig. 6.1) but only indirectly affected by the density of treated weed plants (Tab. 6.2, 6.4, 6.6; Fig. 6.2). A correlation between development, intensity and expression of damage symptoms of GS and GR soybean plants and death of glyphosate treated weed potentially indicate that glyphosate release from treated weed roots might occur in two phases involving (a) exudation of glyphosate from living roots as well as (b) release of glyphosate from decaying root material. In line with this interpretation, results of various studies indicate, release of glyphosate from living roots within first few hours after application of glyphosate on weed plants (Neumann *et al*., 2006; Laitinen *et al*., 2007; Tesfamariam, 2009). However, Tesfamariam (2009) detected also evidence for increased damage of sunflowers in case of 14 days waiting time compared to 7 days waiting indicating a second peak of glyphosate release from roots of glyphosate treated weed plants during complete die off/decay of weed residues in soils.

In the present study, glyphosate-induced symptoms of plant damage during early growth stage were significantly stronger expressed in plants grown on the Regosol compared to the Arenosol (Tab. 6.1, 6.2; Fig. 6.1, 6.2)
In comparison between the two contrasting soils, the Regosol had considerably higher soil organic matter and most likely higher microbial activity. Potentially, higher microbial activity on the Regosol has contributed to a more rapid degradation of glyphosate treated root residues leading to a massive release of glyphosate in a short time span and therefore to higher damage of GS and GR soybean in early growth stages.

A correlation between damage of soybean plants and death of glyphosate treated weeds (Tab. 6.1-6.6; Fig. 6.1, 6.2) might indicate allelopathic effects by decaying weed residues or transfer of soil-borne pathogens from pre-cultured rye grass to crops as potential causes for damage of GS and GR soybean in the present study. Several studies reported increase of infection of crops with fungal pathogen, when total herbicides were used to control weeds shortly before seeding of cereals (Smiley *et al*., 1992; Descalzo *et al*., 1998; Powell and Swanton, 2008). Similarly, it is known that residues of weed plants in soil can cause allelopathic effects on crop plants (Dudai *et al*., 2009). However, in the present study damage of GS soybean was significantly increased in comparison to GR soybean in controls but at the same time significantly smaller in comparison to damage of GS soybean (Tab. 6.3; Fig.6.2-6.4). Studies investigating sensitivity of GR crops to soil-borne pathogens or allelopathic effects revealed no differences compared to GS crops (Northsworthy, 2004; Kremer *et al*., 2005) Therefore, it

is unlikely that allelopathic effects of weed residues or soil-borne pathogens played a role for crop damage observed in the present study.

Glyphosate damage of GR soybean

Damage of GR soybean after indirect exposure to glyphosate by pre-crop application on weed plants has not been reported so far (Tab. 6.1, 6.3, 6.5; Fig. 6.1, 6.3, 6.4). However, according to Nandula *et al*. (2007) GR soybean are not fully resistant to glyphosate but about 50x less sensitive to glyphosate than non-resistant plants. Expression of damage symptoms and decline in plant biomass of GR and GS soybean observed in the present study confirms these results (Tab. 6.1, 6.3, 6.5; Fig. 6.1, 6.3, 6.4). According to various authors, damage of GR soybean after glyphosate application is most likely caused by toxicity of aminomethylphosphonic acid (AMPA) as phytotoxic metabolite of glyphosate (Reddy *et al*., 2004; Nandula *et al*., 2007; Zobiole *et al*., 2010a, 2010b). By contrast, Pline *et al*. (2002a) showed growth depression and accumulation of shikimate in GR cotton seedlings after root exposure to glyphosate. In this study the quantity of glyphosate-resistant EPSP synthase was 4.7 and 6.6 times greater in cotyledons compared to roots and tissues from dark-grown GR cotton seedlings contained 1.2–2.1 times less EPSP synthase than their light-grown counterparts. As accumulation of shikimate in root tissue of GR soybean was detectable in the present study, it seems plausible that damage of GR soybean was caused by direct glyphosate toxicity.
Interestingly, damage symptoms highly similar to those observed in the present study in GR soybean (Fig. 6.3) have been reported by extension services in the United States after glyphosate application in GR soybean fields (Taylor, 2002), but were attributed to drift of growth-regulator herbicides or re-mobilisation of these herbicides by glyphosate formulations in spray tanks. However, in the present study effects of growth regulator herbicides can be ruled out. Therefore it seems plausible that symptoms such as cupping and rolling of young trifoliar GR soybean leaves are caused by a secondary effect of glyphosate on the hormonal status of plants, which has been discussed already by Cole (1985).

Nutritional status of crops

Since glyphosate is a known chelator for divalent cations (e.g. Mn, Fe, Zn, Ca, Mg), competitive interactions limiting acquisition, uptake, translocation and intra-cellular utilisation of cationic nutrients have been discussed as putative causes for glyphosate-induced nutrient limitation (Sprankle *et al*., 1975a; Subramaniam and Hoggard, 1988; Eker *et al*., 2006; Ozturk *et al*., 2008; Cakmak *et al*., 2009). By contrast, several publications concluded that impaired nutritional status of GS and GR plants is most likely a secondary effect of glyphosate toxicity due to impaired root growth or effects on photosynthesis (Duke *et al*., 1983; Neumann *et al*., 2006; Tesfamariam, 2009; Zobiole *et al*., 2010a).
In the present study similar negative effects on plant growth were observed on the Regosol and the Arenosol in case of micronutrient deficiency as well as sufficiency (Fig. 6.4, 6.5), while calculations of nutrient contents in shoots revealed glyphosate-induced general impairment of anionic as well as cationic nutrients acquisition by GS and GR soybean. Therefore impaired nutritional status of plants was rather a secondary effect of glyphosate toxicity to GS- and GR soybean plants.

6.6 Conclusions

Results of the present study highlight the importance of roots residues of glyphosate treated weed plants as storage pool for glyphosate which is associated with increasing and prolonging phytotoxic activity in soils and potential damage of crops in case of short waiting time between application and sowing. A correlation between development, intensity and expression of damage symptoms of crop plants and death of glyphosate treated weed suggest a close connection between risk for crop damage induced by glyphosate transfer from root residues of treated weeds to subsequently cultivated crops and the biotic and abiotic growth conditions. The connection between risk for glyphosate-induced crop damage and environmental growth conditions might explain why glyphosate damage of crops after application on weed plants is frequently but not generally observed.

For a better understanding of risks for crop damage associated to glyphosate stored in residues of treated weeds and to achieve better recommendations for farmers, factors like growth season (spring or fall application), temperature, water content of soils and soil microbial activity which might shorten or enhance the time window for crop damage caused by transfer of phytotoxic glyphosate in the rhizosphere need to be evaluated in future model experiments but also under field conditions.

7 Important factors for rhizosphere transfer of glyphosate: II. Role of differences in sensitivity of crops to glyphosate

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Own contribution: set-up of experiments, plant cultivation, harvest and sample preparation, analysis of nutritional status of plants (support of students in 2 of 2 experiments) ,analysis of shikimate by HPLC, manuscript preparation

7.1 Abstract

Several recent publications indicated crop damage induced by rhizosphere transfer of glyphosate from roots of treated target weeds to subsequently grown crop in model experiments and under field conditions. Differences between plant species in sensitivity to glyphosate in the rhizosphere might influence the probability and severity of crop damage caused by glyphosate rhizosphere transfer from weeds to crops. However, this has not been studied in detail so far.

To assess potential differences in sensitivity to glyphosate in the rhizosphere between crop species, soybean, maize and wheat seedlings were cultivated for 24 h in 5, 10 or 30 µM glyphosate in deionised water. Subsequently plants were transferred to nutrient solution and cultivated for additional 21 days. Visual symptoms of glyphosate toxicity, plant biomass, intracellular shikimate accumulation as physiological indicator for glyphosate toxicity and the plant nutritional status were determined to evaluate potential sensitivity to glyphosate.

Assessments of these indicators for glyphosate revealed consistently significant differences between the plant species with wheat being most susceptible and soybean most resistant to glyphosate in the rhizosphere. Interestingly wheat and maize, but not soybean showed visually detectable symptoms of glyphosate toxicity on shoots, while initial glyphosate damage of roots was comparable in all plant species.

Results indicate that differences between plant species in sensitivity to glyphosate in the rhizosphere are potentially related to differences in translocation of glyphosate from roots to shoots, in glyphosate uptake by roots and/or to the ability for internal detoxification of glyphosate by conversion to its less phytotoxic metabolite aminomethylphosphonic acid (AMPA). Furthermore, results suggest a strong connection between sensitivity of plant species to glyphosate and the abiotic and biotic growth conditions as determining factors for the severity of glyphosate induced crop damage and the potential for recovery of crops.

Key words: glyphosate, toxicity, rhizosphere, plant species, sensitivity, soybean (*Glycine max* L.), maize (*Zea maize* L.), winter wheat (*Triticum aestivum* L.)

Abbreviations:

AMPA aminomethylphosphonic acid

DAT days after transfer

EPSP 5-enolpyruvylshikimate-3-phosphate

GS: glyphosate-sensitive

7.2 Introduction

Due to low production costs and high efficiency, glyphosate (N-phosphonomethylglycine) is the most widely used herbicide on global scale (Baylis, 2000). The primary mechanism of action of glyphosate is the competitive inhibition of the 5-enolpyruvylshikimate-3-phosphate (EPSP) synthase enzyme in the shikimic acid pathway leading to impaired conversion of shikimic acid to chorismic acid thereby causing impairment of general metabolic processes, such as synthesis of aromatic amino acids, protein synthesis and photosynthesis (Geiger *et al*., 1986). Therefore, glyphosate application frequently induces intracellular accumulation of shikimate, which can be used as a sensitive physiological indicator for glyphosate toxicity (Henry *et al*., 2007).

Risks of glyphosate toxicity to non-target plants in soils are generally considered as marginal, since glyphosate in the soil solution is prone to rapid microbial degradation or almost instantaneous inactivation by sorption to the soil matrix (Giesy *et al*., 2000). However, an additional potential pool of glyphosate in soils, which has not been widely considered so far, might be present in plant residues of treated weeds. As systemic herbicide, glyphosate is translocated throughout the plant and organs with high metabolic activity and growth rates such as nodules, root tips and shoot apex represent a high sink activity for glyphosate. In many plant species, glyphosate is not readily metabolised and considerable amounts can accumulate particularly in young tissues (Reddy *et al*., 2004). Under field conditions Laitinen and Rämö (2005) detected 40 days after application glyphosate concentrations of up to 2.7 mg kg dry weight^{-1} in roots of glyphosate treated weeds, while glyphosate concentrations in 0–5 cm and 5–35 cm soil layers were 0.17 and 0.07 mg kg dry weight^{-1}. Recently, Doublet *et al*. (2009) reported that accumulation of herbicides in plants and in particularly in root residues delayed their subsequent soil-degradation, and particularly in case of glyphosate persistence in soil could increase two to six times and should be considered for environmental risk assessments.

In line with this, significant damage of crop plants most likely caused by a transfer of glyphosate from treated roots and/ or root residues of treated weeds to subsequently sown crops was repeatedly observed in model experiments under controlled conditions (Rodrigues *et al*., 1982; Neumann *et al*., 2006; Tab. 4.1, 4.2, 5.2; Fig 4.3, 4.4, 5.4), but also by farmers frequently but not always in fields (Römheld *et al*., 2008; Tab. 5.1; Fig. 5.3).

This suggests that risks associated to glyphosate toxicity in the rhizosphere might be influenced by abiotic and biotic factors at the field site and potentially most importantly by the plant-specific sensitivity to phytotoxic glyphosate in the rhizosphere. However, differences between crops in their sensitivity to glyphosate in rhizosphere have not been studied so far.

In contrast to this, results of numerous studies investigating effects of glyphosate drift on crop plants revealed significant differences in sensitivity of plant species as well as the growth stage at exposure to glyphosate (Ellis *et al*., 2003; Roider *et al*., 2007; Al-Kathib and Peterson, 1999; Ellis and Griffin, 2002). In these studies, gramineous plant species like wheat (*Triticum aestivum* L.), maize (*Zea maize* L.) and rice (*Oryza sativa* L.) responded with

significant yield reduction when glyphosate was applied in early growth stages (Ellis *et al*., 2003; Roider *et al*., 2007), while particularly in conventional soybean (*Glycine max* L.) and in some extent also cotton (*Gossypium hirsutum* L.) appear to be more resistant to glyphosate after drift exposure (Al-Kathib and Peterson, 1999; Ellis and Griffin, 2002; Norsworthy, 2004a).

On background of repeated observations of crop damage induced by glyphosate in the rhizosphere (Rodrigues *et al*., 1982; Neumann *et al*., 2006; Tab. 4.1, 4.2, 5.2; Fig 4.3, 4.4, 5.4) and the known differences in sensitivity of crop species to simulated glyphosate drift (Ellis *et al*., 2003; Roider *et al*., 2007; Al-Kathib and Peterson, 1999; Ellis and Griffin, 2002; Norsworthy, 2004a) the present study was initiated to evaluate the hypothesis of significant differences between crop species in their sensitivity to glyphosate in the rhizosphere. For this purpose, in a series of hydroponic experiments under controlled environmental conditions soybean, maize and winter wheat were compared in their response to a short term root exposure of different µM concentrations of glyphosate. During a culture period of 21 days visual symptoms of glyphosate toxicity, plant biomass, intracellular shikimate accumulation as physiological indicator for glyphosate toxicity and the plant nutritional status were determined to detect potential differences between the plant species in sensitivity to glyphosate.

7.3 Material and Methods

Growth conditions

Hydroponic experiments were performed in a growth chamber under controlled environmental conditions with a light/dark regime of 14/10 h at 24/20 °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.

In all experiments seeds of GS soybean cv. Conquista, GS winter wheat (*Triticum aestivum* L. cv. Isengrain-B) and GS hybrid maize were sterilised for 5 min in 30 % H_2O_2, soaked for 5 h in 10 mM $CaSO_4$ and germinated in upright position for 4 days in an incubator at 24 °C in rolls of filter paper (MN 710, Macchery & Nagel, Düren, Germany) soaked with 2.5 mM $CaSO_4$.

Application of glyphosate

To evaluate toxic effects of root supplied glyphosate six seedlings of winter wheat, soybean and maize were transferred to 2.8 L plastic pots (diameter: 18 cm, depth: 16 cm). Different concentrations of glyphosate (as Roundup UltraMax®) were supplied to the pots resulting in glyphosate concentrations of 0 (control), 5, 10 and 30 µM in the pots. To avoid any complexation/inactivation of glyphosate by di- or trivalent cationic mineral nutrients, glyphosate was supplied for 24 h only in deionised water within 2.8 L pots. Afterwards plants were transferred to new pots (6 plants per pot) containing continuously aerated full nutrient solution (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, 1 µM H_3BO_3 for wheat and maize and 10 µM H_3BO_3 for soybean, 0.5 µM $ZnSO_4$, 0.5 µM $MnSO_4$, 0.2 µM $CuSO_4$ and 0.01 µM $(NH_4)_6Mo_7O_{24}$).

Evaluated parameters

Parameters of shoot development such as determination of shoot height, developmental speed of plants based on BBCH, chlorosis scoring (SPAD-value) and visual scoring of symptoms of potential glyphosate- and/or AMPA phytotoxicity were repeatedly conducted throughout the growth period of 21 days.

Additionally 24, 48 and 72 h after transfer to nutrient solution root growth and root morphology of soybean, maize and wheat were assessed non-destructively. For this purpose, plants were carefully removed from the pots and placed in a transparent waterbath for scanning of the root system. Subsequently scanned images of roots were analysed using the WinRhizo Pro® (Regent Instruments, Quebec, Canada) digital imaging software.

At day 7 after transfer to aerated nutrient solution, a first set of 3 plants was removed from the pots. Roots and shoots were separated and fresh weights of all plant parts were determined. Subsequently, shoots and roots were frozen in liquid nitrogen and stored at -20 °C for analysis of accumulated shikimate in plant tissue as physiological indicator of glyphosate toxicity. In each pot, three plants were kept and further cultivated until final harvest 21 days after transfer. At final harvest, fresh and dry weights after oven-drying at 60 ^{o}C of all plant parts (roots and shoot) were determined. Subsequently dried shoots were grinded for analysis of nutritional status of plants.

Shikimate analysis

Shikimate in acidic tissue extracts was analysed with modifications of the methods described by Singh and Shaner (1998) and Neumann *et al*. (2006). The frozen plant tissue was homogenised 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 20.000 x g) and the supernatant was used for HPLC analysis after appropriate dilution with the HPLC mobile phase. HPLC separation was performed by ion exclusion chromatography using an Aminex 87H column (Bio-Rad, Richmond, CA, USA) designed for organic acid analysis. A sample volume of 20 µL was injected into the isocratic flow (0.5 mL min^{-1}) of the eluent (2.5 mM H_2SO_4, 40 °C) and organic acids were detected spectrophotometrically at 210 nm. Identification and quantification of shikimate were conducted by comparing the retention times, absorption spectra and peak areas with a known standard.

Analysis of mineral nutrients

One hundred milligram of dried shoot material was ashed in a muffle furnace at 500 °C for 5 h. After cooling, the samples were extracted twice with 1 mL of 3.4 M HNO_3 and evaporated until dryness to precipitate SiO_2. The ash was dissolved in 1 mL of 4 M HCl, subsequently diluted ten times with hot deionised water, and boiled for 2 min to convert meta- and pyro-phosphates to orthophosphate. 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). Spectrophotometrical determination of orthophosphate was conducted after addition of molybdate-vanadate colour reagent according to the method of Gericke and Kurmis (1952). Determination of Mg was conducted by atomic absorption spectrometry, while K and Ca were measured by flam photometry.

Statistics

Experiments were conducted in a completely randomised block design with three 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).

7.4 Results

Assessment of symptoms of potential glyphosate damage on shoots of soybean, maize and winter wheat showed considerable differences between the plant species in terms of response to glyphosate in the rhizosphere. In comparison to control, a visual scoring of potential glyphosate toxicity revealed for soybean a slight reduction of plant height (data not shown) but no other symptoms such as chlorosis in meristematic tissue of leaves. In contrast to this, in maize typical symptoms of glyphosate toxicity described in literature were observed. For winter wheat no typical symptoms of glyphosate toxicity such as a bright chlorosis in young leaf tissue and no decline in SPAD-value was observed (Tab. 7.1). However, a visual assessment of plant damage revealed for wheat expression of stripe chlorosis and in case of high levels of glyphosate indications for accumulation of anthocyanin in leaves (data not shown).

Corresponding to this, determination of speed of plant development based on the BBCH code revealed for maize and wheat a significantly impaired plant development which increased with glyphosate concentrations applied during 24 h. By contrast, no significant delay in development was detectable for soybean (Fig. 7.1).

By contrast to shoots, analysis of growth and morphology of roots revealed a similar pattern of glyphosate damage in all plant species. Primary effect of glyphosate on root growth and morphology was inhibition of elongation of the main root (Fig. 7.2) while formation of lateral roots, numbers of root tips and root surface area was considerably less affected (data not shown). Plants differed in their root susceptibility to phytotoxic glyphosate with wheat being most sensitive while maize roots were most resistant. Soybean roots showed lowest relative growth increase particularly in case of medium and high glyphosate levels applied (Tab. 7.2; Fig. 7.2).

Tab. 7.1 SPAD-values of the youngest fully developed leaf of soybean, maize and winter wheat plants depending on glyphosate root supply

Soybean (*Glycine max* L.), maize (*Zea maize* L.) and winter wheat (*Triticum aestivum* L.) plants were grown for seven days under hydroponic conditions after of short-term (24 h) root exposure to 0 (control), 5-, 10-, or 30 µM glyphosate in deionised water. Data represent means and standard deviations of 3 independent replicates. Significant differences ($p<0.05$) are indicated with different characters.

	SPAD-Value		
treatment	**soybean**	**maize**	**wheat**
Control	**26** ±2 ^{A}	**27** ±4 ^{A}	**32** ±2 ^{A}
5µM Gly	**28** ±1 ^{A}	**23** ±3 ^{A}	**30** ±3 ^{A}
10µM Gly	**29** ±1 ^{A}	**18** ±2 ^{B}	**34** ±8 ^{A}
30µM Gly	**27** ±1 ^{A}	**14** ±2 ^{B}	**29** ±5 ^{A}

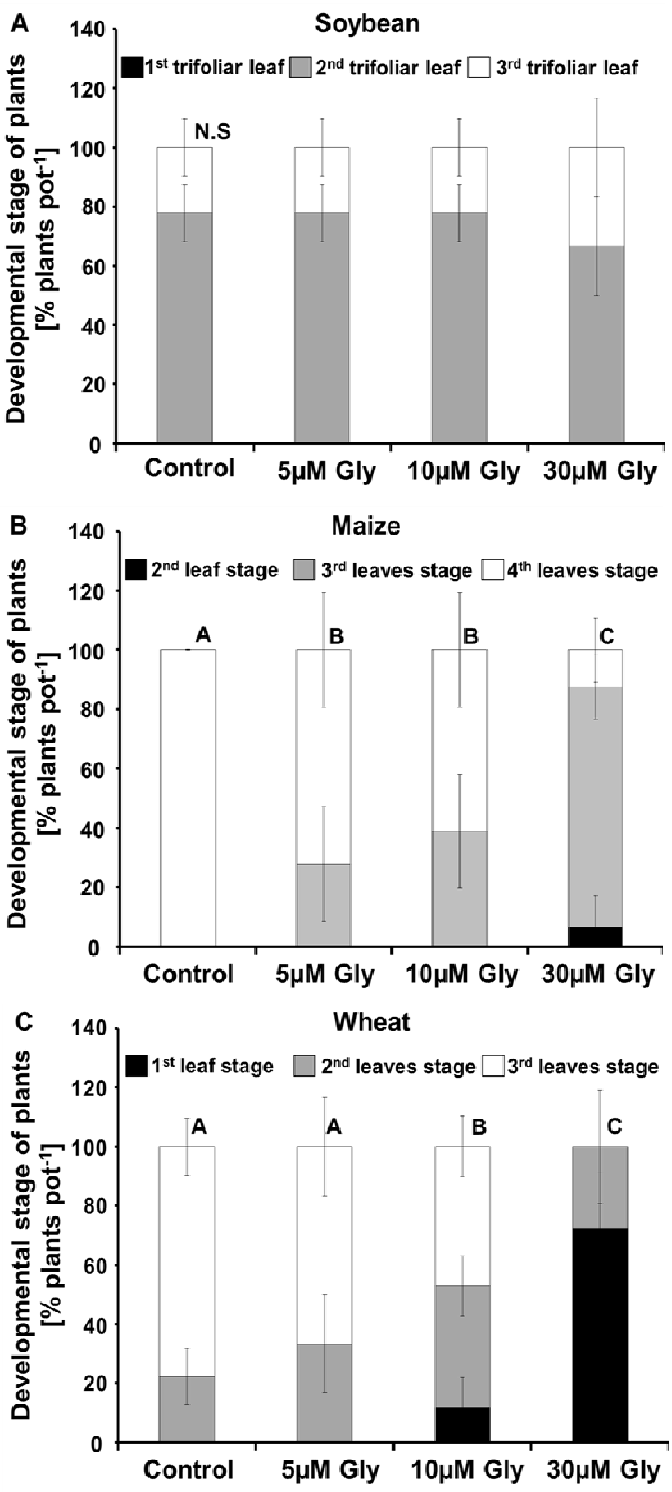

Fig. 7.1: Speed of development of soybean, maize and winter wheat depending on glyphosate root supply

Assessment of developmental speed (based on BBCH code) were measured on soybean (*Glycine max* L.), maize (*Zea maize* L.) and winter wheat (*Triticum aestivum* L.) plants grown for seven days under hydroponic conditions after short-term (24 h) root exposure to 0 (control), 5-, 10-, or 30 µM glyphosate (Gly) to the nutrient solution. Data represent means and standard deviations of 3 independent replicates. Significant differences ($p<0.05$) are indicated with different characters.

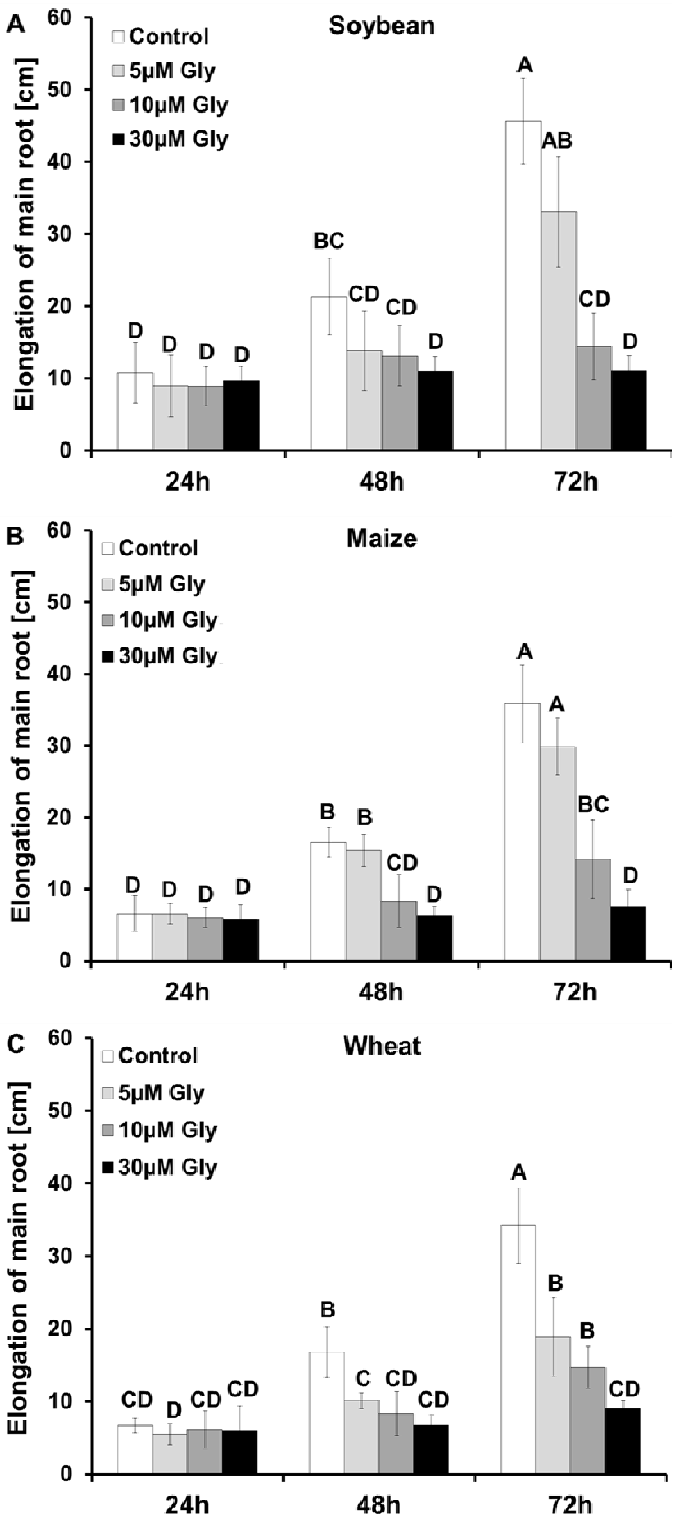

Fig. 7.2: Elongation of main roots of soybean, maize and winter wheat plants depending on glyphosate root supply

Soybean (*Glycine max* L.), maize (*Zea maize* L.) and winter wheat (*Triticum aestivum* L.) were cultivated under hydroponic conditions for 24, 48 or 72 h after short-term (24 h) root exposure to 0 (control), 5 10, or 30 µM glyphosate in deionised water. Data represent means and standard deviations of 3 independent replicates. Significant differences ($p<0.05$) are indicated with different characters.

Determination of plant biomass at the first harvest after 7 days showed in all plant species significantly impaired shoot and root biomass by glyphosate treatments in comparison to controls. In all plant species biomass gradually declined with increasing glyphosate concentrations applied. However, there were differences in sensitivity to glyphosate between plant species, with winter wheat being significantly more susceptible compared to maize and particularly to soybean. Increasing glyphosate concentrations induced in soybean a similar decline in shoot compared to root biomass, which was however slightly stronger expressed in roots. By contrast, in wheat inhibition of shoot biomass was significantly higher compared to root biomass inhibition, especially in case of high glyphosate levels (Tab. 7.2).

Tab. 7.2 Shoot and root biomass of soybean, maize and winter wheat plants depending on glyphosate root supply

Soybean (*Glycine max* L.), maize (*Zea maize* L.) and winter wheat (*Triticum aestivum* L.) plants were grown for seven days (DAT) under hydroponic conditions after short-term (24 h) root exposure to 0 (control), 5, 10, or 30 µM glyphosate in deionised water. Data represent means and standard deviations of 3 independent replicates. Significant differences ($p<0.05$) are indicated with different characters.

	Plant biomass (g FW plant $^{-1}$)							
	Shoot				Root			
	Control	5µM Gly	10µM Gly	30µM Gly	Control	5µM Gly	10µM Gly	30µM Gly
7 DAT								
soybean	**1.82**±0.1^{A}	**1.78**±0.1^{A}	**1.48**±0.0^{B}	**1.01**±0.2^{C}	**1.26**±0.0^{A}	**1.14**±0.1^{A}	**0.97**±0.1^{B}	**0.58**±0.1^{C}
% of control		98±6	81±9	56±13		91±12	77±3	46±12
maize	**0.97**±0.1^{A}	**0.95**±0.2^{A}	**0.66**±0.2^{B}	**0.39**±0.1^{C}	**0.78**±0.1^{A}	**0.77**±0.0^{A}	**0.60**±0.2^{AB}	**0.44**±0.1^{C}
% of control		99±27	70±23	40±11		100±14	79±30	57±12
wheat	**0.38**±0.1^{A}	**0.29**±0.0^{B}	**0.13**±0.0^{C}	**0.07**±0.0^{D}	**0.34**±0.0^{A}	**0.29**±0.0^{B}	**0.16**±0.0^{C}	**0.11**±0.0^{C}
% of control		77±11	34±4	19±5		85±9	47±13	33±8

In all plant species application of glyphosate induced a gradual increase of shikimate concentrations in root tissue. However, increase of shikimate concentrations in root tissue of soybean were generally smaller compared to maize and wheat and in comparison to control only significantly increased at high levels of glyphosate applied. By contrast, in maize and wheat significantly increased concentrations of shikimate were already observed in case of low glyphosate application level (5µM) (Fig. 7.3).

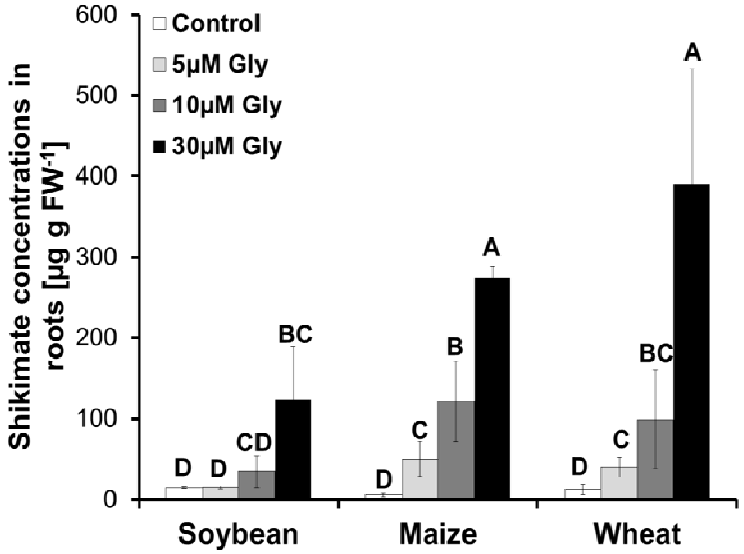

Fig. 7.3: Accumulation of shikimate in roots of soybean, maize and winter wheat plants depending on glyphosate root supply

Accumulation of shikimate as specific physiological indicator of glyphosate toxicity was measured in root tissue of soybean (*Glycine max* L.), maize (*Zea maize* L.) and winter wheat (*Triticum aestivum* L.) plants grown for seven days under hydroponic conditions after short-term (24 h) root exposure to 0 (control), 5, 10, or 30 µM glyphosate in deionised water. Data represent means and standard deviations of 3 independent replicates. Significant differences ($p<0.05$) are indicated with different characters.

Determination of shoot and root biomass at final harvest 21 days after short-term glyphosate application indicated also differences between plant species in terms of recovery from plant damage induced by phytotoxic glyphosate in the rhizosphere. In comparison to the first harvest, soybean plants had completely recovered from plant damage induced by low or medium levels glyphosate toxicity and showed no significant difference in shoot and root biomass compared to control. By contrast, maize and wheat only recovered at low glyphosate levels. Similarly to first harvest, soybean showed significantly less impaired shoot and root biomass compared to wheat, which showed strongest decline at all glyphosate levels. By contrast to first harvest, also maize shoot- and root biomass was significantly lower in comparison to soybean even so still significantly larger compared to wheat (Tab. 7.3).

Determination of micro- and macronutrient concentrations (Mn, Zn, Fe, Cu, Ca, K) in shoots of soybean, maize and wheat exposed to low or medium levels of glyphosate in the nutrient solution revealed generally no significant differences compared to control. However, short-term cultivation at high levels of glyphosate caused in maize and wheat but not soybean significantly lower K (data not shown) and Zn and Mn concentrations (Tab. 7.4) in shoots which were however still above the deficiency thresholds. By contrast, in wheat shoot concentrations of Fe-, Cu- and Ca were significantly increased in case of short term application of 30 µM glyphosate compared to control. Exposure to high levels of glyphosate in the nutrient solution induced in all plant species significant decline of shoot contents of all nutrients compared to controls due to a strong growth inhibition (data not shown).

Tab. 7.3 Shoot and root biomass of soybean, maize and winter wheat plants depending on glyphosate root supply

Soybean (*Glycine max* L.), maize (*Zea maize* L.) and winter wheat (*Triticum aestivum* L.) plants were grown for 21 days (DAT) under hydroponic conditions after short-term (24 h) root exposure to 0 (control), 5, 10, or 30 µM glyphosate in deionised water. Data represent means and standard deviations of 3 independent replicates. Significant differences ($p<0.05$) are indicated with different characters.

Plant biomass (g FW plant $^{-1}$)

	Shoot				Root			
	Control	**5µM Gly**	**10µM Gly**	**30µM Gly**	**Control**	**5µM Gly**	**10µM Gly**	**30µM Gly**
21 DAT								
soybean	**7.41**±0.4^{A}	**7.33**±0.5^{A}	**7.05**±0.7^{A}	**4.84**±0.6^{B}	**3.24**±0.2^{A}	**3.33**±0.4^{A}	**3.52**±0.2^{A}	**2.22**±0.4^{B}
% of control		99±5	95±7	65±7		104±19	109±10	69±11
maize	**5.11**±0.4^{A}	**5.42**±0.7^{A}	**3.34**±0.2^{B}	**0.77**±0.1^{C}	**2.27**±0.3^{A}	**2.48**±0.4^{A}	**1.81**±0.2^{B}	**0.42**±0.1^{C}
% of control		107±19	66±7	15±2		110±14	82±19	19±2
wheat	**1.86**±0.2^{A}	**1.76**±0.1^{A}	**0.64**±0.3^{B}	**0.13**±0.0^{C}	**1.13**±0.2^{A}	**1.15**±0.1^{A}	**0.49**±0.3^{B}	**0.11**±0.0^{C}
% of control		95±14	35±19	7±1		104±23	46±32	9±3

Tab. 7.4 Micronutrient concentrations in shoots of soybean, maize and winter wheat plants depending on glyphosate root supply

Concentrations of Manganese (Mn), Zinc (Zn), Iron (Fe) and Cupper (Cu) (µg g DW^{-1}) were measured in shoots of soybean (*Glycine max* L.), maize (*Zea maize* L.) and winter wheat (*Triticum aestivum* L.) plants were grown for 21 days under hydroponic conditions after short-term (24 h) root exposure to 0 (control), 5, 10, or 30 µM glyphosate in deionised water. Data represent means and standard deviations of 3 independent replicates. Significant differences ($p<0.05$) are indicated with different characters.

	Micronutrient concentrations in shoots [µg g DW^{-1}]			
	Mn	**Zn**	**Fe**	**Cu**
soybean				
Control	**55** ± 8 $^{N.S}$	**34** ± 3 $^{N.S}$	**102** ± 19 $^{N.S}$	**9** ± 0 $^{N.S}$
5µM Gly	**58** ± 2 $^{N.S}$	**42** ± 11 $^{N.S}$	**66** ± 26 $^{N.S}$	**8** ± 1 $^{N.S}$
10µM Gly	**61** ± 8 $^{N.S}$	**35** ± 3 $^{N.S}$	**104** ± 35 $^{N.S}$	**8** ± 1 $^{N.S}$
30µM Gly	**52** ± 7 $^{N.S}$	**33** ± 5 $^{N.S}$	**85** ± 12 $^{N.S}$	**7** ± 1 $^{N.S}$
maize				
Control	**83** ± 5 ^{A}	**83** ± 14 $^{N.S}$	**58** ± 2 $^{N.S}$	**14** ± 1 $^{N.S}$
5µM Gly	**77** ± 12 ^{A}	**69** ± 4 $^{N.S}$	**50** ± 7 $^{N.S}$	**13** ± 1 $^{N.S}$
10µM Gly	**89** ± 10 ^{A}	**85** ± 15 $^{N.S}$	**57** ± 4 $^{N.S}$	**15** ± 1 $^{N.S}$
30µM Gly	**46** ± 24 ^{B}	**71** ± 18 $^{N.S}$	**59** ± 5 $^{N.S}$	**14** ± 2 $^{N.S}$
wheat				
Control	**160** ± 11 ^{A}	**77** ± 7 ^{A}	**94** ± 3 ^{B}	**16** ± 0 ^{B}
5µM Gly	**146** ± 18 ^{A}	**75** ± 3 ^{A}	**91** ± 7 ^{B}	**16** ± 2 ^{B}
10µM Gly	**98** ± 42 ^{AB}	**61** ± 11 ^{AB}	**93** ± 5 ^{B}	**18** ± 2 ^{B}
30µM Gly	**55** ± 5 ^{B}	**48** ± 5 ^{B}	**209** ± 13 ^{A}	**41** ± 19 ^{A}

7.5 Discussion

Differences in susceptibility of plant species to glyphosate

Results of the present study demonstrated that plant species differ significantly in their susceptibility to glyphosate applied to hydroponic culture. Evaluation of visual symptoms of glyphosate toxicity, such as chlorosis in young meristematic tissue of leaves (Tab. 7.1), speed of plant development (Fig. 7.1) and plant biomass production (Tab. 7.2, 7.3) suggest a different sensitivity in the order soybean<maize<wheat. Similarly, determination of shikimate concentrations in roots as physiological indicator of glyphosate toxicity (Fig. 7.3) and evaluation of the nutritional status of plants (Tab. 7.4) indicated a higher sensitivity of maize and wheat in comparison to soybean.

From these data it can be concluded that a similar difference of plant species to glyphosate in the rhizosphere will occur which have not been reported so far. Also, observed crop damage

after glyphosate drift suggest considerable differences between plant species in their sensitivity to glyphosate. In line with the results of the present study, significant damage after glyphosate drift have been reported for wheat, maize or rice, while particularly soybean appear to be more resistant to glyphosate toxicity after drift exposure (Ellis *et al*., 2003; Roider *et al*., 2007; Al-Kathib and Peterson, 1999; Ellis and Griffin, 2002; Norsworthy, 2004a). The exact mechanisms for these differences between plant species in their sensitivity to glyphosate are not fully understood. It has also to be shown by future studies whether these differences between plant species can be generalised if other cultivars will get considered.

Allister *et al*. (2005) demonstrated different glyphosate distribution patterns within plants, depending on leaf or root exposure to glyphosate. In case of foliar application, 80 % of applied glyphosate was translocated to the shoot meristems and young leaves. By contrast, up to 75 % of glyphosate mainly remained in the young root tissues when the herbicide was supplied to the roots. Altered translocation pathways/ limited translocation of glyphosate have been described as mechanism for increased glyphosate resistance in weed plants (Yu *et al*., 2009; Wakelin *et al*., 2004).
In the present study, soybean showed no typical symptoms of glyphosate toxicity such as chlorosis in young meristematic shoot tissue on shoots and only slightly impaired shoot growth, while strongly impaired shoot growth was detected in maize and particularly in wheat (Tab. 7.1, 7.2; Fig. 7.1). Glyphosate induced in maize significant expression of chlorosis in young meristematic tissue (Tab. 7.1), strong delay in developmental speed (Fig. 7.1) and a weak ability for recovery in later growth stages (Tab. 7.2, 7.3) which might be related to strongly impaired photosynthesis. In wheat, glyphosate-induced damage of plants was not associated to typical symptoms of glyphosate toxicity (Tab. 7.1). However, observations of a stripe chlorosis and indications for accumulation of anthocyanin in wheat leaves (data not shown) also hint to glyphosate-induced impairment of general metabolic processes e.g. photosynthesis.
However, analysis of root growth and morphology showed that roots of soybean plants were similarly damaged in comparison to maize and wheat (Fig. 7.2). These findings suggest that differences in mobility of glyphosate in plants e.g. a low translocation from roots to shoots might be responsible for differences in sensitivity to glyphosate toxicity. Limited translocation of glyphosate from roots to shoots (soybean< maize ~ wheat) might induce a lower sensitivity of soybean to glyphosate by limiting disruption of crucial physiological processes for plants taking place in the shoots e.g. in chloroplasts.

There is evidence that glyphosate is one of the few herbicides that crosses the plasma membrane using an active transport system (Gougler and Geiger, 1981; Morin *et al*., 1997; Tilquin *et al*., 2000). Therefore, difference in efficiency between plant species in glyphosate uptake in roots might contribute to difference in sensitivity to glyphosate. On the one hand, in the present study particularly high phytotoxic activity of glyphosate in wheat but also in maize might be caused by higher efficiency of glyphosate uptake inducing higher toxicity (Tab. 7.2, 7.3; Fig. 7.1). It seems also plausible that active root uptake of glyphosate is self-limited due to physiological effects of glyphosate toxicity. In this case, lower sensitivity of soybean to glyphosate toxicity might be induced by low glyphosate uptake due to fast impairment of active glyphosate uptake. Direct or indirect phytotoxic effects of glyphosate on

activity of transporter proteins might also impair phloem- and/or xylem loading of glyphosate inducing limited translocation from roots to shoots.

Differential sensitivity of plant species to phytotoxic glyphosate in the rhizosphere may also be attributed to particularly high ability of soybean for *in planta* conversion of glyphosate to it primary metabolite aminomethylphosphonic acid (AMPA) (Reddy *et al*., 2004; Nandula *et al*., 2007). It has been suggested, that a plant glyphosate oxidoreductase (GOX) or similar type of enzyme catalyses this conversion. Reddy *et al*. (2008) showed in a comparison of plant species, that after foliar application of glyphosate the metabolite AMPA was detectable in six of seven leguminous species, but only in one of four non-leguminous species. Therefore it is possible, that in the present study the high ability of soybean for conversion of glyphosate to AMPA contributed in a low susceptibility to glyphosate toxicity. According to this, maize and wheat plants, which most likely lack the ability for internal glyphosate detoxification by conversion to AMPA, were affected by glyphosate during a prolonged time span and therefore significantly stronger damaged (Tab. 7.2, 7.3). The conversion of glyphosate to AMPA might also explain why shikimate concentrations in roots of soybean were significantly lower compared to maize and wheat (Fig. 7.3).

Phytotoxic effects of glyphosate in the root zone

As shown in the present study glyphosate toxicity comprised a delay in developmental speed of plants (Fig. 7.2), decline of plant growth (Tab. 7.2, 7.3) and/or impaired elongation of main roots and altered root morphology (Fig. 7.3). A delay in developmental speed and effects of root growth and morphology suggest that expression and intensity of crop damage induced by glyphosate in the rhizosphere is also strongly linked to environmental growth conditions. Arguably, biotic and abiotic stress factors, for instance pathogen pressure, allelopathic effects of weed plants, cold or drought stress, limited nutrient supply or oxidative stress due high light intensity will more strongly affect plants impaired in their shoot and/or root development. By contrast, optimal biotic and abiotic growth conditions e.g. nutrient and water availability in later growth stages might also facilitated complete recovery of crops from glyphosate damage.

Nutritional status of plants

Several recent publications have reported a glyphosate-induced impaired plant-nutritional status, particularly of cationic mineral nutrients such as Mn, Zn, Fe and Ca (Neumann *et al*., 2006; Duke *et al*., 1983; Eker *et al*., 2006; Ozturk *et al*., 2008; Cakmak *et al*., 2009). Glyphosate is a potential chelator for many divalent cations (Sprankle *et al*., 1975c; Subramaniam and Hoggard, 1988). Accordingly, limited acquisition, uptake, translocation and intra-cellular utilisation of complexes of mineral nutrients with glyphosate have been discussed as putative causes for nutrient limitation. While Eker *et al*. (2006) reported pronounced decline in root uptake and root-to-shoot translocation of radio-labelled Fe, Zn, and Mn in GS sunflower, Ozturk *et al*. (2008) demonstrated a glyphosate-induced inhibition of iron reductase activity at the plasma membrane of root cells, limiting the iron acquisition of sunflower plants.

In the present study, however, damage of wheat, maize and soybean was not related with micro- or macronutrient deficiencies (Tab. 7.4). Glyphosate caused a declined K concentration in shoots of wheat and maize, while concentrations of Fe and Ca were even significantly increased in wheat, which suggests that glyphosate toxicity on plants was the major limiting factor for nutrient acquisition rather than competitive interactions of glyphosate with certain cationic mineral nutrients.
In line with this, a significant decline of Zn- and/or Mn concentrations in shoots of wheat and maize were only detectable in case of severely impaired shoot and root biomass induced by high glyphosate application level.

7.6 Conclusions

Plant species differ in their susceptibility to glyphosate in hydroponic culture and presumably also in the rhizosphere. These differences in susceptibility most likely increase the potential for severe crop damage induced by glyphosate rhizosphere transfer from roots of treated weeds to subsequently sown crops or re-mobilisation of glyphosate previously fixed to the soil matrix in susceptible crop species like wheat and maize. However, as plants could also recover from glyphosate toxicity in the rhizosphere actual expression of crop damage is not only depending on the sensitivity of plants but most likely strongly influenced by biotic and abiotic growth conditions.

Therefore, for a better understanding of the interactions between glyphosate susceptibility of plants and the biotic and abiotic growth conditions and to evaluate the glyphosate sensitivity of additional crop species model experiments under hydroponic and soil conditions but also field trials are urgently needed.

8 Phytotoxicity of glyphosate soil residues re-mobilised by phosphate fertilisation

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Own contribution: set-up of experiments, plant cultivation, harvest and sample preparation, analysis of nutritional status of plants (support of students in 1 of 5 experiments) ,analysis of shikimate by HPLC, manuscript preparation

8.1 Abstract

It has been repeatedly demonstrated that phosphate (P) and the herbicide glyphosate compete for adsorption sites in soils. Surprisingly, the potential consequences of these interactions for plants e.g. re-solubilisation of phytotoxic glyphosate residues in soils by application of P fertilisers or by root-induced mechanisms for P mobilization have not been investigated so far.

In model experiments under greenhouse conditions, the potential for glyphosate re-mobilisation by P-fertiliser application was evaluated by bio-indication with soybean (*Glycine max* L.) cultivated on five contrasting soils with or without glyphosate application at 10-35 days before sowing. Different levels of P-fertilisation (0, 20, 40, 80, 240 mg P kg^{-1} soil) were supplied at the date of sowing. Visual symptoms of glyphosate toxicity, plant biomass, intracellular shikimate accumulation as physiological indicator for glyphosate toxicity and the plant nutritional status were determined.

On glyphosate-treated soils, P application induced significant plant damage. Expression of damage symptoms declined in the order Arenosol > Acrisol ≈ Ferralsol > Luvisol subsoil > Regosol. On the Arenosol, Ferralsol and Luvisol subsoil plant damage was associated with increased shikimate accumulation in the root tissue. On the Acrisol decline of germination and plant damage in absence of shikimate accumulation indicate toxicity of AMPA (aminomethylphosphonic acid) as the main metabolite of glyphosate in soils. On the Regosol, a growth-stimulating effect of glyphosate soil application (hormesis) was detected. The results suggest that re-mobilisation of glyphosate may represent an additional transfer pathway for glyphosate to non-target plants which is strongly influenced by soil characteristics such as P fixation potential, content of plant-available iron, pH, cation exchange capacity, sand content and soil organic matter.

Key words: Glyphosate, phosphorus, re-mobilisation, rhizosphere, root growth, micronutrients

Abbreviations:

a.e. acid equivalent

AMPA aminomethylphosphonic acid

cv. cultivar

DAS days after sowing

n.d. not determined

N.S not significant

SOM soil organic matter

WHC water holding capacity

8.2 Introduction

Due to low production costs and high efficiency, glyphosate (N-phosphonomethylglycine) is the most widely used herbicide on global scale (Baylis, 2000). Glyphosate is a non-selective herbicide inhibiting the biosynthesis of aromatic amino acids, thereby causing impairment of general metabolic processes, such as protein synthesis, photosynthesis (Geiger et al., 1986) and biosynthesis of aromatic compounds. The herbicidal effect is based on inhibition of the shikimate pathway enzyme 5-enolpyruvylshikimic acid-3-phosphate synthase (EPSPS) (Franz et al., 1997). Therefore, glyphosate application frequently induces intracellular accumulation of shikimate, which can be used as a sensitive physiological indicator for glyphosate toxicity (Henry et al., 2007).
While several recent studies highlighted the potential risk of ground and/or surface water pollution by leaching of glyphosate (Borggaard and Gimsing, 2008; Candela et al., 2010), risks of glyphosate toxicity to non-target plants in soils are generally considered as marginal, since glyphosate in the soil solution is prone to rapid microbial degradation (Giesy et al., 2000) or almost instantaneous inactivation by sorption to the soil matrix (Piccolo et al., 1992; Giesy et al., 2000).

Nevertheless, meanwhile an increasing number of reports suggest negative side effects on non-target plants, supposed to be related with the intensive use of glyphosate herbicides in agriculture. Increased susceptibility of crop plants to soil borne pathogens after glyphosate application has been reported frequently (Smiley et al., 1992; Fernandez et al., 2009; Johal and Huber, 2009; Kremer and Means, 2009). A number of recent studies suggest a risk of glyphosate toxicity to non-target plants due to transient stabilization of the herbicide in root residues of treated weeds, with a subsequent rhizosphere transfer to non-target plants via contact contamination (Neumann et al., 2006; Neumann et al., 2008; Laitinen et al. 2008; Tesfamariam et al., 2009, Doublet et al., 2009). 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 (Gordon , 2007; Zobiole et al., 2010a).

Glyphosate adsorption in soils seems to be mediated by ligand exchange via the phosphonate group of the molecule in a way similar to the adsorption of phosphate (Hance, 1976; Dion et al., 2001; Gimsing and Borggaard, 2002a, 2002b). Accordingly, it has been repeatedly demonstrated that phosphate and glyphosate compete for adsorption sites (Borggaard and Gimsing, 2008; Vereecken, 2005 and references cited therein). Depending on the soil conditions, phosphate concentration is the most important factor determining the amount of glyphosate adsorbed, and in some cases even complete desorption of glyphosate fixed to the soil matrix by phosphate applications has been reported (Borggaard and Gimsing, 2008 and references cited therein, Vereecken, 2005 and references cited therein). Thus, phosphate most likely plays an important role in determining the bioavailability of glyphosate in soils (Cornish, 1992; Laitinen et al., 2008).

While numerous studies have been conducted to investigate the adsorption characteristics and interactions between glyphosate and phosphate in soils, surprisingly, potential consequences

of these interactions for plants e.g. re-solubilisation of phytotoxic glyphosate residues in soils by application of P fertilisers or by root-induced mechanisms for P mobilization have not been investigated so far.

Therefore, this study was initiated to test the hypothesis that P fertilisation can increase the bioavailability of glyphosate residues in soils associated with the risk of phytotoxic effects to non-target plants. In a series of model experiments under greenhouse conditions, the potential for glyphosate re-mobilisation was evaluated by bio-indication with soybean (*Glycine max* L.) cultivated on five contrasting soils with or without glyphosate pre-incubation for 10-35 days. Thereafter, 3-5 levels of P-fertilisation were supplied at the sowing date of the indicator plants. After a culture period of 3 weeks, root and shoot biomass, intracellular accumulation of shikimate as physiological indicator for glyphosate toxicity and the plant nutritional status were determined to assess the potential risk of phytotoxic effects by glyphosate desorption mediated by application of P fertilisers.

8.3 Material and Methods

Soil properties

The characteristics of the used soils are summarized in table 8.1 (Tab. 8.1).

Tab. 8.1 Soil characteristics

Properties and nutritional status of soils investigated in the present study. Phosphorus (P) and potassium (K) were determined after Calcium acetate lactate (CAL) extraction, magnesium (Mg) after Calcium chloride extraction and iron (Fe), manganese (Mn), zinc (Zn), copper (Cu) and boron (B) after Calcium chloride-diethylenetriamine pentaacetic acid (CAT) extraction (VDLUFA, 2004).

soil properties					
	Arenosol	**Acrisol**	**Ferralsol**	**Luvisol**	**Regosol**
pH (CaCl2)	4.5	5.0	5.0	7.4	7.1
clay [%]	<5	42	14	12	40
silt [%]	<5	40	9	45	47
sand [%]	94	18	78	43	13
Corg [%]	0.2	n.d.	0.2	<0.3	3.8
CAL-extractable macronutrients [mg kg^{-1} soil]					
P	3	10	<2	5	550
K	70	19	74.0	52	440
$CaCl_2$-extractable macronutrients [mg kg^{-1} soil]					
Mg	100	180	180	250	250
CAT-extractable micronutrient concentrations [mg kg^{-1} soil]					
Fe	369	115	99	7.8	35
Mn	7.4	71	20	15	7.6
Zn	0.8	0.6	1.7	0.6	5.2
Cu	0.5	0.6	4.1	0.7	1.5
B	0.9	0.13	0.61	0.2	0.54

Soil fertilisation

In all experiments soils were sieved through 2 mm mesh size and fertilized with 100 mg N kg^{-1} soil as $Ca(NO_3)_2$, 50 mg K kg^{-1} soil as K_2SO_4 and 50 mg Mg kg^{-1} soil as $MgSO_4$. For this purpose, the chemical fertilizers was dispersed in an adequate amount of deionized water and sprayed on the soils under continuous mixing to ensure homogenous distribution. Subsequently the soils were sieved a second time (2 mm mesh size) and homogenized by

thorough mechanical mixing. Previous measurements revealed no changes in soil pH after identical fertilizer application to soils.

Glyphosate soil application

Recommended field application rates of commercial glyphosate formulations (applied as Roundup UltraMax®) range from 2-4 L ha^{-1} resulting in an application rate of glyphosate as acid equivalent (a.e.) in the range of 720-1440 g ha^{-1}.

Although a risk of glyphosate leaching from soils has been reported under certain conditions such as heavy rainfall shortly after application (Vereecken, 2005 and references cited therein), due to rapid soil adsorption, most of the applied glyphosate will remain in the uppermost soil layers (2-5 cm).This holds particularly true for minimal- or no tillage systems (Alletto et al., 2010)**.** Thus, a recommended field application rate of 2-4 L ha^{-1} glyphosate in Roundup UltraMax®-formulation (isopropylamine salt; 360 g a.e. L^{-1}) translates into glyphosate concentrations of 2.4 – 4.8 mg a.e. kg^{-1} top-soil. Based on this calculation, in the present study a glyphosate amount of 3.2 mg a.e. kg^{-1} soil was mixed with the different soils together with the N, K and Mg fertiliser solutions.

Subsequently approx. 800 g of glyphosate treated soil was filled into pots (volume: 950 cm^3). Soil moisture was adjusted to 70 % water-holding capacity (WHC) and the pots were incubated at room temperature for a time period of 10-35 days. In control treatments deionized water was applied instead of glyphosate-solution.

Phosphate fertilisation and plant culture

After an incubation time of 10-35 days, P-fertilisation of soils was performed at rates of 0, 20, 40, 80 or 240mg P kg $soil^{-1}$ as $Ca(H_2PO_4)_2$ by applying the fertiliser solution from top to the soil in the plastic pots. Subsequently, six soybean seeds (cv. BR-16 Conquista) were sown into each pot.

Plant culture was conducted under greenhouse conditions with an average day/night temperature of 22–24/14-16ºC. Water loss was determined gravimetrically and replaced by daily applications of de-ionized water.

Analysis of plant growth

Germination rate, deformation of primary- and trifoliate leaves, leaf surface area, shoot height and expression of chlorosis (SPAD-value) were recorded throughout the culture period.

At 10 days after sowing (DAS) (growth stage VC), a first set of 2 - 4 soybean seedlings was removed from the pots. Roots and shoots were separated, frozen in liquid nitrogen and stored at -20°C for shikimate analysis. In each pot, two seedlings were further cultivated until final harvest at 25 DAS (growth stage V2). At final harvest, fresh weights of all plant parts (roots and shoot) were recorded and dry weights of roots and shoots were determined after oven-drying at 60°C.

Analysis of mineral nutrients

One hundred milligrams of dried shoot material was ashed for 5 h in a muffle furnace at 500°C. After cooling, the samples were extracted twice with 1 mL of 3.4 M HNO_3 and

evaporated until dryness to precipitate SiO_2. The ash was dissolved in 1 mL of 4 M HCl, subsequently diluted ten times with hot deionized water, and boiled for 2 min to convert meta- and pyro-phosphates to orthophosphate. 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). Spectrophotometrical determination of orthophosphate was conducted after addition of molybdate-vanadate color reagent according to the method of Gericke and Kurmies (1952). Determination of Mg was conducted by atomic absorption spectrometry, while K and Ca were measured by flame emission photometry .

Shikimate analysis

Shikimate in root tissue was analysed with modifications of the methods described by Singh and Shaner (1998) and Neumann (2006). The frozen plant tissue was homogenised 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 20.000 x g) and the supernatant was used for HPLC analysis after appropriate dilution with the HPLC mobile phase. HPLC separation was performed by ion exclusion chromatography using an Aminex 87H column (Bio-Rad, Richmond, CA, USA) designed for organic acid analysis. A sample volume of 20 µL was injected into the isocratic flow (0.5 mL min^{-1}) of the eluent (2.5 mM H_2SO_4, 40°C) and organic acids were detected spectrophotometrically at 210nm. Identification and quantification of shikimate was conducted by comparing the retention times, absorption spectra and peak areas with a known standard.

Statistics

All experiments were conducted in a randomized block design with four replicates for each treatment. Analysis of variance and the Tukey test for detection of significant differences were performed using the SigmaStat-software (Jandel Scientific, Sausalito, CA, USA).

8.4 Results

Impact of P fertilisation and glyphosate soil application on plant growth and development

In soils without P fertilisation, glyphosate soil application was not associated with any damage of soybean plants (Tab. 8.3-8.7). However, on glyphosate-treated soils, plant damage increased with increasing levels of P fertilisation and was differentially expressed on the different soils (Tab. 8.2, Tab. 8.3). General symptoms of plant damage comprised delayed seedling development, deformations of primary leaves, stunted root growth (evaluation conducted 6 days after germination; growth stage VC), reduced plant height, delayed senescence of cotyledons (evaluation conducted 18 days after germination; growth stage V1/V2) and reduced shoot and root biomass (Tab. 8.2, Tab. 8.3, Fig. 8.1). In general, the expression intensity of damage symptoms declined in the order Arenosol (ARE) > Acrisol (ACR) ≈ Ferralsol (FER) > Luvisol subsoil (LUV) > Regosol (REG; no damage symptoms). On REG, glyphosate soil application even increased shoot biomass production by 27 % in the variants with P fertilisation rates of 0 and 80 mg P kg^{-1} soil (Tab. 8.2).

On ARE, plant damage was associated with anthocyanin formation in the hypocotyls (Fig. 8.1). On all investigated soils, no chlorosis symptoms were detectable on 1st trifoliate leaves of soybean seedlings during a culture period of 18 days (Tab. 8.2).

Germination of soybean generally ranged between 83 and 100 %. However on ACR, P fertilisation of glyphosate-treated soil with 80 and 240 mg P kg^{-1} soil significantly reduced the germination rate by approximately 40 % (Tab. 8.4).

Analysis of shikimate accumulation in the root tissue of soybean seedlings as physiological indicator for glyphosate toxicity revealed no effects on gyphosate treated soils without additional P fertilisation. However, increasing levels of P application significantly increased shikimate accumulation on ARE and to smaller extent also on FER. A similar trend was detectable on LUV. On ARE, a significant increase in shikimate accumulation was inducible already by the lowest level of P fertilisation of 20 mg P kg^{-1} soil. On ACR and REG, P fertilisation of glyphosate-treated soils did not increase shikimate accumulation in the root tissue of soybean seedlings (Tab. 8.5).

Tab. 8.2 Scoring of symptoms of plant damage in soybean grown on five contrasting soils depending on glyphosate soil incubation and P fertilisation

Glyphosate-induced symptoms of plant damage in soybean (cv. Conquista) grown on five contrasting soils with or without soil incubation with glyphosate 10-35 days before sowing. P-fertilisation of soils (40-240mg P kg soil-1) was performed at the sowing date. Scoring of seedling-, primary leaf- and root development was conducted 6 days after germination. Determination of shoot height, chlorosis scoring on first fully developed trifoliar leaves and evaluation of senescence of cotyledons was conducted 18 days after germination. Additionally, leaf surface area was estimated in the Arenosol 18 days after germination but not determined on the other soils (n.d.). Data represent means and standard deviations of 4 independent replicates. Significant differences ($p<0.05$) are indicated with different characters according to the Tukey test (N.S = not significant).

Scoring of symptoms of plant damage

	Arenosol, pH 4.8			Acrisol, pH 5.0		Ferralsol, pH 5.0		Luvisol, pH 7.6		Regosol, pH 7.1	
	40mg P kg^{-1}	80mg P kg^{-1}	240mg P kg^{-1}	80mg P kg^{-1}	240mg P kg^{-1}	80mg P kg^{-1}	240mg P kg^{-1}	80mg P kg^{-1}	240mg P kg^{-1}	80mg P kg^{-1}	240mg P kg^{-1}
treatment	**delayed seedling development [% plants pot^{-1}]**										
- Gly	19 ±14 ^{BC}	10 ±11 ^{C}	18 ±23 ^{BC}	5 ±10 ^{B}	4 ±8 ^{B}	14 ±10 ^{B}	13 ±16 ^{B}	17 ±14 ^{B}	19 ±14 ^{B}	13 ±9 $^{N.S}$	13 ±9 $^{N.S}$
+ Gly	46 ±16 ^{B}	100 ±0 ^{A}	100 ±0 ^{A}	100 ±0 ^{A}	88 ±14 ^{A}	14 ±10 ^{B}	95 ±10 ^{A}	41 ±7 ^{B}	46 ±20 ^{A}	9 ±11 $^{N.S}$	8 ±10 $^{N.S}$
treatment	**morphological disorder of primary leaves**										
- Gly	-	-	-	-	-	-	-	-	-	-	-
+ Gly	++	++	++	-	-	-	++	+	+	-	-
treatment	**impaired root growth**										
- Gly	-	-	-	-	-	-	-	-	-	-	-
+ Gly	+	++	++	+	++	+	++	+	+	-	-

Tab. 8.2 (continued)

Scoring of symptoms of plant damage (continued)

	Arenosol, pH 4.8			Acrisol, pH 5.0		Ferralsol, pH 5.0		Luvisol, pH 7.6		Regosol, pH 7.1	
	40mg P kg^{-1}	80mg P kg^{-1}	240mg P kg^{-1}	80mg P kg^{-1}	240mg P kg^{-1}	80mg P kg^{-1}	240mg P kg^{-1}	80mg P kg^{-1}	240mg P kg^{-1}	80mg P kg^{-1}	240mg P kg^{-1}
treatment					**accumulation of anthocyanin in stems**						
- Gly	-	-	-	-	-	-	-	-	-	-	-
+ Gly	++	++	++	-	-	-	-	-	-	-	-
treatment					**shoot height [cm]**						
- Gly	18 ±1 ^{A}	17 ±1 ^{A}	17 ±1 ^{A}	22 ±3 ^{A}	23 ±3 ^{A}	26 ±2 ^{A}	25 ±3 ^{A}	24 ±2 ^{A}	25 ±3 ^{A}	26 ±2 ^{A}	28 ±3 ^{A}
+ Gly	13 ±1 ^{BC}	10 ±2 ^{CD}	6 ±2 ^{D}	16 ±2 ^{B}	13 ±3 ^{B}	26 ±3 ^{A}	14 ±2 ^{B}	18 ±2 ^{B}	19 ±2 ^{B}	30 ±2 ^{B}	28 ±2 ^{B}
treatment					**senescence of cotyledons**						
- Gly	+	+	+	+	+	+	+	+	+	+	+
+ Gly	-	-	-	-	-	-	-	-	-	+	+
treatment					**chlorosis scoring [SPAD]**						
- Gly	28 ±1 ^{BC}	28 ±1 ^{BC}	25 ±2 ^{BC}	35 ±0 $^{N.S}$	35 ±1 $^{N.S}$	34 ±2 $^{N.S}$	33±2 $^{N.S}$	31 ±2 $^{N.S}$	31 ±2 $^{N.S}$	29 ±2 $^{N.S}$	28 ±2 $^{N.S}$
+ Gly	30 ±1 ^{B}	33 ±3 ^{AB}	34 ±2 ^{A}	34 ±1 $^{N.S}$	35 ±1 $^{N.S}$	34 ±1 $^{N.S}$	36 ±1 $^{N.S}$	30 ±1 $^{N.S}$	30 ±1 $^{N.S}$	28 ±2 $^{N.S}$	28 ±2 $^{N.S}$
treatment					**Leaf surface area [cm^2]**						
- Gly	50 ±2 ^{A}	46 ±3 ^{A}	36 ±3 ^{B}	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
+ Gly	27 ±3 ^{C}	16 ±3 ^{D}	4 ±1 ^{E}	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.

Tab. 8.3 Plant growth of soybean grown on five contrasting soils depending on glyphosate soil incubation and P fertilisation

Shoot and root dry weight and the root/shoot ratio of soybean (cv. Conquista) cultivated for 25 days on five contrasting soils with or without pre-incubation of soils with glyphosate at different P-fertilisation levels. P-fertilisation of soils was conducted at time of seeding. Data represent means of 4 independent replicates. Standard deviations (e.g. 0.0) are not shown. Significant differences ($p<0.05$) are indicated with different characters according to the Tukey test.

	Plant growth					
	shoot dry weight [g]		root dry weight [g]		root/shoot ratio	
soil type, incubation time [days]	- Gly	+ Gly	- Gly	+ Gly	- Gly	+ Gly
Arenosol, 10 days						
0mg P kg^{-1}	**0.42** ABC	**0.44** AB	**0.19** ABC	**0.16** BCD	**0.47** AB	**0.38** ABC
20mg P kg^{-1}	**0.46** AB	**0.34** BC	**0.25** AB	**0.15** CD	**0.55** A	**0.37** ABC
40mg P kg^{-1}	**0.51** A	**0.40** ABC	**0.27** A	**0.14** CD	**0.47** A	**0.32** ABC
80mg P kg^{-1}	**0.50** A	**0.31** C	**0.20** ABC	**0.09** DE	**0.40** ABC	**0.30** BC
240mg P kg^{-1}	**0.42** AB	**0.18** D	**0.19** ABC	**0.04** E	**0.46** AB	**0.23** C
Arenosol, 35 days						
0mg P kg^{-1}	**0.34** CD	**0.31** D	**0.17** AB	**0.12** C	**0.50** A	**0.36** BC
40mg P kg^{-1}	**0.39** AB	**0.25** E	**0.19** AB	**0.07** D	**0.49** A	**0.27** CD
80mg P kg^{-1}	**0.43** A	**0.18** F	**0.19** A	**0.03** E	**0.45** AB	**0.17** D
240mg P kg^{-1}	**0.36** BC	**0.13** G	**0.16** B	**0.02** E	**0.42** AB	**0.20** D
Acrisol, 10 days						
0mg P kg^{-1}	**0.68** A	**0.66** A	**0.22** A	**0.21** A	**0.32** A	**0.32** AB
80mg P kg^{-1}	**0.71** A	**0.54** A	**0.22** A	**0.15** BC	**0.31** AB	**0.28** AB
240mg P kg^{-1}	**0.70** A	**0.29** B	**0.18** AB	**0.09** C	**0.24** B	**0.27** AB
Ferralsol, 10 days						
0mg P kg^{-1}	**0.42** B	**0.43** B	**0.18** BC	**0.17** BC	**0.44** A	**0.38** AB
80mg P kg^{-1}	**1.01** A	**0.95** A	**0.29** A	**0.22** BC	**0.28** CD	**0.24** D
240mg P kg^{-1}	**0.96** A	**0.40** B	**0.25** AB	**0.12** D	**0.26** D	**0.35** BC
Luvisol, 10 days						
0mg P kg^{-1}	**0.54** B	**0.53** B	**0.25** B	**0.20** BC	**0.49** A	**0.41** AB
80mg P kg^{-1}	**0.83** A	**0.58** B	**0.30** A	**0.18** C	**0.39** B	**0.34** C
240mg P kg^{-1}	**0.85** A	**0.58** B	**0.30** A	**0.18** C	**0.38** B	**0.34** C
Regosol, 10 days						
0mg P kg^{-1}	**0.76** C	**0.97** B	**0.25** N.S.	**0.25** N.S	**0.34** A	**0.28** B
80mg P kg^{-1}	**0.84** C	**1.07** A	**0.25** N.S.	**0.27** N.S	**0.31** AB	**0.27** B
240mg P kg^{-1}	**0.95** B	**0.95** B	**0.26** N.S.	**0.24** N.S	**0.29** AB	**0.27** B

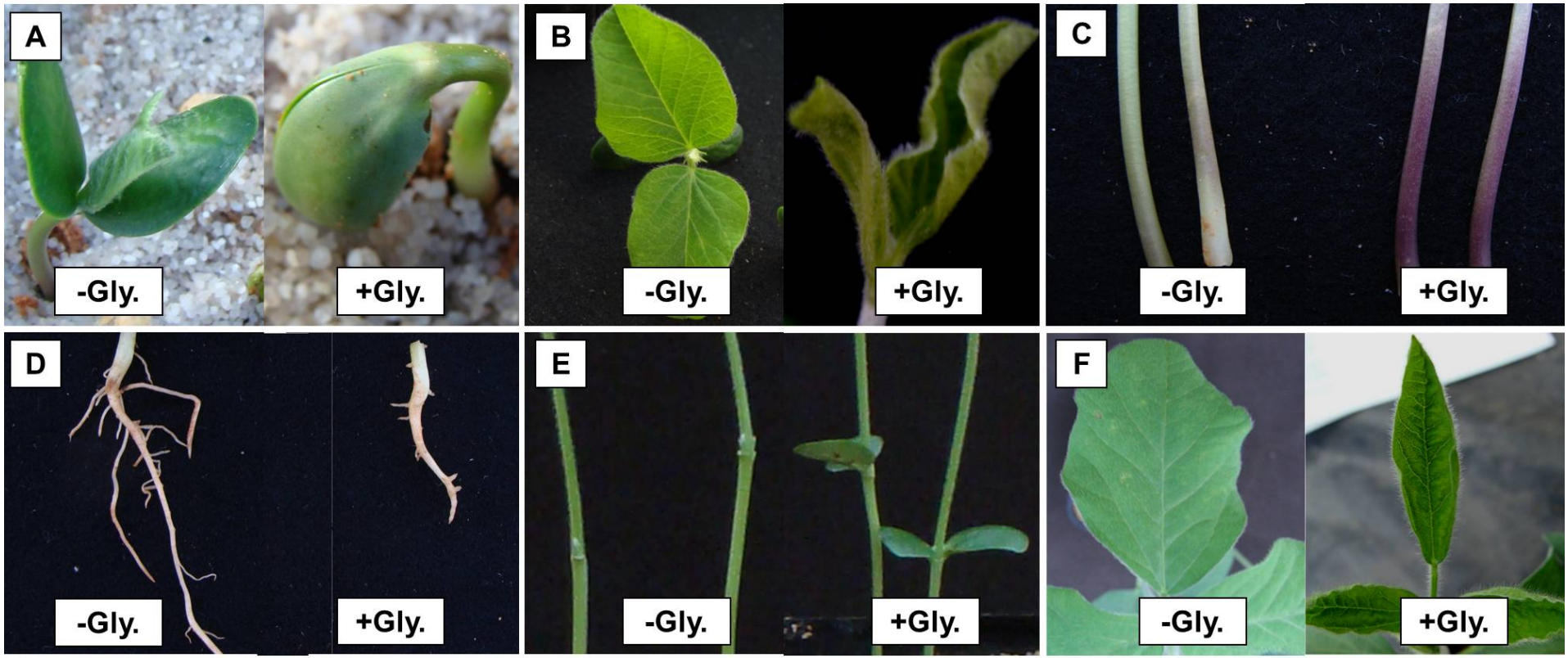

Fig. 8.1: Visual symptoms of soybean depending on glyphosate soil incubation, induced by P fertilisation

Glyphosate-induced delayed seedling development (A), Deformations of primary leaves (B), increased formation of anthocyanin in the hypocotyls (C), impaired root development (D), delayed senescence of cotyledons (E) and deformation of trifoliate leaves (F) in comparison with undamaged control plants. Soybean plants (cv. Conquista) were cultivated on an acidic sandy Arenosol with or without soil incubation with glyphosate 35 days before sowing with a P-fertilisation of 80mg P kg soil^{-1}.

Tab. 8.4 Germination of soybean grown on five contrasting soils depending on glyphosate soil incubation and P fertilisation

Germination of soybean (cv. Conquista) grown on five contrasting soils with or without soil incubation with glyphosate 10-35 days before seeding. P-fertilisation of soils (20-240mg P kg soil-1) was performed at date of seeding while evaluation of germination conducted 4 days after germination of first seedlings. Data represent means and standard deviations of 4 independent replicates. Significant differences ($p<0.05$) are indicated with different characters according to the Tukey test (N.S = not significant).

	Germination [% of seeds pot^{-1}]									
	Arenosol [10-35 days]		**Acrisol [10 days]**		**Ferralsol [10 days]**		**Luvisol [10 days]**		**Regosol [10 days]**	
P fertilisation	- Gly	+ Gly	- Gly	+ Gly	- Gly	+ Gly	- Gly	+ Gly	- Gly	+ Gly
[mg P kg soil^{-1}]										
0	88±16 $^{N.S}$	92±10 $^{N.S}$	92±10 ^{A}	88±8 ^{A}	92±10 $^{N.S}$	92±10 $^{N.S}$	92±17 $^{N.S}$	88±8 $^{N.S}$	96±8 $^{N.S}$	100±0 $^{N.S}$
20	92±10 $^{N.S}$	96±8 $^{N.S}$	-	-	-	-	-	-	-	-
40	92±10 $^{N.S}$	96±8 $^{N.S}$	-	-	-	-	-	-	-	-
80	96±8 $^{N.S}$	92±10 $^{N.S}$	92±10 ^{A}	50±14 ^{B}	83±14 $^{N.S}$	88±16 $^{N.S}$	100±0 $^{N.S}$	92±10 $^{N.S}$	96±8 $^{N.S}$	83±14 $^{N.S}$
240	92±10 $^{N.S}$	92±10 $^{N.S}$	88±8 ^{A}	42±10 ^{B}	88±8 $^{N.S}$	92±10 $^{N.S}$	88±16 $^{N.S}$	88±16 $^{N.S}$	92±10 $^{N.S}$	88±16 $^{N.S}$

Tab. 8.5 Accumulation of shikimate in root tissue of soybean grown on five contrasting soils depending on glyphosate soil incubation and P fertilisation

Accumulation of shikimate as indicator for glyphosate toxicity in root tissue of soybean (cv. Conquista) cultivated for 7 days on five contrasting soils with or without pre-incubation of soils with glyphosate at different P-fertilisation levels. P-fertilisation of soils was conducted at time of seeding. Data represent means and standard deviations of 4 independent replicates. Significant differences ($p<0.05$) are indicated with different characters according to the Tukey test (N.S = not significant).

Accumulation of shikimate in root tissue

	shikimate concentration in roots [µg g FW^{-1}]	
soil type, incubation time [days]	**- Gly**	**+ Gly**
Arenosol, 10 days		
0mg P kg^{-1}	**7.4** ±2 ^{D}	**4.6** ±3 ^{D}
20mg P kg^{-1}	**7.6** ±2 ^{D}	**17.5** ±4 ^{C}
40mg P kg^{-1}	**8.1** ±4 ^{D}	**538.0** ±145 ^{B}
80mg P kg^{-1}	**8.7** ±2 ^{D}	**579.3** ±165 ^{B}
240mg P kg^{-1}	**12.2** ±8 ^{CD}	**1994.6** ±497 ^{A}
Arenosol, 35 days		
0mg P kg^{-1}	**6.3** ±2 ^{D}	**91.4** ±10 ^{C}
40mg P kg^{-1}	**8.0** ±4 ^{D}	**1043.0** ±278 ^{B}
80mg P kg^{-1}	**7.7** ±3 ^{D}	**1299.1** ±144 ^{B}
240mg P kg^{-1}	**19.3** ±13 ^{D}	**3813.0** ±713 ^{A}
Acrisol, 10 days		
0mg P kg^{-1}	**6.2** ±1 $^{N.S}$	**7.4** ±2 $^{N.S}$
80mg P kg^{-1}	**7.7** ±2 $^{N.S}$	**9.5** ±9 $^{N.S}$
240mg P kg^{-1}	**8.9** ±2 $^{N.S}$	**8.7** ±1 $^{N.S}$
Ferralsol, 10 days		
0mg P kg^{-1}	**10.5** ±3 ^{B}	**11.6** ±6 ^{B}
80mg P kg^{-1}	**18.5** ±6 ^{B}	**10.4** ±3 ^{B}
240mg P kg^{-1}	**11.9** ±7 ^{B}	**152.5** ±112 ^{A}
Luvisol, 10 days		
0mg P kg^{-1}	**10.6** ±3 $^{N.S}$	**11.3** ±3 $^{N.S}$
80mg P kg^{-1}	**12.5** ±5 $^{N.S}$	**57.2** ±45 $^{N.S}$
240mg P kg^{-1}	**15.1** ±6 $^{N.S}$	**59.8** ±60 $^{N.S}$
Regosol, 10 days		
0mg P kg^{-1}	**10.2** ±6 $^{N.S}$	**9.6** ±3 $^{N.S}$
80mg P kg^{-1}	**11.4** ±4 $^{N.S}$	**8.8** ±2 $^{N.S}$
240mg P kg^{-1}	**10.1** ±3 $^{N.S}$	**10.3** ±3 $^{N.S}$

Plant nutritional status

Macronutrients

On all investigated soils, biomass production of soybean plants responded to P fertilisation and with exception of REG, optimum responses were achieved at P application levels of 80 mg P kg^{-1} soil. On REG, shoot biomass production continuously increased up to a P fertilisation level of 240 mg kg^{-1} soil. On all soils without P fertilisation, shoot P concentrations were in the critical range (ARE) or below the P deficiency threshold. This was associated with an increase of the root/shoot biomass ratio as a typical response to P limitation. Total shoot P content was significantly affected by glyphosate applications on ARE, ACR and FER particularly at higher levels of P fertilisation (Tab. 8.6). Calcium, Mg and K supply to the plants was sufficient for all treatments with the exception of LUV which was characterised by a critical K status (Tab. 8.7).

Tab. 8.6 Phosphorus status of soybean plants grown on five contrasting soils depending on glyphosate soil incubation and P fertilisation

Phosphorus concentrations and contents in shoots of soybean (cv. Conquista) cultivated for 25 days on five contrasting soils with or without pre-incubation of soils with glyphosate at different P-fertilisation levels. P-fertilisation of soils was conducted at time of seeding. Data represent means of 4 independent replicates. Standard deviations (e.g. 0.0) are not shown. Significant differences ($p<0.05$) are indicated with different characters according to the Tukey test (N.S = not significant).

	Phosphorus status of plants			
	P concentration [mg g DM^{-1}]		P contents [mg pot^{-1}]	
Soil type/ P fertilisation	- Gly	+ Gly	- Gly	+ Gly
Arenosol (pH 4.8)				
0mg P kg^{-1}	2.3 ^{E}	2.4 ^{E}	0.8 ^{D}	0.7 ^{D}
40mg P kg^{-1}	3.2 ^{D}	4.9 ^{C}	1.3 ^{C}	1.2 ^{C}
80mg P kg^{-1}	4.9 ^{C}	5.5 ^{C}	2.1 ^{B}	1.0 ^{CD}
240mg P kg^{-1}	7.6 ^{B}	9.6 ^{A}	2.8 ^{A}	1.2 ^{C}
Acrisol (pH 5.0)				
0mg P kg^{-1}	1.5 ^{C}	1.7 ^{C}	1.0 ^{B}	1.1 ^{B}
80mg P kg^{-1}	2.0 ^{BC}	2.3 ^{B}	1.4 ^{B}	1.3 ^{B}
240mg P kg^{-1}	4.3 ^{A}	3.9 ^{A}	2.9 ^{A}	1.1 ^{B}
Ferralsol (pH 5.0)				
0mg P kg^{-1}	0.7 ^{C}	0.7 ^{C}	0.3 ^{C}	0.3 ^{C}
80mg P kg^{-1}	2.3 ^{B}	2.3 ^{B}	2.6 ^{B}	2.3 ^{B}
240mg P kg^{-1}	5.9 ^{A}	6.6 ^{A}	6.4 ^{A}	2.9 ^{B}
Luvisol (pH 7.4)				
0mg P kg^{-1}	0.9 ^{D}	0.6 ^{D}	0.5 ^{B}	0.3 ^{B}
80mg P kg^{-1}	1.8 ^{C}	1.8 ^{C}	1.5 ^{B}	1.1 ^{B}
240mg P kg^{-1}	4.8 ^{B}	6.5 ^{A}	4.0 ^{A}	3.9 ^{A}
Regosol (pH 7.1)				
0mg P kg^{-1}	1.4 ^{E}	1.6 ^{DE}	1.0 ^{C}	1.6 ^{C}
80mg P kg^{-1}	1.8 ^{CD}	2.0 ^{C}	1.5 ^{BC}	2.2 ^{B}
240mg P kg^{-1}	3.2 ^{B}	3.6 ^{A}	3.0 ^{A}	3.4 ^{A}

Micronutrients

The Mn nutritional status was generally above the deficiency threshold in all treatments. Particularly low Mn concentrations of around 20 mg kg^{-1} DM were observed on REG, while high levels between 200-500 mg kg^{-1} DM were detected on FER. Manganese shoot concentrations declined in response to glyphosate applications on ARE and ACR. The Zn status was sufficient for ARE and FER. Critical levels were detected for ACR and REG and Zn deficiency on LUV. However, there was no clear relationship between glyphosate application and Zn status of the plants. Iron was sufficient for all treatments but critical levels were detected on the glyphosate-treated LUV (Tab. 8.7).

Tab. 8.7 Nutrient concentrations in shoots of soybean grown on five contrasting soils depending on glyphosate soil incubation and P fertilisation

Nutrient concentrations in shoots of soybean (cv. Conquista) cultivated for 25 days on five contrasting soils with or without pre-incubation of soils with glyphosate at different P-fertilisation levels. P-fertilisation of soils was conducted at time of seeding. Data represent means and standard deviations of 4 independent replicates. Significant differences ($p<0.05$) are indicated with different characters according to the Tukey test (N.S = not significant).

	Nutrient concentrations in shoots of plants											
	Mn-concentration [µg g DM^{-1}]		Zn concentration [µg g DM^{-1}]		Fe concentration [µg g DM^{-1}]		Ca concentrations [mg g DM^{-1}]		Mg concentrations [mg g DM^{-1}]		K concentrations [mg g DM^{-1}]	
Soil type	**- Gly**	**+ Gly**	**- Gly**	**+ Gly**	**- Gly**	**+ Gly**	**- Gly**	**+ Gly**	**- Gly**	**+ Gly**	**- Gly**	**+ Gly**
P fertilisation												
Arenosol (pH 4.8)												
0mg P kg $soil^{-1}$	108±10 ^{BC}	149±9 ^{A}	74±6 ^{A}	72±2 ^{AB}	144±48 ^{A}	125±46 ^{AB}	12±1 ^{B}	11±0 ^{BC}	6.1±0 ^{AB}	6.1±0 ^{AB}	34±1 ^{BC}	32±1 ^{C}
40mg P kg $soil^{-1}$	125±15 ^{AB}	105±18 ^{BC}	73±5 ^{A}	63±5 ^{BC}	104±24 ^{AB}	95±32 ^{AB}	15±1 ^{A}	11±1 ^{BC}	6.3±0 ^{A}	5.2±1 ^{BC}	36±1 ^{AB}	36±2 ^{AB}
80mg P kg $soil^{-1}$	125±5 ^{AB}	84±21 ^{C}	70±4 ^{AB}	57±9 ^{C}	102±28 ^{AB}	74±6 ^{B}	15±0 ^{A}	9±1 ^{CD}	5.5±0 ^{ABC}	4.7±1 ^{C}	38±2 ^{A}	37±1 ^{AB}
240mg P kg $soil^{-1}$	95±7 ^{C}	39±5 ^{D}	73±5 ^{AB}	58±10 ^{BC}	74±6 ^{B}	65±6 ^{B}	16±0 ^{A}	9±1 ^{D}	5.0±1 ^{C}	3.4±0 ^{D}	38±1 ^{A}	38±3 ^{A}
Acrisol (pH 5.0)												
0 mg P kg $soil^{-1}$	62±2 ^{AB}	67±6 ^{AB}	25±2 ^{ABC}	32±6 ^{A}	96±38 $^{N.S}$	113±9 $^{N.S}$	17±1 ^{B}	18±1 ^{B}	6.0±0 ^{AB}	5.9±0 ^{AB}	25±2 ^{C}	27±3 ^{BC}
80mg P kg $soil^{-1}$	66±2 ^{AB}	70±9 ^{AB}	24±3 ^{ABC}	20±4 ^{BC}	107±17 $^{N.S}$	106±5 $^{N.S}$	19±1 ^{B}	18±1 ^{B}	6.0±0 ^{AB}	6.2±0 ^{AB}	28±1 ^{BC}	30±1 ^{AB}
240mg P kg $soil^{-1}$	74±3 ^{A}	59±7 ^{B}	27±4 ^{AB}	18±1 ^{C}	125±13 $^{N.S}$	118±10 $^{N.S}$	21±1 ^{A}	18±1 ^{B}	6.4±0 ^{A}	5.7±0 ^{B}	34±3 ^{A}	34±3 ^{A}

Tab. 8.7 (continued)

	Nutrient concentrations in shoots of soybean (continued)											
	Mn-concentration [µg g DM^{-1}]		Zn concentration [µg g DM^{-1}]		Fe concentration [µg g DM^{-1}]		Ca concentrations [mg g DM^{-1}]		Mg concentrations [mg g DM^{-1}]		K concentrations [mg g DM^{-1}]	
Soil type	**- Gly**	**+ Gly**	**- Gly**	**+ Gly**	**- Gly**	**+ Gly**	**- Gly**	**+ Gly**	**- Gly**	**+ Gly**	**- Gly**	**+ Gly**
P fertilisation												
Ferralsol (pH 5.0)												
0mg P kg soil^{-1}	193±31 ^{B}	283±79 ^{B}	64±5 ^{AB}	68±8 ^{A}	148±48 ^{AB}	167±37 ^{A}	13±1 ^{AB}	14±1 ^{AB}	5.1±0 $^{N.S}$	5.0±0 $^{N.S}$	19±1 ^{C}	22±4 ^{C}
80mg P kg soil^{-1}	178±58 ^{B}	225±80 ^{B}	54±5 ^{BC}	57±8 ^{ABC}	92±16 ^{B}	99±17 ^{AB}	12±1 ^{B}	13±1 ^{AB}	4.9±0 $^{N.S}$	5.3±0 $^{N.S}$	30±2 ^{B}	31±3 ^{B}
240mg P kg soil^{-1}	223±46 ^{B}	531±49 ^{A}	52±3 ^{C}	68±3 ^{A}	82±12 ^{B}	109±25 ^{AB}	12±1 ^{B}	15±1 ^{A}	4.8±0 $^{N.S}$	4.8±0 $^{N.S}$	31±1 ^{B}	39±1 ^{A}
Luvisol (pH 7.4)												
0mg P kg soil^{-1}	107±10 ^{B}	110±22 ^{B}	15±0 ^{A}	13±3 ^{AB}	60±6 ^{BC}	57±12 ^{ABC}	17±1 ^{B}	17±2 ^{B}	6.4±0 ^{C}	6.4±1 ^{C}	14±1 ^{A}	10±2 ^{B}
80mg P kg soil^{-1}	100±9 ^{B}	116±23 ^{B}	11±1 ^{BC}	9±2 ^{BC}	66±3 ^{A}	53±7 ^{BC}	18±1 ^{B}	20±4 ^{AB}	7.6±0 ^{BC}	8.2±1 ^{B}	13±1 ^{AB}	9±2 ^{B}
240mg P kg soil^{-1}	105±23 ^{B}	169±24 ^{A}	11±1 ^{BC}	9±1 ^{C}	64±4 ^{AB}	53±4 ^{C}	18±1 ^{B}	23±1 ^{A}	8.2±0 ^{B}	10.0±0 ^{A}	13±1 ^{AB}	10±3 ^{AB}
Regosol (pH 7.1)												
0mg P kg soil^{-1}	21±1 ^{C}	25±1 ^{A}	18±1 ^{AB}	20±1 ^{A}	120±42.2 ^{A}	76±5 ^{AB}	18±0 ^{D}	21±0 ^{BC}	5.0±0 ^{D}	5.3±0 ^{CD}	22±1 ^{B}	23±0 ^{AB}
80mg P kg soil^{-1}	22±2 ^{BC}	23±1 ^{AB}	19±0 ^{AB}	19±1 ^{AB}	85±6.7 ^{AB}	71±6 ^{B}	19±0 ^{CD}	22±1 ^{AB}	5.6±0 ^{BC}	5.9±0 ^{AB}	24±1 ^{AB}	26±2 ^{A}
240mg P kg soil^{-1}	23±2^{ABC}	25±0 ^{A}	16±2 ^{B}	17±1 ^{B}	82±3.8 ^{AB}	81±6 ^{AB}	19±0 ^{CD}	23±1 ^{A}	5.8±0 ^{AB}	6.0±0 ^{A}	25±1 ^{A}	26±1 ^{A}

8.5 Discussion

Phytotoxic effects by re-mobilisation of glyphosate residues in soils

No phytotoxic effects were detectable in response to glyphosate soil incorporation on all investigated soils without P fertilisation (Tab. 8.2, Tab. 8.3, Tab. 8.4). These results support the concept of rapid inactivation and detoxification of glyphosate in soils by adsorption to phosphate binding sites, such as Fe/Al-oxides and hydroxides, precipitation as calcium salts, and rapid microbial degradation of free glyphosate in the soil solution (Sprankle et al., 1975a,b; Giesy et al., 2000).
In contrast, with the exception of REG, application of P-fertiliser significantly impaired seedling growth and development on soils pre-incubated with glyphosate (Tab. 8.2, Tab. 8.3, Fig. 8.1). With the exception of ACR, expression of plant damage was associated with increased accumulation of shikimate in the root tissue as a physiological indicator of glyphosate phytotoxicity (Tab. 8.2, Tab. 8.3, Tab. 8.5). Damage and stress symptoms comprised stunted root and shoot growth, leaf deformation, anthocyanin formation (also reported by Jursík et al., 2010) and delayed senescence of cotyledons. Interstingly, no chlorosis was detectable in young leaves, which is usually one of the first toxicity symptoms after foliar glyphosate spray applications. However, Allister et al. (2005) demonstrated different glyphosate distribution patterns within plants, depending on leaf or root exposure to glyphosate. In case of foliar application, 80% of applied glyphosate was translocated to the shoot meristems and young leaves. By contrast, up to 75% of glyphosate remained mainly in the young root tissues when the herbicide was supplied to the roots. This may also imply a different expression of plant damage symptoms. While foliar glyphosate application leads to direct expression of toxicity symptoms such as chlorosis and necrosis of young leaves within several days, more indirect symptoms can be expected after root exposure to glyphosate, mainly as a consequence of an impairment of root function, e.g. limited acquisition and translocation of water (Zobiole et al. 2010b) and nutrients (Neumann et al., 2006) or of hormonal signals such as cytokinins (Sergiev et al. 2006).

Similar to the symptoms of plant damage, intracellular shikimate accumulation was also differentially expressed on different soils and declined in the order ARE > FER > LUV > REG (no damage symptoms, no shikimate accumulation). These findings suggest a differential capacity for glyphosate immobilisation / detoxification of the investigated soils and a glyphosate re-mobilisation potential of P fertilisers (Tab. 8.1, Tab. 8.2, Tab. 8.4), probably based on competitive soil adsorption of phosphate and glyphosate (Hance, 1976; Dion et al., 2001; Gimsing and Borggaard, 2002a, 2002b).

On ACR, induction of plant damage by P fertilisation of the glyphosate-treated soil was not associated with increased intracellular shikimate accumulation. Moreover, germination rate was affected only on ACR but not on the other investigated soils (Tab. 8.4). The major metabolite of glyphosate accumulating in soils is AMPA (aminomethylphosphonic acid). Compared with glyphosate, AMPA exhibits similar adsorption characteristics in soils, a lower phytotoxicity and no induction potential for shikimate accumulation in plant tissues (Giesy et al., 2000; Reddy et al., 2004; Laitinen et al., 2008). Recently, a Monsanto patent on

production of AMPA-resistant crops reported an inhibitory effect of AMPA on wheat in an embryo germination test (Barry, 2009). Accordingly, our own investigations revealed an inhibitory effect of AMPA but not of glyphosate on germination of winter wheat seeds (Bott et al., 2010). Therefore, it seems to be likely that on ACR the conversion of glyphosate to AMPA proceeded more rapidly than on the other investigated soils. Thus, AMPA was probably the main phytotoxic compound re-mobilised by P fertilisation, associated with typical symptoms of AMPA toxicity such as suppression of germination without induction of shikimate accumulation.

Plant nutritional status

An increasing number of publications have reported a glyphosate-induced impaired plant-nutritional status particularly of cationic mineral nutrients such as Mn, Zn, Fe and Ca (Sprankle et al., 1975c; Duke et al., 1983; Subramaniam and Hoggard, 1988; Neumann et al., 2006; Gordon et al., 2007; Eker et al., 2006; Ozturk et al., 2008; Bott et al., 2008; Tesfamariam et al., 2009; Cakmak et al., 2009; Zobiole et al., 2010a). Since glyphosate is a potent metal chelator for many of these divalent cations, competetive interactions limiting acquisition, uptake, translocation and intra-cellular utilisation of cationic nutrients have been discussed as putative causes for nutrient limitation (Sprankle et al., 1975c;; Subramaniam and Hoggard, 1988; Eker et al., 2006; Ozturk et al., 2008; Cakmak et al., 2009). Additionally, Ozturk et al. (2008) demonstrated a glyphosate-induced inhibition of iron reductase activity at the plasma membrane of root cells, limiting the iron acquisition of sunflower plants. In the present study, however, plant-damage on glyphosate-treated soils was not related to a certain cationic nutrient deficiency (Tab. 8.7). On different soils, different nutrients were affected by the glyphosate treatments. This finding suggests that in case of root exposure of plants to glyphosate in our study, the impairment of root growth by glyphosate toxicity rather than competitive interactions of glyphosate with certain cationic nutrients was the major limiting factor for nutrient acquisition. Since root growth determines the spatial acquisition particularly of sparingly soluble nutrients, the differential solubility of nutrients in different soils can explain the expression of variable nutrient deficiencies by glyphosate treatments depending on soil type. However, depending on the organs primarily exposed to glyphosate (e.g. leaf application versus root uptake) also differential interactions with mineral nutrients may be expected.

Factors determining glyphosate resolubilisation

Similar to phosphate adsorption in soils, the main sorption sites of glyphosate and AMPA, are found on surfaces of iron and aluminum oxides, poorly ordered aluminum silicates and edges of layer silicates, while sorption of glyphosate by permanent charge layer silicates seems to be limited (Borggaard and Gimsing, 2008; Vereecken, 2005 and references cited therein). Competitive desorption of glyphosate by phosphate on iron and aluminum oxides (e.g. goethite, α-FeOOH) has been shown in numerous studies (Barja and Dos Santos Afonso, 2005; Gimsing and Borggaard, 2002a,b; Gimsing et al., 2004; Vereecken, 2005 and references cited therein).

The induction of plant damage by P fertilisation on glyphosate-treated soils indicates a P-induced competitive desorption of glyphosate from phosphate binding sites also in the present study. Differential expression of glyphosate plant damage on the investigated soils may be related to differences in the fixation potential for P and glyphosate. Phosphate fixation in soils can also be a limitation for the plant availability of fertiliser P. Therefore, the P fixation potential of the investigated soils may be reflected by the responsiveness of the plant P-nutritional status to P fertilisation. Accordingly, glyphosate-induced plant growth inhibition was positively correlated with P availability for soybean on the five different soils ($r^2 = 0.58$), calculated according to the responses of the P-nutritional status to P fertilisation. Excluding the ACR where plant damage was potentially caused by re-mobilisation of AMPA, the correlation of glyphosate-induced plant damage and the P availability for soybean rises to $r^2=0.97$ (Fig. 8.2a).

A similar correlation was observed for glyphosate-induced inhibition of plant growth and the CAT-extractable Fe fraction of the investigated soils ($r^2 = 0.57$), representing the chelator-exchangeable Fe pool (VDLUFA, 2004) that is considered to be plant available (Fig. 8.2b). These findings suggest that a significant proportion of glyphosate (and AMPA on the ACR) may be adsorbed to more labile/ plant-available Fe fractions of the investigated soils. Low pH has been reported to increase the amount of glyphosate adsorbed to pure iron/ aluminium oxides and also in soils (Sheals et al., 2002; Barja and Dos Santos Afonso, 2005). Lowering the pH increases the pool of labile ferric iron available for adsoprtion of free glyphosate. Based on the assumption that phosphate and glyphosate are bound by the same adsorption sites, competitive desorption of high amounts of glyphosate from labile ferric iron by application of P fertilisers would explain the strong expression of plant damage particularly on the three acidic soils (ARE, ACR, FER) (Tab. 8.3, Tab. 8.4, Tab. 8.5).

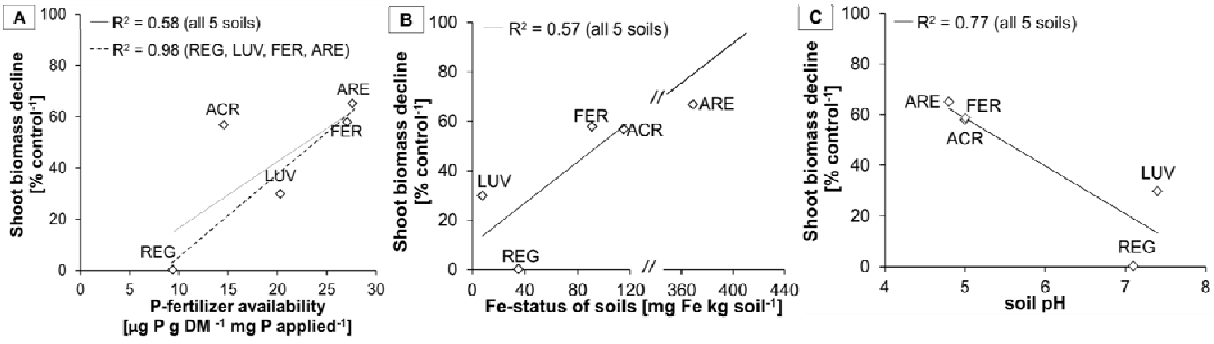

Fig. 8.2: Correlation between glyphosate-induced damage and soil characteristics

Glyphosate-induced shoot biomass decline of soybean (cv. Conquista) cultivated on an Arenosol (ARE), Acrisol (ACR), Ferralsol (FER), Luvisol (LUV) and Regosol (REG) pre-incubated with glyphosate for 10-35 days before sowing and application of 240mg P kg soil^{-1} in correlation with P-fertiliser availability (A), Fe-availability of the soil (B) and the soil pH (C). The P-fertiliser availability as indicator for the strength of soil P-fixation was calculated as: (Shoot P-conc$_{(240mg\ P)}$ – Shoot P-conc$_{(0mg\ P)}$ / 240mg P-fertiliser kg soil^{-1}. Data points represent means of 4 independent replicates. The coefficient of determination (R^2) is indicated.

A weaker correlation (r^2=0.46) exists between glyphosate-induced plant damage and sand content of all investigated soils (Fig. 8.3a). However, excluding the ACR where plant damage was potentially caused by re-mobilisation of AMPA the correlation glyphosate-induced plant damage and sand content rise to a significant positive correlation (r^2=0.98). Similarly, a significant positive correlation (r^2=0.99) between glyphosate-induced plant damage and the cation exchange capacity was detectable for all soil (Fig. 8.3b). This confirms earlier reports on potential risks for non-target plant damage on light sandy soils (Cornish, 1992), probably caused by weak adsorption of glyphosate due to a limited number of adsorption sites, mainly located on silt-clay minerals and/or low microbial degradation of glyphosate (Vereecken, 2005; Borggaard and Gimsing, 2008 and references cited therein).

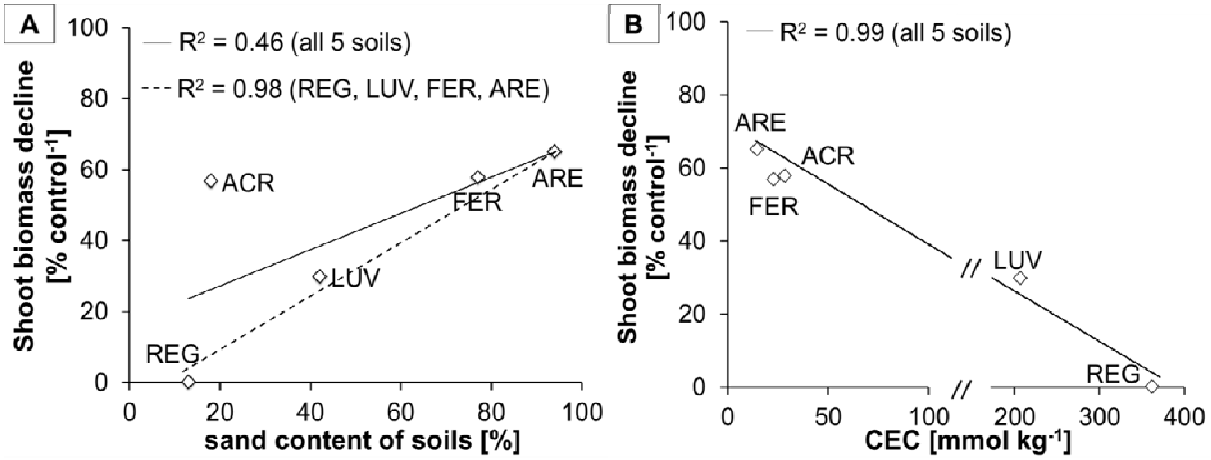

Fig. 8.3: Correlation between glyphosate-induced damage and soil characteristics

Glyphosate-induced shoot biomass decline of soybean (cv. Conquista) cultivated on an Arenosol (ARE), Acrisol (ACR), Ferralsol (FER), Luvisol (LUV) and Regosol (REG) pre-incubated with glyphosate for 10-35 days before sowing and application of 240mg P kg $soil^{-1}$ in correlation with the sand content (A) and the cation exchange capacity (CEC) of the soils (B). Data points represent means of 4 independent replicates. The coefficient of determination (R^2) is indicated.

On LUV the high calcium carbonate content (23.3%) may also limit the plant availability of glyphosate (and phosphate) by complexation and formation of insoluble salts with Ca^{2+} (Sprankle et al., 1975a, 1975b) explaining the comparatively low potential for glyphosate-induced plant damage after application of P fertilisers (Tab. 8.3, Tab. 8.4, Tab. 8.5). In all treatments, no plant damage was observed on REG. This soil is characterised by an comparably high organic matter content (SOM=3.8 %) (Tab. 8.1). Various studies reported a sorption potential for glyphosate also for humic compounds (Piccolo et al.,1992, 1994) and SOM (Albers et al., 2009), which is not the main soil fraction for P adsorption. Therefore; glyphosate associated with SOM may not be available for competitive desorption with P fertilisers. Moreover, a high SOM content is frequently associated with a high soil-microbial activity contributing to rapid microbial degradation of glyphosate (Schnürer et al., 1985; Franz et al., 1997; Borggaard and Gimsing, 2008).

Various plant species are able to modify the rhizosphere chemistry to improve the acquisition of sparingly soluble nutrients such as P, Fe, Mn and Zn by root-induced changes of rhizosphere pH, redox potential and release of organic chelators (Neumann and Römheld, 2002; 2007). In principle, these root-induced-chemical changes may also influence the solubility of glyphosate in the rhizosphere as a factor contributing to the risk of plant damage by re-mobilisation of glyphosate residues in soils. In the present study, phosphate deficiency was detectable in all soybean plants cultivated on soils without additional P fertilisation (Tab. 8.6). However, under these conditions no plant damage was induced on glyphosate-treated soils (Tab. 8.3, Tab. 8.4, Tab. 8.5). This finding suggests, that the soybean plants did not express adaptive rhizosphere-chemical changes in response to P limitation with a potential for co-mobilisation of glyphosate. Accordingly, results of Tesfamariam (2003) also revealed no indications for P deficiency-induced alterations of rhizosphere chemistry in soybean. However, it remains to be established whether the well-documented adaptations for P and Fe acquisition in various other plant species (Neumann and Römheld, 2007) bear a risk for re-mobilisation of glyphosate.

Hormesis effects

At P fertilisation levels of 0 and 80 mg P kg^{-1} soil, glyphosate application to REG even caused a significant increase in shoot biomass of the soybean indicator plants (Tab. 8.3). Growth-stimulating effects of subtoxic glyphosate doses (so-called hormesis) have been reported for different plant species, although the underlying mechanisms remain poorly understood (Velini et al., 2008). Hormesis is likely to be related to the molecular target of glyphosate, since the effect was not seen in glyphosate-resistant plants. (Velini et al., 2008). Among all investigated soils of the present study, the highest potential for P fixation was detected on REG, reflected by the lowest response of the plant P-nutritional status to P application. Therefore, a strong fixation potential can be expected also for glyphosate. As a consequence, only trace amounts of glyphosate, responsible for the hormesis effect may be mobilized by competitive adsorption with P fertilisers. Accordingly, the hormesis effect disappeared at the highest level of P fertilisation (240 mg P kg^{-1} soil).

8.6 Conclusions

The results of the present study suggest that re-mobilisation of glyphosate residues in soils by addition of P fertilisers should be considered as additional potential pathway for glyphosate toxicity to non-target plants, which is strongly influenced by soil characteristics related to the soil capacity for glyphosate adsorption/degradation.

Soils with a low or moderate fixation capacity for glyphosate and phosphate, low potential for glyphosate degradation, frequent applications of glyphosate and P fertilisers as well as cropping systems with limited soil perturbation are potential candidates for increased risk of crop damage due to glyphosate re-mobilisation. These conditions are likely to occur in no tillage or minimal-tillage systems with glyphosate pre-crop application or in cropping systems with a rotation of glyphosate-resistant and non-resistant crops particularly on sandy and/or

acidic soils. This applies particularly to cropping systems in the tropics and subtropics on soils with low nutrient availability requiring high input of fertiliser P. With the increasing cropping area of glyphosate-resistant plants, even more intense use glyphosate can be expected in the future, increasing also the potential risks of detrimental side effects to non-target plants. Since damage symptoms do not resemble commonly known toxicity symptoms of glyphosate, recognition under field conditions might be difficult.

Competitive desorption of glyphosate with P fertilisers may be of particular relevance, since both compounds are concentrated in the uppermost soil layers. Moreover, phosphate has a potential to attract root growth into the soil zones with the highest P accumulation (Drew, 1975) and therefore also into the regions with the highest concentrations of glyphosate residues. Of special interest is also the question whether root-induced alterations of rhizosphere chemistry for acquisition of sparingly soluble forms of P and Fe, expressed in various plant species can also contribute to mobilization of glyphosate residues in soils. Since the phenomenon of glyphosate re-mobilisation by application of P fertilisers is currently only investigated in model experiments under controlled environmental conditions, field studies are urgently required to evaluate the potential relevance for agricultural production systems.

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

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Own contribution: set-up of experiments, plant cultivation, harvest and sample preparation, analysis of nutritional status of plants (support of students in 1 of 4 experiments), manuscript preparation

9.1 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 immobilisation 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:

cv. cultivar

GM genetically modified

GR glyphosate-resistant

LMW low molecular weight

HMW high molecular weight

9.2 Introduction

Due to low production costs and high herbicidal efficiency, glyphosate is the most widely used wide-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 reported 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 and Hansen, 2004; Huber and McCay-Buy, 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-labelled 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 sub-lethal 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 suggest 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) mobilisation (Huber, 2006; Neumann *et al*., 2006); and (2) intracellular accumulation of phytotoxic glyphosate metabolites, such as amino-methylphosphonic 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 utilisation 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 immobilisation of the investigated micronutrients in young leaves of glyphosate-treated plants by metal complexation with glyphosate (Sprankle *et al*., 1975c), 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 conducted 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).

9.3 Materials and methods

Plant material and growth conditions

Soybean (*Glycine max* L.) seeds of the Glyphosate-resistant (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 sterilised 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; Corg [%] <0.3) and a sandy acidic Aphorizon of an Arenosol (pH ($CaCl_2$) 4.5; Corg [%] 0.16). Calcium chloride-diethylenetriamine pentaacetic 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 fertilised 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 fertilisation, the soils were sieved again to guarantee homogeneous distribution of the fertilisers. Previous measurements showed no profound changes in soil pH after identical fertiliser 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 fertilised 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/ 14–16 ºC. Water loss was determined gravimetrically and replaced by daily applications of de ionised 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 analysed 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 deionised 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 homogenised 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 analysed 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 randomised block design with four replicates per treatment. Nutrient solution experiments were conducted in a completely randomised 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.

9.4 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 (Tab. 9.1). Similar trends were also detected for shoot biomass of glyphosate-treated plants although the differences were not significant (Tab. 9.1).

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. 9.1).

Tab. 9.1 Plant biomass of glyphosate-sensitive (GS) and glyphosate-resistant (GR) soybean depending on Manganese supply and glyphosate application

Root- and shoot dry matter production were measured for the GR soybean (*Glycine max* L.) cv. Valiosa and the non-resistant parental line Conquista, grown for two weeks in hydroponic culture with sufficient [0.5 µM] and low [0.1 µM] Mn supply. 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.

Mn supply	0.1 µM		0.5 µM	
	Dry matter production (mg DM pot^{-1})			
Treatment	Root	Shoot	Root	Shoot
Conquista	**218** a	**1002** a	**201** a	**1009** a
Valiosa –Gly	**181** a	**1027** 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

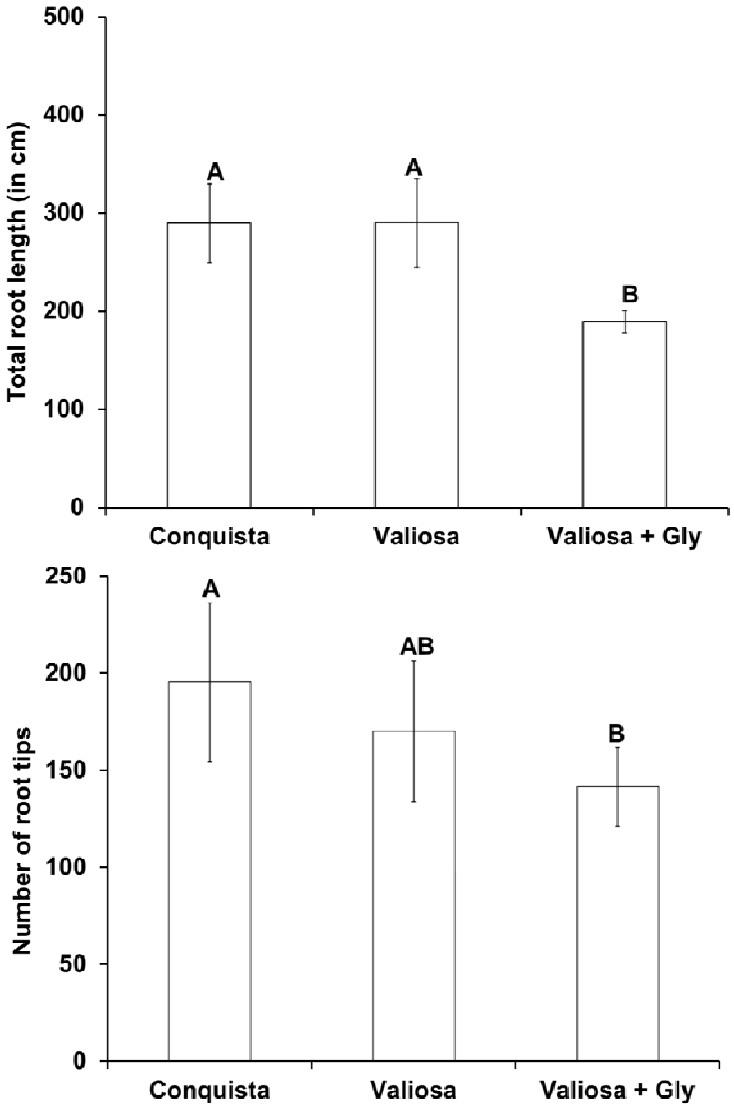

Fig. 9.1: Root morphology of glyphosate-sensitive (GS) and glyphosate-resistant (GR) soybean depending on glyphosate application

Root length (above) and number of root tips (below) were determined for the GR soybean (*Glycine max* L.) cv. Valiosa and its nonresistant 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

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. 9.2). 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 nontransgenic 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.

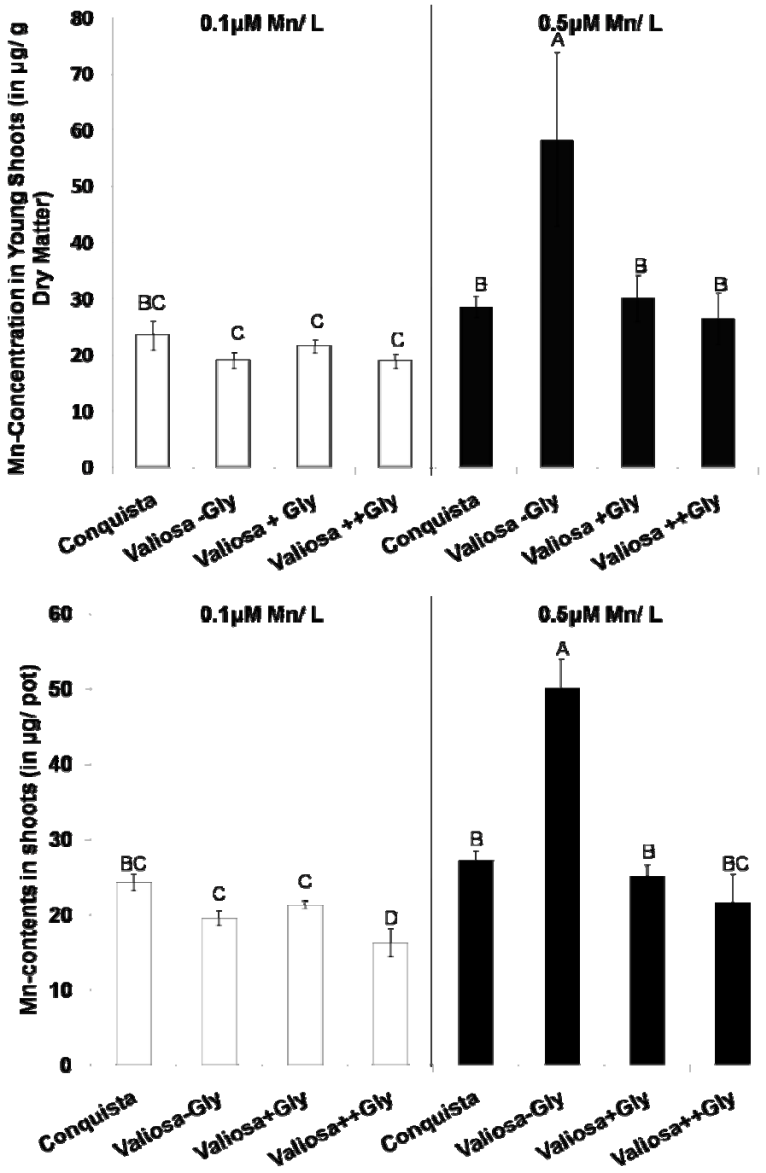

Fig. 9.2: Manganese concentration in shoots of glyphosate-sensitive (GS) and glyphosate-resistant (GR) soybean depending on Manganese supply and glyphosate application

Manganese concentration (A) and total Mn content (B) were measured in young leaves of the GR soybean (*Glycine max* L.) cv. Valiosa and its non-resistant parental line cv. Conquista after two 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.

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. 9.3). 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. 9.3) or root elongation on both soils.

Glyphosate significantly reduced the concentration of Zn in young leaves of cv. Valiosa (Tab. 9.2) in two independent replications of the experiment, while no significant differences were detectable for Mn (Tab. 9.2). 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 (Tab. 9.2).

9.5 Discussion

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 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 and Hansen, 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 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 (Tab. 9.1; Fig. 9.1, 9.3). 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 (Tab. 9.1; Fig. 9.1). 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 soybean 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. 9.3).

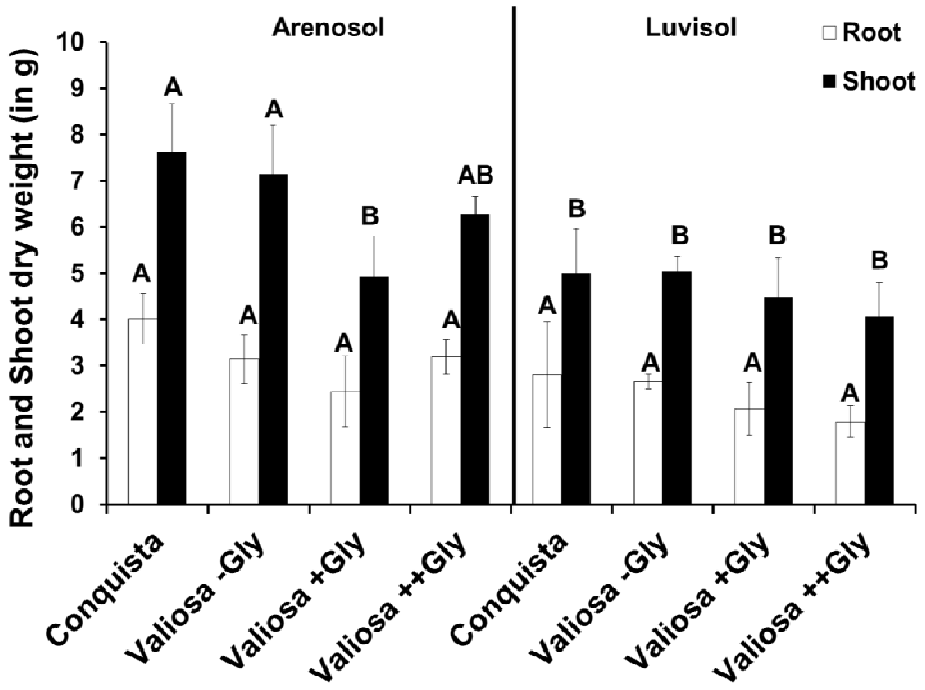

Fig. 9.3: Plant biomass of glyphosate-sensitive (GS) and glyphosate-resistant (GR) soybean depending on soil type and glyphosate application

Root-, and shoot biomass production were measured for the GR soybean (*Glycine max* L.) cv. Valiosa and its non-resistant 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.

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. 9.2) and of Zn in plants grown in soil culture (Tab. 9.2). 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. 9.2), while other micronutrients, such as Zn and Fe remained unaffected (data not shown). This may be a consequence 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 (Tab. 9.1; Fig. 9.1,) 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. 9.2).

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 immobilisation 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.

Tab. 9.2 Soluble and insoluble micronutrient (Mn, Zn, Fe) fractions in leaves of glyphosate-sensitive (GS) and glyphosate-resistant (GR) soybean depending on soil type and glyphosate application

Concentrations of Manganese (Mn), Zinc (Zn) and Iron (Fe) in the 80 % ethanol-soluble (LMW) and insoluble fractions (HMW) were obtained from young leaves of the GR soybean (*Glycine max* L.) 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			
	Conquista	**Valiosa**	**Val. +Gly**	**Val.++Gly**	**Conquista**	**Valiosa**	**Val. +Gly**	**Val.++Gly**
soluble Mn	**1.2** ^{A}	**0.9** ^{A}	**1.3** ^{A}	**1.2** ^{A}	**5.1** ^{B}	**5.7** ^{B}	**5.6** ^{B}	**6.4** ^{B}
[µg g^{-1} DM]	(±0.29	(±0.2)	(±0.2)	(±0.4)	(±2.3)	(±1.3)	(±1.1)	(±1.5)
insoluble Mn	**53.2** ^{A}	**48.3** ^{A}	**53.6** ^{A}	**56.3** ^{A}	**85.7** ^{B}	**81.6** ^{B}	**93.0** ^{B}	**92.9** ^{B}
[µg g^{-1} DM]	(±2.7)	(±7.6)	(±4.6)	(±5.1)	(±18.4)	(±8.5)	(±16.0)	(±15.0)
Total Mn	**54.4** ^{A}	**49.2** ^{A}	**54.9** ^{A}	**57.5** ^{A}	**90.9** ^{B}	**87.4** ^{B}	**98.7** ^{B}	**99.4** ^{B}
[µg g^{-1} DM]	(±2.9)	(±7.6)	(±4.4)	(±4.8)	(±20.6)	(±9.5)	(±16.6)	(±14.4)
soluble Zn	**41.3** ^{A}	**28.1** ^{B}	**27.5** ^{B}	**22.2** ^{B}	**28.1** ^{A}	**27.7** ^{A}	**30.3** ^{A}	**32.4** ^{A}
[µg g^{-1} DM]	(±5.7)	(±7.7)	(±6.3)	(±9.9)	(±8.9)	(±3.7)	(±4.9)	(±2.6)
insoluble Zn	**85.5** ^{A}	**68.6** ^{A}	**39.1** ^{B}	**38.2** ^{B}	**40.5** ^{A}	**35.1** ^{A}	**35.8** ^{A}	**38.0** ^{A}
[µg g^{-1} DM]	(±35.0)	(±18.2)	(±2.6)	(±10.8)	(±11.2)	(±1.8)	(±7.3)	(±9.7)
Total Zn	**126.8** ^{A}	**96.8** ^{A}	**66.6** ^{B}	**68.8** ^{B}	**68.6** ^{A}	**62.8** ^{A}	**66.3** ^{A}	**70.4** ^{A}
[µg g^{-1} DM]	(±35.2)	(±25.5)	(±8.5)	(±16.7)	(±15.8)	(±3.2)	(±4.1)	(±8.8)
soluble Fe	**21.6** ^{A}	**14.2** ^{A}	**27.2** ^{B}	**13.6** ^{A}	**16.1** ^{A}	**15.1** ^{A}	**18.5** ^{A}	**20.9** ^{A}
[µg g^{-1} DM]	(±8.6)	(±5.3)	(±6.8)	(±3.8)	(±4.8)	(±5.2)	(±9.6)	(±11.7)
insoluble Fe	**134.4** ^{A}	**124.9** ^{A}	**114.8** ^{A}	**114.8** ^{A}	**122.8** ^{A}	**124.2** ^{A}	**105.2** ^{A}	**111.7** ^{A}
[µg g^{-1} DM]	(±42.8)	(±22.8)	(±25.7)	(±18.8)	(±25.0)	(±23.5)	(±29.7)	(±36.4)
Total Fe	**155.9** ^{A}	**139.1** ^{A}	**141.9** ^{A}	**128.4** ^{A}	**138.9** ^{A}	**139.2** ^{A}	**123.7** ^{A}	**132.5** ^{A}
[µg g^{-1} DM]	(±44.9)	(±22.5)	(±28.2)	(±21.9)	(±24.0)	(±24.7)	(±22.7)	(±33.5)

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*., 1975c) 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 (Tab. 9.2). 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 immobilisation in the root tissue by complexation with glyphosate, which may limit the translocation of micronutrients to the shoots, still needs to be investigated.

9.6 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 characterise responsible factors and to investigate underlying mechanisms of action and their degree of expression under field conditions.

10 General discussion

Presently, risks of glyphosate toxicity for conventional and genetically modified glyphosate-resistant crops are predominantly discussed in terms of contamination of crop plants by glyphosate via the air during drift events or via a pathway from soil particles to the plant. Although the risk of glyphosate toxicity due to drift is acknowledged, because of rapid inactivation of glyphosate by adsorption to the soil matrix and microbial degradation of glyphosate in soil solution, risks of glyphosate toxicity to non-target organisms in soils are generally considered as marginal. Similarly, protection provided by the glyphosate-resistant 5-enolpyruvylshikimate-3-phosphate synthase are seen as factor causing low risk of crop damage during application of glyphosate to GR crops.

However, results of a series of bioassays conducted with sunflower (Chapter 4), wheat (Chapters 5, 7) and soybean (Chapters 6, 7, 8) revealed the potential relevance of glyphosate transfer via the rhizosphere from root tissue of treated weed plants to subsequently grown crops (Chapters 4, 5 and 6) and a re-mobilisation of glyphosate from the soil matrix (Chapter 8) as two possible pathways of glyphosate in soils, which were not widely considered so far. Both pathways are connected with a distinct but partly overlapping set of factors (risk factors), influencing the probability of damage of crop plants caused by glyphosate application. In case of pre-crop glyphosate application on weed plants and subsequent release of glyphosate into the rhizosphere, most important risk factors identified in the present study comprised the waiting time between glyphosate application and subsequent sowing of crops and the density of glyphosate-treated weed plants acting as storage pool for phytotoxic glyphosate (Chapters 4, 5 and 6). In case of possible re-mobilisation of glyphosate from the soil matrix, most important risk factors were the ad- and desorption characteristics of a soil for glyphosate and the soil specific potential for glyphosate degradation (chapter 8). Results of the present study revealed impaired nutritional status of non-resistant and glyphosate-resistant (GR)-crops as important secondary effect of glyphosate damage of plants (Chapters 4-9). However, in the absence of phytotoxic effects also growth stimulating effects of sub-lethal doses of glyphosate were observed (Chapter 8).

The following discussion highlights the most important findings of this thesis and discusses them in broader context of agricultural plant production and their use for an improved application of glyphosate for weed control.

10.1 Transfer pathways of glyphosate in the rhizosphere

Poor establishment and growth of glyphosate-treatment succeeding crops has been repeatedly reported by farmers and scientists when glyphosate or other non-selective herbicides have been used to kill weeds before sowing of crops in no tillage or conservation tillage systems (Smiley *et al.*, 1992; Descalzo *et al.*, 1998; Römheld *et al.*, 2008). In line with these observations, preliminary field trials at two locations in no-tillage systems in the vegetation period 2007/2008 revealed strong damage of winter wheat in case of short waiting time between glyphosate application to self-sown wheat (*Triticum aestivum* L.) or alfalfa (*Medicago sativa* L.) and sowing of winter wheat (Römheld *et al.*, 2008).

A rhizosphere transfer of the herbicide during degradation of the weed residues to germinating seeds and seedlings of the subsequent crop, stimulation of root pathogens attracted by the decaying weed residues and the release of allelopathic compounds have been discussed as possible reasons, but the underlying mechanisms are still not clear (Römheld *et al.*, 2008; Smiley *et al.*, 1992; Descalzo *et al.*, 1998; Dudai *et al.*, 2009).

10.1.1 Rhizosphere transfer of glyphosate to crop plants via weed residues

Results of four independent field experiments (Chapter 5) indicated a direct correlation of short waiting time between pre-crop application of glyphosate and sowing of crop, with damage of crops e.g. delayed plant development, impaired shoot growth, chlorosis, decreased density of crop plants, symptoms of damage (Tab. 5.1; Fig. 5.1) and also impaired nutritional status of plants (data not shown).

The results of the present study demonstrated that in comparison between different tillage treatments, damage of crops induced by short waiting time was stronger expressed in case of no-tillage- compared to minimal tillage treatments (Tab. 5.1). This effect might be explained by the increased dispersion and soil mixing of glyphosate containing root material in the minimal tillage treatment causing on the one hand a destruction of zones with high phytotoxic activity of glyphosate and on the other hand an increase in speed of decomposition of glyphosate-treated root residues and subsequently an inactivation of glyphosate by microbial degradation or adsorption to the soil matrix.

Root channels of weed roots potentially represent preferential growth environment for roots of subsequently grown crop plants causing an increased likelihood for crop damage in case of growth of crop roots in root channels of glyphosate-treated weed plants. Minimal soil tillage potentially leads to disruption of such root channels of glyphosate treated roots and additionally might increase the probability for alternative growth pathways for roots of crop plants. Both aspects might reduce the likelihood for root growth of crops in zones of high phytotoxic activity of glyphosate in soils and potentially explain reduced damage of crop plants induced by short waiting time in minimal tillage treatments compared to no-tillage treatments (Tab. 5.1).

In parallel, results of the field trial at Starzach revealed that damage of crops after pre-crop application of glyphosate was in case of short waiting times also directly correlated to density of glyphosate treated weed plants (Fig. 5.3).

Results of the field trials suggested that glyphosate-treated weed residues acted as a transient storage pool of active glyphosate, associated with a risk of contact contamination of crops sown after short waiting times after pre-crop application of glyphosate for weed control.

This conclusion is in accordance with the behaviour of glyphosate *in planta*. According to various reports, after adsorption of glyphosate by leaves of treated plants glyphosate is in most plant species not readily metabolised. Glyphosate is mobile in phloem and long distance

transport can be observed through the plant following the same source-to-sink pattern that occurs for photoassimilates (Sprankle *et al.*, 1975c; Wyrill and Burnside, 1976; Ahmadi *et al.*, 1980; Bingham *et al.*, 1980; Gougler and Geiger 1981). Glyphosate is rapidly translocated to stems, leaves and roots of the entire plant, but preferentially to young plant tissues with high metabolic activity and growth rates, such as root tips and shoot apices, where potentially up to 80 % of the absorbed glyphosate accumulate (Schulz *et al.*, 1990; Franz *et al.*, 1997; Hetherington *et al.*, 1999; Feng *et al.*, 2003; Reddy *et al.*, 2003).

According to Doublet *et al.* (2009), the fate of pesticides in plant residues in soil is under-investigated and basically unknown. Most of the available information originates from studies of glyphosate residues in foliage (Newton *et al*., 1984; Feng and Thompson, 1990; Thompson *et al*., 1994; Reddy *et al*., 2004) and not in roots. Studies with soybean (*Glycine max* L.) and wheat suggested unspecific and non-covalent binding of glyphosate to starch and cell wall components (Komossa *et al.*, 1992). In a study of von Wirén-Lehr *et al.* (1997), release and degradation of ^{14}C-labelled glyphosate in different agricultural soils correlated with the soil-microbial activity but only after direct soil application. No such correlation was observed after soil incorporation of lyophilised soybean tissue cultures contaminated with glyphosate. These findings suggest different mechanisms for degradation of glyphosate adsorbed to the soil matrix or bound in plant residues in soils, respectively. In line with this, Doublet *et al.* (2009) reported that absorption of glyphosate and sulcotrione in plants delays their subsequent soil-degradation and, particularly in case of glyphosate, that persistence in soil could increase 2 to 6 times. Under field conditions, Laitinen and Rämö (2005) found, 40 days after application, glyphosate concentrations of up to 2.7 mg kg^{-1} dry weight in roots of glyphosate-treated weeds, while glyphosate concentrations detected in 0–5 cm and 5–35 cm soil layers were only 0.17 and 0.07 mg kg^{-1} dry weight. Also in a later study, Laitinen *et al.* (2007) reported considerably higher concentrations of glyphosate stored in roots in comparison to soil.
A release of glyphosate by roots was first shown in a study of Coupland and Caseley (1979) who reported that intact quackgrass roots (*Agropyron repens* L.) exuded significant amounts of ^{14}C-glyphosate into the surrounding solution. Neumann *et al.* (2006) investigated the potential transfer of foliar-applied glyphosate, released from roots of weed plants to simultaneously cultivated non-treated indicator plants (sunflower seedlings - *Helianthus annuus* L.) in hydroponics and in soil culture systems. Results of Neumann *et al.* (2006) demonstrated a release of glyphosate via the roots of target plants, which can be subsequently taken up by simultaneously growing non-treated plants, exerting inhibitory effects on the shikimate pathway, uptake of micronutrients (Mn) and plant growth. Similarly, damage of plants after root exposure to glyphosate has been reported by other scientists (Rodrigues *et al.*, 1982; Pline *et al.*, 2002a, 2002b; Guldner *et al.*, 2005; Tesfamariam, 2009). However, the underlying causes and practical relevance of these findings remained unclear due to limitations of the experimental set-up (e.g. growth conditions, glyphosate application).

Results of model experiments conducted in the present study with sunflower (Chapter 4), wheat (Chapter 5) and soybean (Chapters 6) clearly supported the hypothesis that damage of wheat was caused by phytotoxic glyphosate stored in root tissue of glyphosate-treated weed plants.

In sunflower, grown on an acidic sandy Arenosol (pH 4.8) or on calcareous subsoil of a Luvisol (pH 7.4), analysis of intracellular shikimate accumulation, metabolic indicator for glyphosate toxicity, revealed that the risk of toxic effects, induced by pre-crop glyphosate application on rye grass (*Lolium perenne* L.), increases with declining waiting time and can persist up to 3 weeks (Tab. 4.1, 4.2; Fig. 4.3, 4.4).
Toxicity of pre-sowing glyphosate treatments on sunflower seedlings was also strongly dependent on the mode of glyphosate application (Tab. 4.1, 4.2; Fig. 4.1-4.4). When glyphosate was sprayed on pre-cultured rye grass seedlings, detrimental effects on plant growth and the Mn nutritional status, as well as increased intracellular shikimate accumulation in the root tissue were stronger expressed than after direct soil application of the same amount of glyphosate (Fig. 4.3-4.6). In this case, it is clear that glyphosate transfer took place in weed residues but since shoots and roots of glyphosate-treated weed plants remained in the pots, transfer of glyphosate might have occurred in the soil via the rhizosphere but also above soil by leaf contact between weeds and crops.
Model experiments in the present study, simulating conditions of field trials by using different field soils, showed that short waiting time between glyphosate application to wheat as weed plant and sowing of winter wheat as crop plant caused, in comparison to control, significant damage of plants in terms of deformation of leaves, chlorosis, impaired plant biomass production and accumulation of shikimate (Tab. 5.2, 5.3; Fig. 5.4). Interestingly, in these experiments, since shoot biomass of glyphosate-treated weed plants was removed before germination of non-target wheat plants, glyphosate transfer must have occurred through rhizosphere pathway (Tab. 5.2; Fig. 5.4).

Additionally, model experiments with wheat shown a strong correlation between crop damage and the density of glyphosate-treated weed plants. In case of short (0 days) or medium (7 days) waiting time, glyphosate application on high density weed populations induced significantly stronger damage of crop plants compared to application on low densitiy (Tab. 5.3; Fig. 5.4). Results of experiments with soybean cultivated on an Arenosol (pH 4.8) and a Regosol soil (pH 7.1) showed similar results, highlighting that root residues of glyphosate-treated weed plants in soil cause an increased and prolonged phytotoxic activity of glyphosate in soils (Tab. 6.1-6.6; Fig. 6.1-6.5).

Therefore, it is plausible that root tissue of glyphosate-treated weed plants acts as storage pool of glyphosate in the soil, which is released after/during degradation affecting and prolonging the time-window of potential glyphosate phytotoxicity to subsequently sown crop plants.

Furthermore, experiments with soybean showed that a decline in shoot and root biomass, damage symptoms as well as accumulation of shikimate as indicator of glyphosate toxicity were highly correlated to the speed of decay of glyphosate-treated weed plants (Tab. 6.2, 6.4, 6.6; Fig. 6.2, 6.5).

This correlation between development, intensity and expression of damage symptoms of soybean plants and death of glyphosate-treated weed potentially indicate that glyphosate release from treated weed roots might occur in two phases involving (a) exudation of

glyphosate from living roots as well as (b) release of glyphosate from decaying root material. Speed of death and decay of glyphosate treated weed plants is most likely directly connected to factors like growth season (spring or fall application), temperature, water content of soils and/or the tillage treatment which might shorten or increase the time window for crop damage caused by transfer of glyphosate in the rhizosphere under field conditions. As glyphosate adsorption to soils represents an important factor of glyphosate inactivation (Giesy *et al.*, 2000), soil type might also be one of the most important factors influencing the time window for glyphosate-induced crop damage.

Surprisingly, glyphosate application on weed plants grown on eight different soils induced comparable glyphosate damage of sunflower (Chapter 4), wheat (Chapter 5) and soybean plants (Chapter 6). This is suggesting that, in case of short waiting times between pre-crop glyphosate application and sowing of crop plants, transfer of glyphosate from root residues of glyphosate-treated weed plants is primary independent of the soil type.

However, as observed in Chapter 6, soil types can have profound effects on the growth conditions for weed plants influencing the speed of death of glyphosate treated weed plants (Tab. 6.1-6.6; Fig. 6.1, 6.2). Plausibly, differences in soil microbial activity might also shorten or increase the time window for crop damage caused by transfer of phytotoxic glyphosate in the rhizosphere due to differences in degradation of root residues of glyphosate-treated weed plants.

In the context of crop production, conservation or no-tillage systems potentially present several/ all of the identified conditions for damage of crops induced by rhizosphere transfer of glyphosate from weed roots to subsequently cultivated crops. In fact they are characterised by the need for effective weed control (e.g. glyphosate application) shortly before sowing as essential tool to minimise crop production losses caused by high weed infestations (Lyon *et al.*, 1996; Calado *et al.*, 2010). Additionally, minimal disturbance of top soil horizon containing glyphosate-treated root during tillage might increase the probability for contact between these root residues and following crops. Thus, it seems plausible that these crop production systems are particularly at risk for damage of crops after rhizosphere transfer of glyphosate from treated weed residues.

However, actual probability of crop damage caused by short waiting time between glyphosate application on weed plants and direct sowing of crops in conservation/no-tillage systems is likely to be variable due to the influence of abiotic and biotic growth conditions and might not be observed at the same extent every year on field sites.
In addition, short waiting times between glyphosate application and sowing are actually not necessary to avoid yield loss due to competition between crops and weed plants because, as the results of field trials of the present study indicated, the well-known excellent weed control characteristic of glyphosate can allow long waiting times after glyphosate application without yield loss due to high weed pressure.

10.1.2 Rhizosphere transfer of glyphosate to crop plants after re-mobilisation from soils

Compared to other pesticides, glyphosate possesses unique sorption characteristics in soil. Almost all other pesticides are moderately to weakly adsorbed in soils, mainly by soil organic matter, because most of these molecules are dominated by apolar groups, i.e. aliphatic and/or aromatic carbon, and often have only one functional group (Borggaard and Eberling, 2004; Schwarzenbach *et al.*, 1993). In contrast, glyphosate, which is a small molecule with three polar functional groups (carboxyl, amino and phosphonate groups), is strongly adsorbed by soil minerals.

In line with this, results of the present study demonstrated very low soil activity of glyphosate under most conditions. In an acidic sandy Arenosol and a calcareous Luvisol subsoil, only a waiting time of 0 day between glyphosate application to the soil and sowing of sunflower revealed risk of phytotoxic effects of glyphosate on plants as shown by analysis of physiological parameters, like intracellular shikimate accumulation as metabolic indicator for glyphosate toxicity and the micronutrient status (Tab. 4.1, 4.2; Fig. 4.3, 4.4). Similarly, after an application of glyphosate to an Arenosol and a Regosol, no indication for glyphosate-induced damage of soybean was detectable (Tab. 6.1, 6.3, 6.5).

However, glyphosate has been suggested to adsorb to soils and minerals by ligand exchange through its phosphonic acid group in a way similar to the adsorption of phosphate (Piccolo *et al.*, 1992; Hill, 2001; Hance, 1976; Dion *et al.*, 2001; Gimsing and Borggaard, 2002a, 2002b). Accordingly, it has been suggested that phosphate and glyphosate compete for adsorption sites. Various studies showed that the presence of phosphate significantly decreased the adsorption of glyphosate to the soils (Gimsing and Borggaard, 2002a, 2002b; Borggaard and Gimsing, 2008), Therefore, bioavailability of glyphosate to plants can potentially be altered by fertiliser application and/or plant strategies for P mobilisation leading to a re-mobilisation of glyphosate previously fixed to the soil matrix.

In the present study (Chapter 8), a series of bioassays in pot experiments were conducted with conventional soybean cultivated on five contrasting soils with or without pre-incubation of soils with glyphosate 10-35 days before sowing of plants and application of five different levels of inorganic P fertiliser at sowing. On an Arenosol (pH 4.8), Acrisol (pH 5.0), Luvisol (pH 7.4) and a Ferralsol (pH 5.0), application of P fertiliser to soils pre-incubated with glyphosate induced visual symptoms of plant damage, declined shoot and root growth, impaired nutritional status and accumulation of shikimate as indicator of glyphosate toxicity (Tab. 8.2-8.7; Fig. 8.1). First, these results indicated that on a wide range of soil types, re-mobilisation of glyphosate represents a potential pathway for damage of crops. Second, they gave clear indications that inactivation of glyphosate by adsorption to the soil matrix can be a reversible process under specific conditions, such as application of phosphorus fertiliser.

However, in this experiment, soil dependent differences in intensity of damage due to P-induced re-mobilisation of glyphosate were observed and on a Regosol (pH 7.1), no evidence for damage of soybean after re-mobilisation of glyphosate was shown (Tab. 8.2-8.5). These

results can be explained by differences between the soil types in terms of strength of P/glyphosate-fixation (Tab. 8.2-8.7; Fig. 8.2, Fig. 8.3).

In conformity with phosphate chemistry, the main soil sorption sites of glyphosate and aminomethylphosphonic acid (AMPA), the phytotoxic primary metabolite of glyphosate in soils (Reddy et al, 2004, Laitinen et al, 2008), are found on surfaces of iron and aluminium oxides, poorly ordered aluminium silicates and edges of layer silicates, while sorption of glyphosate by permanent charge layer silicates seems to be limited (Barja and Dos Santos Afonso, 2005; Gimsing and Borggaard, 2002a, 2002b; Gimsing *et al.*, 2004; Vereecken, 2005 and references cited therein).

Damage of soybean plants caused by re-mobilised glyphosate was positively correlated to increasing soil levels of plant-available Fe and generally increased on acidic soils (Fig. 8.2). Increasing of pH has been found to decrease the amount of glyphosate adsorbed by iron/aluminium oxides as well as soils (Sheals *et al.*, 2002; Barja *et al.*, 2005). These findings suggest that a significant proportion of phosphate-exchangeable glyphosate may be adsorbed to the plant-available Fe fractions in the investigated soils.

Therefore, expression of plant adaptations for root-induced Fe mobilisation may also be associated with a risk of glyphosate re-mobilisation (Neumann and Römheld, 2007).

Beside this, in these experiments, glyphosate-induced plant damage correlated with increasing sand content (Fig. 8.3) and inversely correlated to increasing soil organic matter contents of the evaluated soils (Tab. 8.1-8.7). Potential risks for non-target plant damage was reported to be increased on light sandy soils due to weak adsorption and/or low microbial degradation of glyphosate (Cornish, 1992). In the present study, big soil particle size on sandy soils might also increase the chance for re-mobilisation of glyphosate due to lower numbers of adsorption sites and therefore higher competition with phosphate.

In comparison to the other soils the Regosol had a considerably higher soil organic matter content and second highest pH, which might have induced lower glyphosate adsorption capacity of the soil (Gimsing *et al.*, 2004). Mineralisation/ degradation of glyphosate in soil has been found to be inversely correlated with the glyphosate sorption capacity of the soil (De Jonge *et al.*, 2001; Wackett *et al.*, 1987), i.e. if adsorption of glyphosate is strong, mineralisation/degradation of glyphosate is low, possibly because bioavailability is low. Thus, potentially soil conditions of the Regosol were favourable for glyphosate degradation during pre-incubation due to low glyphosate adsorption. Investigations have shown that soil sorption of glyphosate is not, or is sometimes negatively, correlated with soil organic matter content which potentially increases the potential for glyphosate degradation. Additionally, higher microbial activity due to higher soil organic matter content might have increased the potential for glyphosate degradation during incubation period.

By contrast, Piccolo *et al.* (1992, 1994) reported very high glyphosate sorption by four different purified humus samples. Chemical interactions between glyphosate and soil organic matter and/or humic substances are possible by the phosphonic acid group as well as the amin-group and/or the carboxyl-group. Thus, it is possible that less competition between glyphosate and phosphorus due to glyphosate-specific binding sites on soil organic matter caused low potential for P-induced re-mobilisation of glyphosate.

Therefore, results of the present study suggested that differences between soils in terms of potential risks for damage of plants caused by re-mobilisation of glyphosate most likely are related to their glyphosate adsorption- and/ or degradation capacity.

In the context of crop production, in contrast to crop damage observed under field conditions in case of short waiting times after glyphosate weeds application, the relevance of glyphosate re-mobilisation as risk factor for crops under field conditions is not entirely clear.
Nevertheless, soils with a low or moderate fixation capacity for glyphosate and phosphate, low potential for glyphosate degradation and frequent applications of glyphosate and P fertilisers, as well as cropping systems with limited soil perturbation are potential candidates for increased risk of crop damage due to glyphosate re-mobilisation. These conditions are likely to occur in no-/ minimal tillage systems with glyphosate pre-crop application or in cropping systems with a rotation of glyphosate-resistant and non-resistant crops particularly on sandy and/or acidic soils. This applies particularly to cropping systems in the tropics and subtropics on soils with low nutrient availability requiring high input of fertiliser P.

However, as showed in the present study, desorption of glyphosate by competition with P can occur also on calcareous high pH soils after P fertilisation (Tab. 8.3-8.5). Therefore, potential re-mobilisation of glyphosate might be also a risk factor in European soil and crop production conditions. Interestingly, severe crop damage has been repeatedly observed in case of long-term glyphosate use in no-tillage winter wheat production system in South Germany (Hirrlingen/Tübingen). However, it is so far uncertain if re-mobilisation of glyphosate induced by competitive ad/desorption of phosphate are responsible for the symptoms observed (Fig. 10.1).
Hypothetically, depending on soil characteristics, the re-mobilisation of glyphosate might also be induced by acidification of the rhizosphere, due for instance to fertiliser application of NH_4^+ with nitrification inhibitors.

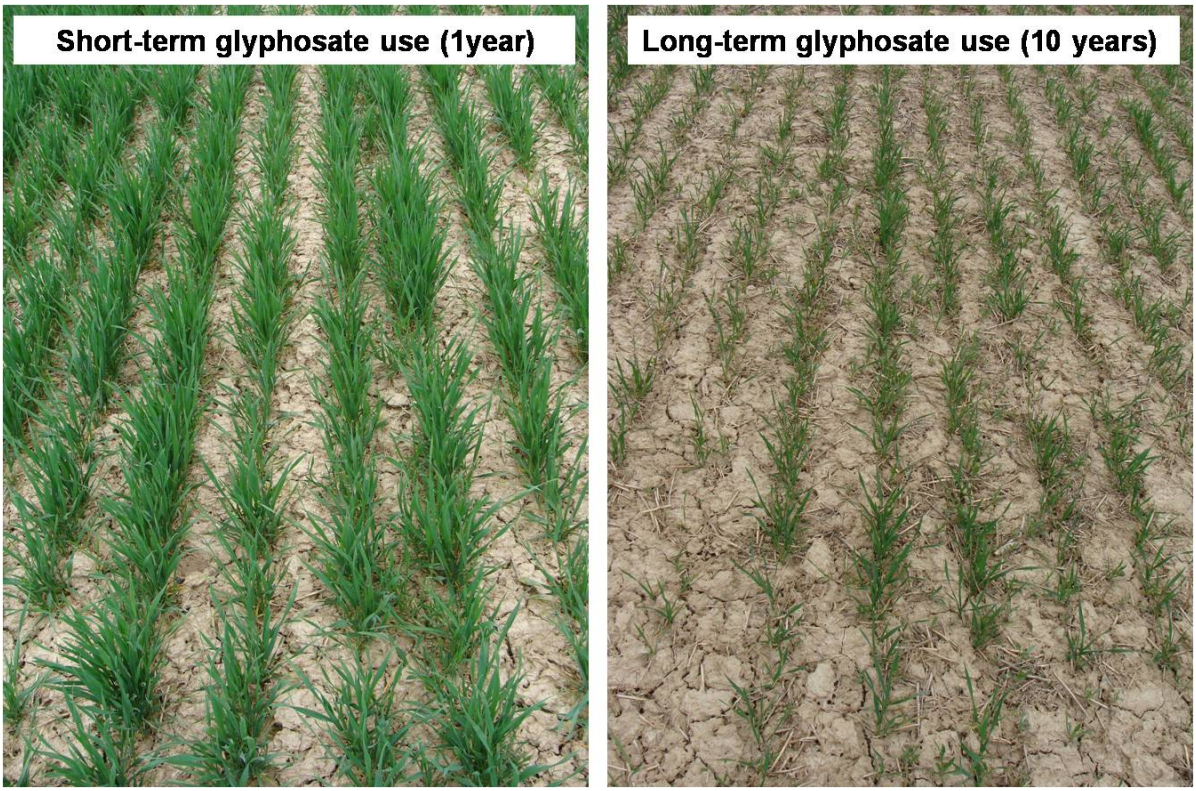

Fig. 10.1: Growth and development of field grown winter wheat depending on time of continuous glyphosate use

Growth and development of field-grown winter wheat (Triticum aestivum L.) growing in a no-tillage cropping system at same field site near Hirrlingen (Southwest Germany) in case of glyphosate pre-crop application for 1 year (left) or 10 years (right). Except of glyphosate application, field site is managed in regard to sowing date, fertilisation, and pesticide application etc. identically for several years.

Recommendations of management practices to counter the risk for potential re-mobilisation of glyphosate are not easy. Farmers in sensitive cropping systems (e.g. no tillage systems) should be informed about the possibility of glyphosate re-mobilisation and cautioned to observe their crops after activities with the potential for a re-mobilisation of glyphosate such as P fertilisation. The most promising strategy to avoid risk for re-mobilisation of glyphosate in sensitive cropping systems seems to be the avoidance of a temporary build-up of glyphosate most likely caused by repeated application within a short time period. In fact, observation of good agricultural practice, e.g. the changing of herbicides to avoid spread of resistance of weeds against specific compounds, might be the most simple and practical tool to avoid risks for crop damage associated to a re-mobilisation of glyphosate from soil matrix.

10.2 Alternative causes for crop damage

As already mentioned (Section 10.1.1), results of four independent field experiments indicated a direct correlation between short waiting time after glyphosate application, damage

of winter wheat (Tab. 5.1) and nutritional status of plants (data not shown). In addition to a rhizosphere transfer of the glyphosate, these results might be due to a stimulation of root pathogens by the decaying weed residues and/or the release of allelopathic compounds (Smiley *et al.*, 1992; Descalzo *et al.*, 1998; Dudai *et al.*, 2009). Moreover, in soils, glyphosate is degraded to its primary metabolite aminomethylphosphonic acid (AMPA), which is also considered as phytotoxic (Reddy *et al.*, 2004). Therefore, observed damage under field conditions might have been induced by one or several of these additional causes.

10.2.1 Toxicity of AMPA as cause for crop damage

Experiments with wheat evaluating the toxicity of glyphosate and AMPA as its main metabolite in soils under hydroponic conditions and in germination tests on filter paper (Chapter 5) gave clear indications that damage of wheat in terms of leaf deformations, impaired shoot and root growth and accumulation of shikimate, as observed in field trials and model experiments, was caused by glyphosate phytotoxicity (Fig. 5.2). In contrast to root exposure, results of seed exposure to glyphosate and AMPA revealed significantly impaired germination in case of exposure to AMPA but not glyphosate (Fig. 5.2).
An effect of AMPA on germination of plants has not been reported so far. The reasons for a differential phytotoxicity of glyphosate and AMPA depending on the developmental stage of plants are not further investigated in the present study and not entirely clear. Interestingly, a decline of germination and/or crop emergence was repeatedly detected when glyphosate was applied to dense weed pre-culture (Tab. 5.3, 6.1, 6.2). Similarly, at the field trial in Starzach, reduced crop density was detectable already during emergence of winter wheat (data not shown). As results of Reddy *et al.* (2004) indicated, AMPA is considered as considerably less phytotoxic compared to glyphosate. Therefore, phytotoxic AMPA levels in the rhizosphere might only occur when a large amount of glyphosate is exuded/ released by a high density of treated weed plants.

The reasons for differences in phytotoxicity of glyphosate and AMPA depending on the developmental stage of plants are not further investigated in the present study and not entirely clear. Due to the potential difference in phytotoxicity of glyphosate and AMPA depending on the developmental stage of plants, heterogeneity of damage symptoms in crop plants may arise under field conditions, depending on whether glyphosate or AMPA or both are present in a damaging amount in the sensitive developmental stage of crop plants.

10.2.2 Increased infection with soil-borne pathogens and/or allelopathic effects of weed residues as cause of crop damage

In winter wheat, the “green bridge” provided by volunteer cereals growing in summer is important for maintaining the life cycle of several pathogens (viruses, bacteria, fungi) and insect pests (Jiang *et al.*, 2005). Several studies reported increase of infection of wheat with fungal pathogens (*Fusarium*, *Phytium*, *Rhizoctonia*), or via the “green bridge”, when total herbicides were used to control weeds shortly before sowing of cereals (Smiley *et al.*, 1992; Descalzo *et al.*, 1998; Powell and Swanton, 2008).

In the present study, infection of winter wheat by soil-borne pathogens (*Fusarium*, *Phytium* and/or *Rhizoctonia*) was detectable by PCR and pathogen-specific primers in damaged plants in case of short waiting time after glyphosate or Basta® application at the field sites of Tauberbischofsheim and Dusslingen. These analyses, conducted by IdentXX, were preliminary investigations using a single, damaged plant per treatment in growth stage BBCH 30-31. Therefore, observed damage of winter wheat in the field trials of the present study might have been caused by pathogen infection (Tab. 5.1; Fig. 5.1, 5.3) but the results have to be used with caution.

The observed increase in pathogen infection could have been caused by one or a combination of following factors:

(a) A transfer of pathogens via an intact green bridge in case of short waiting after pre-crop glyphosate application (Smiley, 1992; Descalzo *et al.*, 1998; Powell and Swanton, 2008).

(b) Toxic effects of glyphosate or its main metabolite AMPA on plants inducing increased susceptibility of plants to soil borne pathogens (Johal and Huber, 2009; Kremer and Means, 2009; Kremer *et al.*, 2005; Yamada *et al.*, 2009; Johal and Rahe, 1984, 1988, 1990).

(c) Effects on soil microflora e.g. direct promotion of soil borne pathogens by glyphosate or AMPA acting as C- or P-source for specific pathogenic microorganisms and/or suppression of microorganisms antagonistic to these pathogens caused by toxic effects of glyphosate or AMPA effects.

(d) Glyphosate-induced impairment of the micronutrient as important physiological co-factors for mechanisms of plant disease resistance.

Beside pathogen infection, damage of winter wheat observed at different field sites in the present study might have been caused by allelopathic effects of decaying plant material of weeds (Tab. 5.1; Fig. 5.1, 5.3). It is known that residues of weed plants in soil can cause allelopathic effects on wheat (Dudai *et al.*, 2009). Similarly, autotoxicity of wheat residues has been reported in wheat monocultures under conventional and no-tillage conditions (Waller *et al.*, 1987).

However, biotic and abiotic factors can influence the production of allelochemicals by plant species and modify the effect of an allelochemical on crop plants. Factors such as soil type, light, nutrient availability, water availability, pesticide treatment and disease can affect the amount of allelochemicals in a plant (e.g. Inderjit & Del Moral, 1997, Reigosa *et al.,* 1999). And even though the production of allelochemicals in a plant increases in response to stress, it is not clear whether a corresponding release of allelochemicals to the environment also occurs (Einhellig, 1996; Inderjit and Del Moral, 1997). In parallel, sensitivity of plants to allelochemicals is typically increased by stress conditions (Einhellig, 1996, Reigosa *et al.,* 1999).

Interestingly, several groups of compounds with known allelopathic potential like benzoxazinoids, glucosinolates and others (e.g. ferulic acid, *p*-hydroxybenzaldehyde, emodin, physcion, *p*-hydroxybenzaldehyde, *p*-hydroxybenzoic acid, oligostilbenes) (Inderjit, 1996) are likely linked to physiological phytotoxic effects of glyphosate via the shikimate pathway. Thus, inhibition of the shikimate pathway by phytotoxic glyphosate in weed plants might affect the allelopathic potential of weed residues due to changes in contents of plant secondary metabolites. Whether inhibition of the shikimate pathway by glyphosate increase the allelopathic potential of weed residues e.g. by accumulation of potentially allelopathic compounds or decrease the allelopathic potential of weed residues due to impaired formation of benzoxazinoids glucosinolates and other compounds is not systematically investigated so far. Potentially, inhibition of the shikimate pathway by glyphosate causes a lower initial allelopathic potential of weed residues but accumulated precursors of allelochemicals in plants tissue might also be subsequently degradated to allelopathic compounds by soil microorganisms during decomposition of weed roots (Inderjit, 2005).

In order to differentiate potential damage caused by allelopathic effects of weeds, soil-borne pathogens or glyphosate transfer in the model experiments of the present study, genetically modified glyphosate-resistant (GR) soybean cultivars have been used as additional controls and cultivated under the same conditions as their near-isogenic glyphosate-sensitive (GS) parents (Chapter 6). In fact, they are approx. 50 times less sensitive to glyphosate than their parental glyphosate-sensitive (GS) genotypes (Nandula *et al.*, 2007) but not significantly different in their sensitivity to allelopathic compounds (Norsworthy, 2004c) or soil-borne pathogens (Johal and Huber, 2009; Kremer *et al.*, 2005; Kremer and Means, 2009).
In GS soybean, significantly reduced plant biomass, intracellular shikimate accumulation as physiological indicator for glyphosate toxicity and a decreased nutritional status of plants were observed in case of glyphosate plant application (Tab. 6.1, 6.3, 6.5; Fig. 6.4). Significantly weaker expressed damage was observed in GR soybean plants (Tab. 6.1, 6.3, 6.5; Fig. 6.4). These results gave further evidence that, at least in the model experiments of the present study (Chapter 4, 5, 6), rhizosphere transfer of glyphosate was the primary cause for plant damage.

Potentially, the observed increase in soil-borne pathogens infection under field conditions was rather a consequence of weak crop plant development due to glyphosate-transfer from weeds (Tab. 5.1; Fig. 5.1, 5.3) than the primary cause of crop damage. However, it should not be ruled out that, short waiting times between glyphosate application of weeds and sowing of crops might directly and/or indirectly increase the potential for crop damage due to pathogen infection via a green bridge or allelopathic effects caused by decaying weed residues.

Under field conditions, again, in conservation-/ no-tillage systems, minimal soil movements cause a build-up of residues of crops and weeds in the growth horizon of crop plants, which is potentially increasing risks of crop damage associated to soil-borne pathogen or allelopathic effects of weed residues (Alleto *et al.*, 2010). Even so the exact interactions between pre-crop glyphosate application shortly before sowing and potential for increased pathogen pressure and/or allelopathic effects remained unclear, farmers should be informed that, under these

conditions, unexpected and atypical damage of crops can occur, which might be related to effects of glyphosate application shortly before sowing.

10.3 Effects of glyphosate in the rhizosphere on crops

10.3.1 Differences in susceptibility of plant species to glyphosate in the rhizosphere

In order to evaluate potential differences between plant species in response of glyphosate in the rhizosphere, important crop plant species such as soybean, maize (*Zea maize* L.) and winter wheat were cultivated under hydroponic conditions after short-term exposure of roots to low glyphosate concentration (Chapter 7). Evaluation of visual symptoms of glyphosate toxicity, such as chlorosis in young meristematic tissue of leaves (Tab. 7.1), speed of plant development (Fig. 7.1) and plant biomass production (Tab. 7.2, 7.3) suggested a sensitivity in the order soybean < maize ≤ wheat. Similarly, determination of shikimate concentrations in roots as physiological indicator of glyphosate toxicity (Fig. 7.3) and evaluation of the nutritional status of plants (Tab. 7.4) indicated a higher sensitivity of maize and wheat in comparison to soybean. Results indicating differences in sensitivity of plant species to phytotoxic glyphosate in the rhizosphere have not been reported so far and the underlying mechanisms remained not fully understood.

Detection of chlorosis and significantly impaired shoot growth in maize and wheat but not in soybean (Tab. 7.1-7.3) potentially indicate that differences in mobility of glyphosate in plants e.g. a low translocation of glyphosate from roots to shoots, were responsible for differences in sensitivity to glyphosate toxicity. Limited translocation of glyphosate from roots to shoots (soybean < maize ~ wheat) might lead to a lower sensitivity of soybean to phytotoxic glyphosate because of a limited disruption of shoot crucial physiological processes e.g. in chloroplasts.
Differential sensitivity of plant species to phytotoxic glyphosate in the rhizosphere may also be attributed to particularly high ability of soybean for *in planta* conversion of glyphosate to it primary metabolite AMPA (Reddy *et al.*, 2004; Nandula *et al.*, 2007). It has been suggested, that a plant glyphosate oxidoreductase (GOX) or similar type of enzyme catalyses this conversion. Reddy *et al.* (2008) showed in a comparison of plant species, that after leaf application of glyphosate, the metabolite AMPA was detectable in six of seven leguminous species, but only one of four non-leguminous species. Therefore it is possible, that in the present study the high ability of soybean for conversion of glyphosate to AMPA contributed to low susceptibility to glyphosate toxicity. Accordingly, maize and wheat plants which most likely lack the ability for internal glyphosate detoxification by conversion to AMPA, were affected by phytotoxic glyphosate during a prolonged time span and therefore significantly stronger damaged than soybean (Tab. 7.2, 7.3). The conversion of glyphosate to AMPA might also explain why shikimate concentrations in roots of soybean were significantly lower compared to maize and wheat (Fig. 7.3).

Beside plant species specific sensitivity to glyphosate toxicity, results of experiments under hydroponic conditions indicated also differences between plant species in their ability to recover after exposure to phytotoxic glyphosate (Tab. 7.2, 7.3). Again, soybean showed a higher ability for recovery compared to maize and wheat, but winter wheat also showed a

considerable ability of recovery, given its high sensitivity and strong damage (Tab. 7.2, 7.3). Results are in line with field observations which frequently reportes only transient damage and complete recovery of soybean until final harvest, in case of foliar exposure to glyphosate by drift (Norsworthy, 2004c).
Even though growth conditions were not absolutely identical, it is interesting to note that wheat (Tab. 5.2; Fig. 5.4) and soybean (Fig. 6.4, 6.5) both considerably recovered after damage caused by glyphosate application on weed plants and reached a biomass of 50-60 % of control plants, while sunflower reached only a biomass of 10% of control (Tab. 4.1, 4.2).

The ability of plants to recover from glyphosate toxicity in the rhizosphere might also explain results of field experiments in the present study, where a small yield loss of in average 10% was measured at final harvest (Fig. 5.5), in contrast to the significant damage observed at juvenile growth stages (Tab. 5.1; Fig. 5.1). However, an additional explanation, based on the observation of lower plant density in case of short waiting times after pre-crop glyphosate application, might be that lower plant density (Fig. 5.5) caused a higher N-availability per plant and thus promoted plant recovery.

Obviously, both aspects, differences in the sensitivity of crops to glyphosate toxicity and in their ability for recovery after initial damage, have potential implications for agricultural crop production. Under field conditions, likelihood and severity of crop damage most likely increase in case of cultivation of potentially highly sensitive crop plant species like wheat or maize. Moreover, similarly to the time window for glyphosate transfer from root residues of glyphosate-treated weed plants, the ability of crop to recover after glyphosate toxicity is strongly influenced by biotic and abiotic conditions present at the field site at time of exposure of crop plants to glyphosate. Arguably, all biotic and abiotic stress factors, for instance pathogen pressure, allelopathic effects of weed plants, cold stress, drought stress, limited nutrient supply or oxidative stress due high light intensity, will have an impact on the ability of crops to recover from glyphosate toxicity and influence the severity of crop damage and yield loss under field conditions.
Again, depending of the transfer pathway of glyphosate via (a) weed roots or (b) re-mobilisation from soil matrix adequate waiting times and good agricultural practice such as a regular change of herbicides used (Powles, 2008) seem to be promising strategies avoid damage of crops by glyphosate toxicity and keep the excellent weed control abilities of glyphosate and thus improve application of an very important tool of modern intensive agriculture.

10.3.2 Expression of damage symptoms and hormonal effects

Results of the present study gave very clear indications that root growth (e.g. elongation of main root) is also primarily affected by glyphosate toxicity, in case of root exposure to glyphosate in the rhizosphere (Tab. 4.1, 4.2, 8.1, 8.2; Fig. 4.1, 7.2, 8.1, 9.1).
Impaired elongation of main roots might prevent roots to grow out of a glyphosate containing phytotoxic soil horizon, while formation of adventivious and lateral roots in this phytotoxic soil horizon might enhance the likelihood for re-intoxification of plants by glyphosate.

Strongly impaired root growth most likely results in impaired nutrient acquisition. Additionally, glyphosate-induced impaired elongation of main roots observed in the present study (Fig. 4.1, 7.2, 8.1) potentially results in shallow root systems and increases susceptibility to water shortage/ drought stress. Therefore, it is expectable that under field conditions drought resistance of crops affected by glyphosate toxicity will be significantly declined. Since scientists predicted increased incidents of drought periods caused by global warming in the near future, there is the potential for increase of crop damage caused by glyphosate-induced inhibition of root growth.

Based on its primary mode of action, glyphosate is generally classified as an aromatic amino acid biosynthesis inhibitor (Hoagland and Duke, 1982; Cole, 1988; Duke and Hoagland ,1985). However, expression of atypical symptoms (e.g. morphology of roots, deformation of leaves and delay of senescence) of glyphosate phytotoxicity observed in the present study in wheat (Fig. 5.1) and soybean (Fig. 6.3, 8.1) indicated hormonal imbalances in plants even after recovery from initially glyphosate toxicity.
Inhibition of the shikimic acid pathway leading to the disruption of synthesis of aromatic aminoacids and phenolic compounds plausibly causes also strong disturbance of the auxin (IAA) status of plants as important secondary effect (Hoagland, 1990; Matschke *et al.*, 2002; Yasuor *et al.*, 2006). Additionally, high accumulation of phytotoxic glyphosate in root tips most likely affects formation of cytokinin. In line with this, Sergiev *et al.* (2006) could show that application of phenylurea cytokinin alleviated symptoms of glyphosate toxicity in maize.

Expression of atypical symptoms of glyphosate phytotoxicity observed in the present study in wheat (Fig. 5.1) and soybean (Fig. 6.3, 8.1) can be potentially explained by altered translocation of glyphosate in plants in case of re-mobilisation of glyphosate in the rhizosphere. Allister *et al.* (2005) demonstrated different glyphosate distribution patterns within plants, depending on leaf or root exposure to glyphosate. In case of foliar application, 80 % of applied glyphosate was translocated to the shoot meristems and young leaves. By contrast, up to 75 % of glyphosate remained mainly in the young root tissues when the herbicide was supplied to the roots. This may also imply a different expression of plant damage symptoms. While foliar glyphosate application leads to direct expression of toxicity symptoms such as chlorosis and necrosis of young leaves within several days, more indirect symptoms can be expected after root exposure to glyphosate, mainly as a consequence of an impairment of root function, e.g. limited acquisition and translocation of water (Zobiole *et al.,* 2010b) and nutrients (Neumann *et al.*, 2006) or of hormonal signals such as cytokinins (Sergiev *et al.,* 2006).

Under field conditions, expression of atypical symptoms in case of glyphosate in the rhizosphere might lead to the misinterpretation of causes of damage. In fact, the attribution of symptoms of damage in GR soybean (e.g. deformation of primary and trifoliar leaves, cupping and coiling of emerging trifoliar leaves, dark green leaves, Fig. 6.3) to re-mobilisation of growth regulator herbicides like Clarity, Banvel or 2,4-D by glyphosate formulations from tanks, if tank cleanout had been inadequate (Taylor, 2002) could be an example for such a misinterpretation.

Potentially, long-lasting effects of glyphosate toxicity on hormonal status of plants are also responsible for the delay of ripening/senescence of crops observed in the present study in case of short waiting times between pre-crop application and sowing of winter wheat under field conditions (data not shown).
Depending on the environmental growth conditions, a delay of senescence/ ripening is not necessarily negative. But a delay in plant development and senescence might cause yield loss under field conditions under distinct circumstances.

10.3.3 Glyphosate-induced impairment of nutritional status of plants

An increasing number of publications have reported glyphosate-induced impaired micro- and/ or macronutrient (e.g. Mn, Zn, Fe and Ca) acquisition, uptake, translocation from roots to shoots, retranslocation within shoots or intracellular utilisation under experimental as well as field conditions in non-resistant as well as glyphosate-resistant plant species (Sprankle *et al.*, 1975c; Duke *et al.*, 1983; Subramaniam *et al.*, 1988; De Ruiter *et al.*, 1996; Neumann *et al.*, 2006; Gordon *et al.*, 2006; Eker *et al.*, 2006; Ozturk *et al.*, 2008; Cakmak *et al.*, 2009; Zobiole *et al.*, 2010a).

In line with this, in the present study, glyphosate-induced impaired acquisition of mineral nutrients was observed in model experiments with sunflower (Fig. 4.5, 4.6), wheat (Tab. 7.4) and GS soybean (Tab. 8.5, 8.6; Fig. 6.5) as well as GR soybean (Tab. 9.2; Fig. 6.4, 9.2).
Based on its chemical properties, glyphosate acts as potent chelator of di- and trivalent cations potentially causing negative effects on nutrient availability for crop plants. According to Eker *et al.* (2006), formation of poorly soluble glyphosate-metal complexes is possibly the main factor responsible for the antagonism between glyphosate and cationic micronutrients. Additionally, Ozturk *et al.* (2008) demonstrated a glyphosate-induced inhibition of iron reductase activity at the plasma membrane of root cells, limiting the iron acquisition of sunflower plants.

Recently, Cakmak *et al.* (2009) showed that glyphosate rates between 0.6 and 1.2 % of the recommended application rate for weed control affected not only micronutrients but also induced a declined concentrations of Ca and Mg in young leaves and seeds of conventional soybean plants. According to Cakmak *et al.* (2009), young leaves, shoot tips and seeds are highly sensitive to small changes in Ca concentrations due to their low transpiration capacity. Accumulation of glyphosate in such sink organs with low Ca concentration would induce physiological Ca deficiency by complexing Ca.

Thus, based on the well-documented ability of glyphosate to form stable complexes with metal cations such as Al, Fe, Zn, Mn and Ca (Sprankle *et al.,* 1975c) may induce internal micronutrient deficiencies, although total micronutrient leaf concentrations might be in the adequate range.

In the present study, in order to assess a possible physiological immobilisation of micronutrients in young leaves of glyphosate-treated plants by metal complexation with glyphosate (Sprankle *et al.,* 1975c), leaf tissue of glyphosate-treated GR soybean was

extracted with 80 % ethanol in order to separate the low molecular weight (LMW) soluble fraction containing potential metal complexes with glyphosate, from high molecular weight (HMW) compounds (Chapter 9). However, micronutrients in the ethanol-soluble LMW fraction of young leaves obtained from glyphosate-treated and non-treated control plants in soil culture were not significantly different (Tab. 9.2). This suggests that, at least in the experiments of this study (Chapter 9), 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 immobilisation in the root tissue by complexation with glyphosate, which may limit the translocation of micronutrients to the shoots still needs to be investigated.

In the present study, negative effects of glyphosate on the nutritional status of plants were consistently observed, but strongly varied between the different culture systems (hydroponics, soil culture, field trials) and different different soils (Tab. 7.4, 8.5, 8.6, 9.2; Fig. 4.5, 4.6, 6.4, 6.5, 9.2). Therefore, these results suggest a strong interrelationship with growth conditions and environmental factors.
Potentially, glyphosate-induced impaired Mn, Zn, Fe, Mg or Ca status of plants observed in various experiments under field-, soil- and hydroponic conditions can limit plant growth due to negative effects on photosynthesis, on plant own defence mechanisms against abiotic and biotic stresses as well as on hormonal metabolism (Marschner, 1995, Cakmak *et al.*, 1996). Based on its phytotoxic mode of action, glyphosate itself targets photosynthesis, plants own defence mechanisms and hormonal status (Zobiole *et al.*, 2010a, Kremer *et al.*, 2005, Sergiev *et al.*, 2006). Thus, the possibility of an additive detrimental effect of glyphosate and/or AMPA on plants due to phytotoxicity and impaired micronutrient bioavailability seems plausible.

In summary, findings of the present study suggest that the declined concentration of mineral nutrients in shoots of plants is rather the consequence than the cause of impaired plant growth induced by glyphosate application. In line with this argument, calculations of nutrient contents frequently revealed also significant negative effects on the K or P status of plants (data not shown), which are generally not considered to be complexing partners of glyphosate.

Thus, impaired nutritional status of plants is most likely an important but secondary effect of glyphosate and/or AMPA toxicity to GS- and GR-crop plants. Under field conditions, glyphosate-induced inhibition of root growth most likely will affect nutrient acquisition of all nutrients leading to potential deficiency of different nutrients depending on their plant availability on a specific field site and therefore leading to a limitation of plant growth. For example, in case of Mn, Zn and Fe as important mineral nutrients, negatively affected by glyphosate as the present study (Tab. 8.6; Fig. 4.5, 4.6, 6.4, 6.5), plant availability is particularly limited in calcareous soils.

10.3.4 Hormesis effects of glyphosate

Growth-stimulating effects of sub-lethal doses of glyphosate (hormesis) have been reported for different plant species even so the underlying mechanism(s) remain poorly understood (Wagner *et al.,* 2003; Cedergreen, 2008; Cedergreen *et al,.* 2009; Cedergreen and Olesen, 2010; Velini *et al.*, 2008).
In the present study, a growth stimulating effect of glyphosate in the rhizosphere was observed on a Regosol soil (Tab. 8.2, 8.3, 8.4). According to Velini *et al.* (2008), the hormesis effect of glyphosate is likely to be related to the molecular target of glyphosate, since the effect was not seen in glyphosate-resistant plants and shikimate levels were enhanced in plants with stimulated growth. In contrast to this, in the present study, only an increase in growth but no accumulation of shikimate was detectable (Tab. 8.2, 8.3, 8.4).

Very low/ non-toxic doses of glyphosate are most likely frequently present under field conditions. Thus, under certain conditions glyphosate hormesis might be a factor for better plant growth and higher yields. Results of field trials of Cedergreen *et al.* (2009) with barley (*Hordeum vulgare* L.) indicate increase in grain yield in case of exposure to simulated glyphosate drift shortly before grain filling. However, hormesis effects of glyphosate on plant growth are, according to Cedergreen *et al.* (2009), generally not sustained over time. Because of this, it is unlikely that glyphosate hormesis caused by pre-crop application of glyphosate before sowing of crops will have a substantial positive effect on yields.

10.4 Conclusions

As conclusion, results of the field trials particularly demonstrated the excellent weed control efficiency of glyphosate which is needed in agricultural crop production to achieve highest possible yields. However, results of the present study also highlighted potential risks for crops associated to the little investigated aspect of glyphosate in the rhizosphere e.g. in roots of glyphosate-treated weed plants as pool of phytotoxic glyphosate in soils.

According to the results of the study, these risks could be minimized by simple management tools such as the observation of waiting times between application of glyphosate and sowing of crops and the observation of good agricultural practice e.g. alternation of herbicides to prevent not only risk for re-mobilisation of glyphosate but also problems associated to glyphosate-resistant weeds.

According to the results of the present study, risks of crop plants associated to glyphosate toxicity in the rhizosphere is, in more than one way, strongly influenced by biotic and abiotic factors. The independency and interactions between these factors are so far not entirely clear and should be investigated in future studies to improve the understanding of the behaviour of glyphosate in the rhizosphere, the prevention of risks for crop plants and the application of the most extensively used herbicide. Therefore, in the next section, a short recommendation of future research steps is presented.

10.5 Outlooks

According to the results of the present study, several aspects of glyphosate in the rhizosphere should be better investigated to improve the understanding of factors potentially inducing and/or contributing to increased risks for crop damage and yield losses in farmers practice and to achieve a better and safer application under field conditions in the near future.

10.5.1 Evaluation of abiotic and biotic factors influencing the speed of death and decay of glyphosate treated weed residues

As mentioned above (section 10.1.1), release of glyphosate from treated weed roots and the time window of potential crop damage most likely depend on biotic and abiotic factors influencing the duration for glyphosate release by living roots (exudation) and release during degradation of root residues. Factors such as temperature and/or soil moisture might be evaluated in bioassays in model experiments with different waiting times between glyphosate application and sowing of crops on field soils with different soil moisture levels and using a cooling device to control root zone temperature. Soil moisture levels might be varied in terms of different moisture levels for growth of weed plants before application and/or for growth of crop plants after application of glyphosate to weed plants.

Similarly to abiotic conditions, biotic factors, such as microbial activity and degradability of glyphosate treated weed residues, might affect the time window for potential crop damage by glyphosate transfer from weeds to crops through the rhizosphere. These parameters might be also evaluated in bioassays with crop plants as indicator of glyphosate toxicity on different fresh field soils. Analog to the experiments carried out by Tesfamariam *et al.* (2009), glyphosate might be applied to different weed plant species pre-cultured under hydroponic conditions. Shoot or root tissue of pre-cultured weed plants might be incorporated after different waiting times before sowing of crops to the different soils. To observe speed of degradation of glyphosate-treated weed residues experiments might be carried out in rhizoboxes with root observation windows.

10.5.2 Evaluation of factors contributing to damage of crops induced by glufosinate (Basta®) application on weed plants observed under field conditions

Field trials investigating parameters influencing the risk of crop damage induced by short waiting time between pre-crop glyphosate application and sowing of plants, need appropriate control treatments. According to the experiences of the present study, this cannot be achieved without effective tools for weed control in controls application because of the weed pressure.

As the results of field trials of the present study indicate, not only short waiting time between pre-crop application of glyphosate but, in contrast to expectations, also application of glufosinate (Basta®), which was used as control treatment, caused significant damage of crops. Basta® is frequently considered to be a semi-systemic herbicide. However, there are also scientific reports indicating a translocation of Basta® to the roots of plants. Thus, the causes for damage in case of short waiting time between application of glufosinate (Basta®) and sowing of crops, remained unclear, but are potentially caused by a rhizosphere transfer of

Basta® to crop plants or by contact contamination of crops during emergence within Basta®-treated weed shoots.

Potentially, model experiments under soil conditions, with an experimental design analog to the model experiments of the present study, investigating transfer pathways of glyphosate from treated weed to crops plants might be conducted to evaluate the possibility of transfer of Basta® from leaf- or root tissue of weeds to crop plants.

10.5.3 Evaluation of allelopathic effects as source of damage of crop plants

Particularly under field conditions, damage of crops might have been induced by allelopathic effects of glyphosate-treated weed residues. To evaluate this aspect, weed plants might be pre-cultured under hydroponic conditions. Shoots and roots of weed plants treated and untreated with glyphosate or stressed might be harvested, frozen in liquid nitrogen and applied to the soil. Potential controls would include application of identical amounts of glyphosate to the soil and/or to weed plants cultivated under soil conditions in the same pots, soil without glyphosate application and mechanical weeding of weed plants cultivated in the pots.

10.5.4 Evaluation of interactions glyphosate and soil-borne pathogens

As mentioned above (section), the causal relationship of potential increase in infection of crops with soil-borne pathogens like *Fusarium* and short waiting time between glyphosate application on weeds and sowing of crops, are not entirely clear. During his Ph.D research, Yusran (2009) was able to establish conditions for successful investigation of the ability of plant growth promoting rhizobacteria (PGPRs) for suppression of soil-borne pathogen *Fusarium oxysporum* in tomato plants. Potentially, his growth conditions could be used to investigate effects of glyphosate soil application on the severity of damage of tomato caused by the pathogen as well as the effect of glyphosate on ability of PGPRs for suppression of *Fusarium oxysporum*.

Additionally, field soil infected with take all (*Gaeumannomyces graminis*) exists at one of the experimental field stations of the University Hohenheim. Model experiments with different waiting times between pre-crop application of glyphosate on “self-sown” winter wheat as weed plants and sowing of winter wheat as crop plant might be used to evaluate the effect of the green bridge as transfer pathway of pathogens and damage of subsequently grown crops.

If this system is established, application of glyphosate to the soil and/or the application of shoot and root residues of glyphosate treated weed plants might be used to evaluate whether potential of infection of plants is increased by promotion of pathogens by promotion of soil borne pathogens by glyphosate directly or not.

10.5.5 Evaluation of glyphosate re-mobilisation from the soil matrix and long-term effects of glyphosate

As the present study showed, re-mobilisation of glyphosate from the soil should be considered as a possible pathway for glyphosate toxicity to crop plants. Therefore, in line with the experiments of the present study, additional soils e.g. field soils from sites where damage was previously observed, should be evaluated for potential for re-mobilisation of glyphosate induced by application of P fertilisers.

Additionally, the potential for re-mobilisation of glyphosate by expression of root-induced mechanisms for phosphorus or iron mobilisation in the rhizosphere, reported for various plant species and cultivars (Neumann and Römheld, 2002) might be conducted on different soils according to the experimental design of the present study (Chapter 8). Plant strategies of P-/ Fe mobilisation potentially can be simulated by application of synthetic root exudates or by cultivation of P-/Fe-efficient plants under P-/Fe-limiting soil conditions. Additionally, in similar experiments, it could be possible to evaluate the potential for re-mobilisation by acidification of the rhizosphere by application of NH_4^+ fertilisers.

As observations of damage under field conditions near Tübingen (South Germany) have indicated, long-term application of glyphosate might induce significant damage. However, the underlying mechanisms and their relationship to potential re-mobilisation of glyphosate are not clear.
As a first step, glyphosate-concentrations in soil solution of damaged an undamaged field sites might give indications whether glyphosate could be extracted from the soils in a damaging amount. Secondly, germination- and/or bio-assays might be performed with exposure of plants to soil solution extracted for soil from sites showing substantial damage of crops.

10.5.6 Evaluation of differences in sensitivity of crop plant species to glyphosate toxicity and factors contributing to enhanced recovery

Differences in sensitivity of crops to glyphosate toxicity in the rhizosphere and their ability for recovery after exposure phytotoxic glyphosate can have potentially important implications for agricultural crop production. Therefore, in continuation of the approach of the present study, it seems worthwhile to evaluate systematically the sensitivity of important crop plants to phytotoxic glyphosate in the rhizosphere.

Furthermore, in a second step, experiments could be conducted to evaluate factors contributing to higher ability of plant recovery. Since hormonal imbalances are potentially the physiological most long-lasting negative effects of glyphosate toxicity, it seems interesting to evaluate the possibility whether application of synthetic phytohormons can mitigate damage and symptoms of plant damage. Similarly, leaf application of specific mineral nutrients like Mn or Zn might enhance the ability of plants to recover.

In line with this, long-term pot experiments under soil conditions might offer possibilities for studying potential management strategies for enhanced recovery of crop plants after exposure

to glyphosate. For instance, as field trials and model experiments indicated, short waiting time between pre-crop glyphosate application and sowing of crops, frequently causes delayed development and decline in crop plant densities. Since it is known that nitrogen can have a profound effect on tillering and plant development, both aspects might by manageable by timing and form of applied N fertilisation. Loss of plants caused by glyphosate toxicity might be partly or completely compensated by enhanced tillering and better development of remaining plants. Interestingly, the form of N fertilisation most likely has also an effect on the hormonal status of plants and might be a useful tool to alleviate negative effects of glyphosate on the hormonal status of plants.

10.5.7 Evaluation of factors contributing to damage of crops induced by glyphosate application on weed plants under field conditions

Results of field trials of the present study gave clear indications for increased damage and yield losses in case of short waiting time between pre-crop application of glyphosate and sowing of winter wheat. However, many questions related to the exact causes, interrelationship between several potential causes, frequency/ likelihood of damage and the relationship to factors like site-specific abiotic and biotic conditions, tillage practice and the timing of application (e.g. fall vs. spring cropping), remain not properly understood. Beside this, the practical relevance of several factors identified in the present study (for instance, difference in sensitivity of plant species to phytotoxic glyphosate in the rhizosphere) is not clear so far. Therefore, for evaluation of these parameters, additional multi-seasonal field trials under real farming conditions are needed.

As mentioned earlier, for a successful evaluation of risk factors associated to glyphosate transfer from treated weed root residues to crop plants, appropriate controls are a necessary prerequisite. As the present study showed, both application of alternative herbicides like Basta® as well as plots without weed control proved to be problematic as control treatments. Therefore alternative solutions need to be considered.
Control treatments with thermal weed control, mechanical weed control or coverage of control plots with plastic foil might be an option. However, there is a potential that these control treatments have profound effects on soil microorganisms and/or mineralisation of nutrients in the soil, which potentially limits the comparability of treatments. Depending on the plot size, weeding by hand is most likely too laborious and time consuming. Application of alternative non-systemic herbicides might be another possibility. However as results of the Basta®-control treatments indicate, potentially unexpected negative effects might occur.

Potentially, better control treatments might not only include one or several of the possibilities descripted above, but in addition a positive control in which most likely glyphosate damage of crops can be observed. This positive control might be achieved by glyphosate application before crop emergence but after starting of germinating of crop seeds, which is because of risks for glyphosate-induced crop damage not allowed in agricultural practice.

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08/2009: S. Bott, U. Lebender, D.-J. Yoon, T. Tesfamariam, Volker Römheld, Günter Neumann (2010) Evidence for glyphosate damage of winter wheat depending on waiting-times after pre-crop glyphosate application and density of desiccated weed plants under field and experimental conditions *Proceedings of the XVI. International Plant Nutrition Colloquium*, University of Califonia (Davis) (http://www.escholarship.org/uc/item/25v599pr?display=all#page-2)

02/2009: T. Tesfamariam, S. Bott, I. Cakmak, V. Römheld, G. Neumann „Glyphosate in the rhizosphere – Role of waiting times and different glyphosate binding forms in soils for phytotoxicity to non-target plants", European Journal of Agronomy. 31:126–132

08/2008: S. Bott, T. Tesfamariam, H. Candan, I. Cakmak, V. Römheld, G. Neumann „Glyphosate-induced impairment of plant growth and micronutritional status of glyphosate-resistent soybean (Glycine max L.)", Plant and Soil 312:185-194

PUBLICATIONS (not peer-reviewed):

05/2010: S. Bott, T. Tesfamariam, G. Neumann, V. Römheld (2010) Glyphosatherbizide in der Rhizosphäre – Risiken bei der Vorssatbehandlung? In Steuerungsfaktoren von Rhizosphärenprozessen. 19. Borkheider Seminar zur Ökophyiologie des Wurzelraumes. Verlag Grauer, Stuttgart

08/2008: V. Römheld, S. Bott, T. Tesfamariam, K. Weiss, G. Neumann"Fehler mit Totalherbiziden vermeiden" DLZ-Magazin, Ausgabe 09/2008, S. 44-47

PARTICIPATIONS TO CONGRESSES

09/2009: Poster: Bott, U. Lebender, D.-J. Yoon, T. Tesfamariam, Volker Römheld, Günter Neumann (2010) Evidence for glyphosate damage of winter wheat depending on waiting-times after pre-crop glyphosate application and density of desiccated weed plants under field and experimental conditions *Proceedings of the XVI. International Plant Nutrition Colloquium*, University of Califonia (Davis)

09/2008: Presentation: S. Bott, T. Tesfamariam, V. Römheld, G. Neumann „Glyphosatherbizide in der Rhizosphäre – Risiken in der Vorssaatbehandlung", im Rahmen der "Wissenschaftlichen Arbeitstagung zur Ökophysiologie des Wurzelraumes", 24. September 2008, Speyer, Deutschland

09/2008: Poster: S. Bott, T. Tesfamariam, V. Römheld, G. Neumann „Glyphosatherbizide in der Rhizosphäre – Risiken in der Vorssaatbehandlung", Jahrestagung der „Deutschen Gesellschaft für Pflanzenernährung" (DGP), 23.-24. September 2008, Speyer, Deutschland

08/2007: Poster: S. Bott, T. Tesfamariam, H. Candan, I. Cakmak, V. Römheld, G. Neumann „Glyphosate-induced impairment of plant growth and micronutritional status of glyphosate-resistent soybean (Glycine max L.)". 26.-31. August 2007, "Rhizosphere 2", Montpellier, Frankreich

08/2006: Poster: S. Bott, T. Tesfamariam, V. Römheld, G. Neumann „Effects of different waiting times after pre-crop glyphosate application on non-target plants". "Plant Nutrition meets Plant Breeding – 1st Joint Conference of the German Society for Plant Nutrition (DGP) and the Research Centre Biotechnology and Plant Breeding"in Stuttgart-Hohenheim

SUPERVISING EXPERIENCES

03/2010: Supervision of undergraduate students on „Diagnostic of nutritional disorder in plants"

07-08/2009: Supervision of three students in the program I.A.E.S.T.E. (International Association for the Exchange of Students for Technical Experiences)

10/2008-06/2009: Supervision of two Master students

07-08/2008: Supervision of two students in the program I.A.E.S.T.E. (International Association for the Exchange of Students for Technical Experiences)

04-10/2007: Supervision of a Master student

04-07/2006: Supervision of Bachelor- and Master- students on „Ecological execices in plant nutrition"

OTHER EXPERIENCES

06/2006: Participation to IP-Socrates Euroleague for Life Science Summer University (University for Soil Science, Vienna, Austria)

09/2004-02/2005: Visit at China Agricultural University (CAU) for Master (Beijing, V.R. China)

LANGUAGES AND COMPUTER SCIENCE

Computer science:
Microsoft Office softwares: excellent
Adobe softwares: very good
Microsoft software WinRhizo Pro: very good
Statistics software SigmaStat: very good

Languages:
German: mother tongue
English: fluent
8-week school exchange in Melbourne (Australia)