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GLYPHOSATE USE IN AGRO-ECOSYSTEMS: Identification of key factors for a better risk assessment.

Dissertation
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AMPA	aminomethylphosphonic acid
BS	Bare soil
CA	Citric acid
cv	Cultivar
d	day
DAA	Days after application
DAS	Days after sowing
DMPP	3,4-Dimethylpyrazole phosphate
EPSPS	5-enolpyruvylshikimate-3-phosphate synthase
GR	Glyphosate Resistant
HPLC	High Pressures Liquid Chromatography
hr	Hour
IAA	Indole-3-acetic Acid
MW	Mechanical weeding
mM	Mill mole
µM	Micromolar
MPN	Most Probable Number
NC	Sodium citrate
n.d.	Not determined
POEA	Polyoxyethaline-alkylamine
SPAD	Soil Plant Analyses Development
YFEL	Youngest fully expanded leaves

1.0. GENERAL INTRODUCTION

Glyphosate (N-(phosphonomethyl) glycine) is a broad-spectrum, non-selective, post-emergence systemic herbicide, applied through the leaves to desiccate all annual and perennial weed species. It can effectively control 76 of the world´s 78 worst weeds (Franz, 1985). It is the world´s biggest-selling chemical used for weed control in agricultural, silvicultural and urban environments (Baylis, 2000). It is sold in different formulation but regardless of the product, the active ingredient that actually kills plants, glyphosate, is the same. Every glyphosate product is composed of three parts: the parent acid (N-(Phosphonomethyl) glycine): the active ingredient that kills plants), salt (such as isopropylamine, trimethylsulfonium etc.: to stabilize the product, make it easier to handle, and allow it to mix well with other products), and proprietary (e.g. Polyoxyethaline-alkylamine (POEA): to enhance foliar penetration of glyphosate and make the product more convenient to handle). Glyphosate exhibits many unique biological properties. The rapid translocation of glyphosate from the foliage of treated plants to the roots, rhizomes and apical meristems is one of its most important characteristics. This systemic property results in the total destruction of hard-to kill perennial weeds and accelerated the large-scale adoption of glyphosate as total herbicide all over the world. Glyphosate-resistant crops (GR) were created by stable integration of a transgene that codes a glyphosate insensitive EPSPS (Padgette *et al*., 1996). Expression of the GR EPSPS helps to maintain normal aromatic amino acid levels in GR crops treated with glyphosate. GR crops are grown in several countries, and their rapid adoption has led to a large increase in the use of glyphosate. The rapid increase in glyphosate use also initiated alarming interest in scientific research regarding its behavior and potential side effects.

1.2. Glyphosate discovery and development

Glyphosate was first discovered to have herbicidal activity in 1970 by John Franz, while working for Monsanto (Baird *et al*., 1971). The compound was found during a study of the herbicidal effects of more than 100 tertiary aminomethylphosphonic acids derived from various primary and secondary amines (Moedritzer and Irani, 1966). Only two of these compounds, known as compound 4 and 5, prepared from iminodiacetic acid and

glycine, respectively, showed any herbicidal activity, but both had very low unit activities. However, the plant growth regulatory properties of the compounds led to the introduction of one of them, glyphosine, as a sugar cane ripening agent (Polaris ®, Monsanto Co.). Attempts to find other tertiary aminomethylphosphonic acids with improved herbicidal activity failed. As a last resort, it was suggested that degradation of the two compounds might give rise to a common, active metabolite (contrary to the general trend that metabolism reduces toxicity). Glyphosate was among the possible metabolites of the two compounds, and was found to have extremely high herbicidal activity (Franz, 1985).

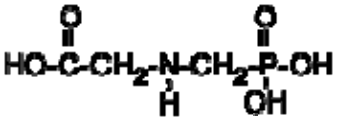
The original Roundup® herbicide, containing the active ingredient glyphosate, was first introduced to the market by Monsanto in 1974 (Monsanto backgrounder, 2005). Today, Roundup®WeatherMax, Roundup®UltraMax, and other glyphosate agricultural herbicides produced by Monsanto are among the world´s most widely used herbicides. Monsanto´s glyphosate products are registered in more than 130 countries and are approved for weed control for more than 100 crop species (Monsanto backgrounder, 2005).

Chronologically, the market growth of glyphosate can be characterized into four stages. Initially, it was sold for control of perennials. In the second stage, price elasticity was determined and it was introduced into residential and other non-farm applications. The thirds stage was the growth of conservation tillage and use in pre-harvest application to aid in dry down for easier harvest. The final stage was the introduction of ROUNDUP READY® crops (Magin, 2002).

1.3. Biochemistry of glyphosate

Glyphosate is an aminophosphonic analogue of the natural amino acid glycine, the name is a construct of glycine, phosphor- and *-ate*. It´s chemical formula is “N-(phosphonomethyl) glycine“. Glyphosate or N-(phosphonomethyl)glycine has an empirical formula of $C_3H_8NO_5P$ and is a white crystalline solid which exists as a zwitterionic species *1a* in the solid state (Knuuttila and Knuuttila, 1979). Pure glyphosate has relatively low solubility in water (1.2 – 8% at 25-100°C) and is insoluble

in other organic solvents apparently due to strong intermolecular hydrogen bonds stabilizing the crystal lattice but various salts of glyphosate have much higher solubilities and do not lose any of the herbicidal properties of the parent compound (Franz, 1985).



glyphosate
N-(phosphonomethyl)glycine

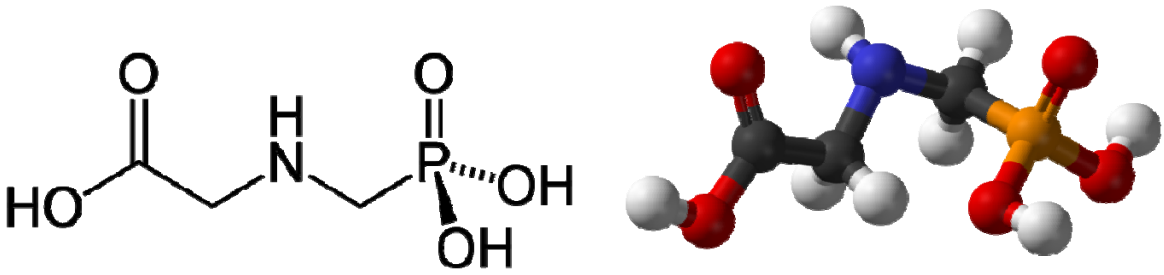


Fig. 1. 1. Molecular structure of glyphosate.

1.4. Herbicidal mode of action

As a broad-spectrum and systemic post-emergence herbicide which is applied through the leaf, glyphosate is phloem mobile and is readily translocated throughout the plant (Franz *et al*., 1997). From the leaf surface, glyphosate molecules are absorbed into the plant cells where they are translocated to meristematic tissues (Laerke, 1995). Glyphosate´s primary site of action is the inhibition of the enzyme 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS), a chloroplast localized enzyme in the shikimic acid pathway of plants (Della-Cioppa *et al*., 1986). EPSPS catalyzes the reaction of shikimate-3-phosphate (S3P) and phosphoenolpyruvate to form 5-enolpyruvyl-shikimate-3-phosphate (ESP). ESP is subsequently dephosphorylated to chrosmate, an essential precursor in plants for the synthesis of aromatic amino acids, such as phenylalanine, tyrosine and tryptophan (Fig. 1.2). Inhibition of EPSPS by glyphosate has been shown to proceed through the formation of an EPSPS-S3P-

glyphosate ternary complex and the binding is ordered with glyphosate binding to the enzyme only after the formation of a binary EPSPS-S3P complex. Binding of glyphosate to EPSPS has been shown to be competitive with PEP and uncompetitive with respect to S3P (Kishore, 1988).

Therefore, glyphosate prevents the production of chorismate, thereby biosynthesis of essential aromatic amino acids which are used by plants in protein synthesis and to produce many secondary plant products such as growth promoters, growth inhibitors and lignin (Franz et al., 1997). Besides inhibiting aromatic amino acid biosynthesis in sensitive plants, the interaction between glyphosate and EPSPS interferes with the production of secondary compounds derived from aromatic amino acids. The biosynthesis of proteins, auxins, pathogen defence compounds, phytoalexins, folic acid, precursors of lignins, flavonoids, plastoquinone and hundreds of other phenolic and alkaloid compounds may all be affected by EPSPS inhibition (Bentley 1990). Injury symptoms are often slow in developing from treatment with glyphosate. The symptoms are thought to occur primarily as a result of starvation of the plant for amino acids, proteins and secondary plant products derived from chorismate and a deregulation of the shikimate pathway (Pline-Srnic, 2005).

Although EPSPS is the only known enzyme target of glyphosate, it affects many physicochemical and physiological processes (Cole, 1985). Among these are reductions in photosynthesis and degradation of chlorophyll; inhibition of auxin transport and enhancement of auxin oxidation. These aspects of the mode of action of glyphosate are either a direct consequence of the blockage of the shikimate pathway (through which some 30% of assimilated carbon is estimated to pass) or a result of feedback mechanisms (Singh, 1991).

Unlike many contact herbicides, phytotoxic symptoms of glyphosate injury often develop slowly. Chlorosis is followed by necrosis, and eventual plant death can take two weeks and even longer, particularly at low temperature conditions. Visible effects on most annual weeds occur within two to four days and may not occur for 7 days or more on most perennial weeds. Extremely cool or cloudy weather following treatment may slow

activity of glyphosate and delay development of visual symptoms. The chloroplasts of the young apical leaves begin to swell between 16 and 20 hrs after treatment of the plants with a sublethal glyphosate dose and burst after 4 days (Mollenhauer *et al*., 1987).

Visually visible symptoms of glyphosate damage vary between different plant species and include: chlorosis and distortion to basal leaves, stunting, leaf death, complete shoot death, production of deformed leaves and blossoms with reduced in size and delayed in opening, deformed shoot with typically elongated leaves and up-rolled margins and in woody plants causes wound in bark (Atkinson, 1985). The death cycle of glyphosate treated plant normally starts with a gradual wilting and yellowing of the plant which advances to complete browning of above-ground growth and deterioration of underground plant parts.

1.4.1. The shikimic acid pathway

The shikimic acid pathway participates in the biosynthesis of most plant phenolics. The shikimic acid pathway converts simple carbohydrate precursors derived from glycolysis and the pentose phosphate pathway to the aromatic amino acids (Herrmann and Weaver, 1999). One of the pathway intermediates is shikimic acid, which has given its name to this whole sequence of reactions. Hence, as systemic herbicide, glyphosate kills plants by blocking a step in this pathway (Fig.1.2). Due to EPSPS inhibition by glyphosate, shikimic acid and shikimate 3-phosphate levels increase rapidly in sensitive plants (Holländer-Czytko and Amrhein,1983; Lydon and Duke, 1988; Mollenhauer *et al*., 1987). On the other hand, synthesis of the shikimic acid pathway end products, such as phenylalanine, tyrosine and tryptophan is restricted, thereby the content of their processor compounds (phenolic compounds eg. lignin; glycocide and phytohormones eg. IAA) is reduced in plants which leads to plant death (Fig. 1.2).

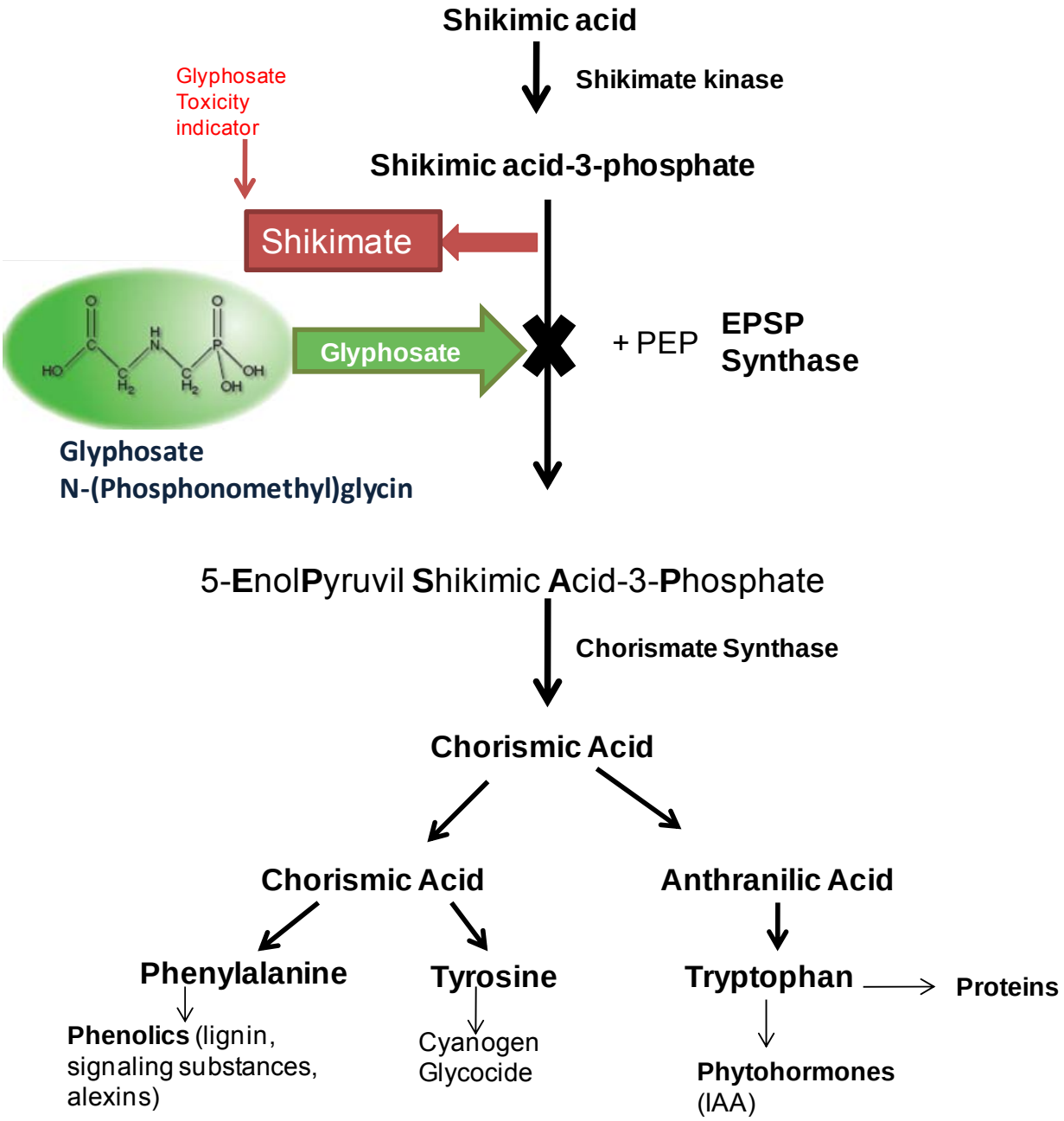


Fig. 1. 2. Schematic presentation of shikimic acid pathway and the inhibition by glyphosate (adapted from Dill, 2005 with minor modification).

1.5. Glyphosate metabolism in plants

Glyphosate is degraded relatively fast in soils by microbial process (Franz *et al*., 1997; Laitinen *et al*., 2006). The most frequently detected degradation product is aminomethylphosphonic acid (AMPA). In most plant species, glyphosate is not readily

metabolized and is preferentially translocated to young growing tissues of roots and shoots, where it can accumulate in millimolar concentrations (Reddy *et al*., 2004). Little is know about the enzyme(s) involved in the degradation of glyphosate to AMPA in plants, though it has been detected on some plant species such as RR and non-RR soybeans (*Glycine max*), cowpea (*Vigna unguiculata*), sicklepod (*Senna obtusifolia*), coffee senna (*Cassia occidentalis*), Illinois bundleflower (*Desmanthus illinoensis*), kudzu (*Pueraria lobata)*, and horseweed (*Conyza Canadensis*) (Reddy *et al*., 2008). AMPA have been also detected as major metabolite in seeds of canola, wheat, field pea, barley, flax and RR soybean treated with glyphosate (Cessna *et al*., 1994; 2000; 2002; Duke, 2003). Similar to microorganisms, in plants, two metabolic pathways have been considered through which glyphosate can be metabolized to AMPA i.e., one that involves the oxidative cleavage of the C-N bond to yield AMPA and the other one as breaking of C-P bond by a C-P lyase to generate sarcosine (Franz *et al*., 1997).

1.6. Dissipation mechanisms in soil

Glyphosate is moderately persistent in soil, with reported half-lives ranging from 1 to 174 days (Wauchope *et al*., 1992). Glyphosate is metabolized in soils and to a minor extent in plants to AMPA, which is also a moderately persistent metabolite (Roy *et al*., 1989). The degradation of glyphosate in soil is primarily by microbial metabolism (Rueppel *et al*., 1977; Torstensson, 1985). Characterization of the sorption properties of a substance may provide valuable information about its mobility. Generally, a high adsorption tendency of a substance on the soil matrix is related to a reduced mobility. Two steady rates of glyphosate degradation have been identified i.e. more rapid rate of degradation that represents the metabolism of the unbound glyphosate molecules, while the slower rate represents the metabolism of glyphosate molecules bound to soil particles (Nomura and Hilton, 1977; Rueppel *et al*., 1977).

Glyphosate is believed to be fixed on clay minerals, soil oxides and hydroxides and soil organic matter. Sprankle *et al*. (1975b) found a stronger adsorption of glyphosate on a clay loam soil than a sandy soil, suggesting that clay minerals were responsible for adsorption. The addition of cations such as Ca^{2+}, Mn^{2+}, Zn^{2+}, Mg^{2+}, Fe^{3+} or Al^{3+} to

bentonite clay increased the adsorption of glyphosate. Miles and Moye (1988) indicated that the main mechanism of glyphosate sorption is caused by H-bonding and ion-exchange mechanisms in the case of cation saturated clays.

Nomura and Hilton (1977) found that adsorption of glyphosate varied inversely with pH and directly with organic matter. It also has been shown that glyphosate interacted strongly with iron–humic acid complexes (Piccolo *et al*., 1995). These interactions were stronger than the adsorption observed solely on the humic acid. This suggests that the presence of organic–mineral complexes may explain the adsorption of glyphosate on soil organic matter.

Glyphosate sorption study of five Hawaiian volcanic soils cropped with sugarcane indicated a parallel between inorganic phosphate fixation and glyphosate sorption in these soils, whereby glyphosate fixation was determined by the presence of oxides and hydroxides and clays, probably associated with the phosphonic acid moiety of glyphosate (Nomura and Hilton, 1977). Experiment done with four typical European soils by Piccolo *et al*. (1994) demonstrated that glyphosate mainly interacted with the iron and aluminium hydroxides. They concluded that glyphosate sorption is far from being permanent and leaching to lower soil horizons may occur under certain conditions. Other studies also indicate that the interaction of glyphosate with soils is mainly governed by amorphous iron and aluminium oxides and organic matter (Morillo *et al*., 1999). De Jonge *et al*. (2001) quantified the variation in glyphosate adsorption and desorption in a sandy and sandy loam soil with varying phosphorus content and pH. They found that increased phosphate concentrations led to a decrease in the extended Freundlich adsorption coefficient for glyphosate. Liming of the coarse sandy soil resulted in a stronger adsorption due to an increase in reactive amorphous aluminium and iron hydrous oxides. They concluded that competition of glyphosate with phosphate for adsorption sites may lead to a higher mobility of glyphosate.

Generally this higher sorption behavior of glyphosate leads to a slower degradation rate of the molecule in soils with higher adsorption capacity. Microbial degradation rate is also affected by the particular microbial community of each soil (Carlisle and Trevors,

1999; Malik *et al*., 1989). While chemical decomposition and photolysis play a minor role in glyphosate degradation (Torstensson, 1985; Mallat and Baeceló, 1998). Adsorption of glyphosate being a reversible process, glyphosate adsorbed on soil was also reported to have a residual activity towards some plant species (Salazar and Appleby, 1982).

1.7. Interaction of glyphosate with other soil minerals

Glyphosate has a strong tendency to be sorbed on minerals by bonding with surface metals through its metal-coordinating functional groups. However, this same chemical process can potentially mobilize sorbed trace metals by chelation and sorbed anions such as phosphate by displacement. The application of a commercial RoundUp spray solution to long-contaminated soils containing elevated concentrations of heavy metals and phosphate resulted in a significant increase in leaching of Cu, Zn, Al, Ni, P, Si, and As (Barrett and McBride, 2006). An additional factor that potentially increases the stability of glyphosate in soils is the formation of stable complexes with Al^{3+}, Fe^{3+}, Ca^{2+}, Mg^{2+} and Cu^{2+} (Subramaniam and Hoggard, 1988; McBride and Kung, 1989; McBride, 1991). Saturating clay minerals with various cations increases glyphosate adsorption, in particular with divalent and trivalent cations. Thus, Glass (1987) reported that the adsorption of glyphosate by a cation-saturated montmorillonite increased in the order: $Na^{+}<Ca^{2+}<Mg^{2+}<Cu^{2+}<Fe^{3+}$. Such formation of stable metal-glyphosate complexes in soils will reduce the potential of a microbial degradation of glyphosate with implications for glyphosate dissipation.

On the basis of the chemical structure, with an active phosphonate group at the end of the molecule, glyphosate can form an inner-sphere complex with Al and Fe oxides in a soil similar to phosphate. This similarity implies that glyphosate and phosphate compete for the same sorption sites in soils. Hence many research findings confirm this phenomenon of a decreased glyphosate adsorption at a higher phosphate status of a soil (Sprankle *et al*., 1975a; de Jongle *et al*., 2001; Laitinen *et al*., 2008). Furthermore, adsorption of glyphosate and phosphate by goethite clearly demonstrated such competition with phosphate sorption preference. In such case, presorption of phosphate

eliminated glyphosate sorption and presorbed glyphosate was mobilized by phosphate addition (Gimsing and Borggaard, 2001).

1.8. Implications of glyphosate discovery

The direct consequence of glyphosate discovery as herbicide and its unique behavior was the conception of genetically modified plants to be tolerant to glyphosate and its easy use in agriculture. Perhaps the most important aspect of the success of glyphosate was the introduction of transgenic, glyphosate-resistant crops in 1996. Almost 90% of all transgenic crops grown worldwide are glyphosate resistant and the adoption of these crops is increasing at a steady pace (Duke and Powles, 2008).

The largest land area of glyphosate-resistant crops are occupied by soybean (54.2 million ha), maize (13.2 million ha), cotton (5.1 million ha), canola (2.3 million ha) and alfalfa (0.1 million ha). Currently, the USA, Argentina, Brazil and Canada have the largest plantings of these crops (Dill *et al*., 2008).

Adoption of glyphosate-resistant soybeans has been rapid in the USA, Argentina and Brazil. Almost 90% of the acreage in the USA (ca. 30 million ha) planted with such varieties in 2004 (Antonio *et al*., 2006). In Argentina, the adoption of glyphosate-resistant soybean was even more rapid than in the USA, reaching almost 90% (ca. 14 million ha) within 4 years after introduction (Panna and Lema, 2003).

Furthermore, increased adoption of glyphosate-resistant crops resulted in an increased use of glyphosate. In turn this resulted in a reduction of other herbicides including the ACCase inhibitors, ALS inhibitors, and Protox inhibitors (Shaner, 2000). This concentration of herbicide use to glyphosate will lead towards a shift in weed communities. The glyphosate-based weed management tactics used in glyphosate-resistant crops imposes the selection pressure that supports such a weed population shifts. Examples of weed population shifts in glyphosate-resisitant crops include common waterhemp (*Amaranthus tuberculatus* (Moq ex DC) JD Sauer), horseweed (*Conyza canadensis*), giant ragweed (*Ambrosia trifida*) and other relatively new weed problems (Owen, 2008).

1.8.1. Strategy for obtaining glyphosate resistant crops

Parallel to the fast adoption of glyphosate as systemic herbicide, extraordinary effort has been done to produce resistant crops to facilitate the use of glyphosate. Many ways of basic strategies have been evaluated in order to introduce glyphosate resistance into crops: (i) impaired uptake of the herbicide, (ii) over-expression of the sensitive target enzyme, (iii) detoxification of the glyphosate molecule within the plant and (iv) expression of an insensitive form of the target enzyme (Dill, 2005; Cogginns, 1989). However, only the last approach has been successfully utilized to develop commercially glyphosate-resistant crops (Fig. 1.3). The first mechanism, glyphosate-resistant forms of the EPSPS enzyme, is currently used in all commercial GR crops. Two forms of glyphosate-resistant EPSPS enzymes have been commercialized to date. The GA21 event in transgenic glyphosate-resistant maize contains a resistant form of the maize EPSPS enzyme with two mutations conferring resistance, T102I and P106S (Dams *et al*., 1995; Lebrun *et al*., 1997 and 2003). The CP4-EPSPS gene isolated from *Agrobacterium* spp shares low homology with native plant EPSPS, but plants containing this enzyme exhibit high level of glyphosate resistance (Barry *et al*., 1992). The CP4-EPSPS enzyme is in all currently commercialized GR soybean, cotton, sugar beet and some maize cultivars.

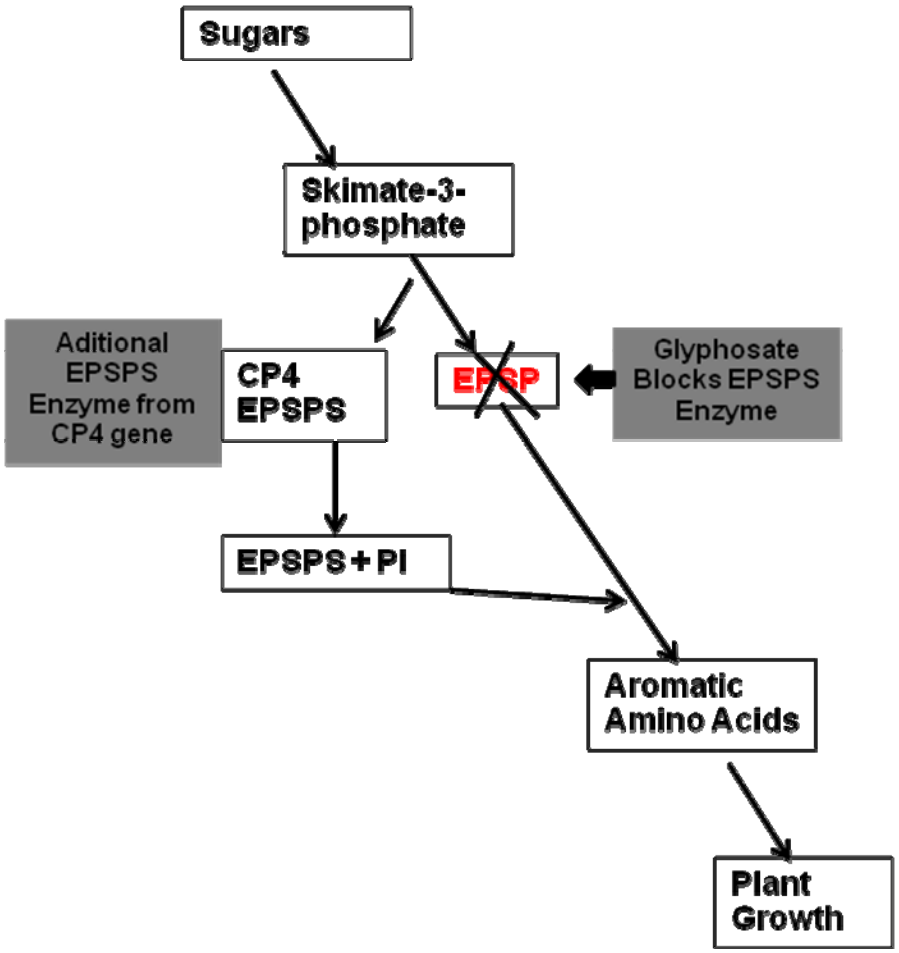


Fig.1. 3: Strategy for the development of glyphosate resistant crops (adapted from Dill, 2005).

1.9. Glyphosate intoxication of non-target organisms

Accidental damage by glyphosate can occur either by drift contamination during weed control operation within a crop or by drift from one field to another. Glyphosate molecules conserved in weed residues treated by glyphosate are also potential reservoir pools for intoxication of subsequent crops. Glyphosate contamination to non-target organism carried by run-off water from treated fields is also very often discussed side effect concern. Stachowski-Haberkorn *et al*. (2008) reported marine microbial community disturbance under field condition exposed to 1 µg L^{-1} RoundUp concentration, a value typical of those reported in coastal waters during a run-off event.

1.9.1. Routes of drift contamination to non-target organism

In general, movement of a pesticide through unwanted drift is unavoidable and drift contamination to non-target organisms by glyphosate can not be exceptional. Due to its easy mobility in plants, glyphosate drift, however, is particularly more significant because drift damage is likely to be much more extensive and more persistent than with many other herbicides (Atkinson, 1985). Lange *et al*. (1975) found that glyphosate drift caused fewer immediate symptoms but more eventual plant damage than other translocated herbicides in peach, plum and seedless grapevine. Damage to perennial plants when not exposed to enough glyphosate to kill them is persistent, with some symptoms lasting several years (Atkinson, 1985).

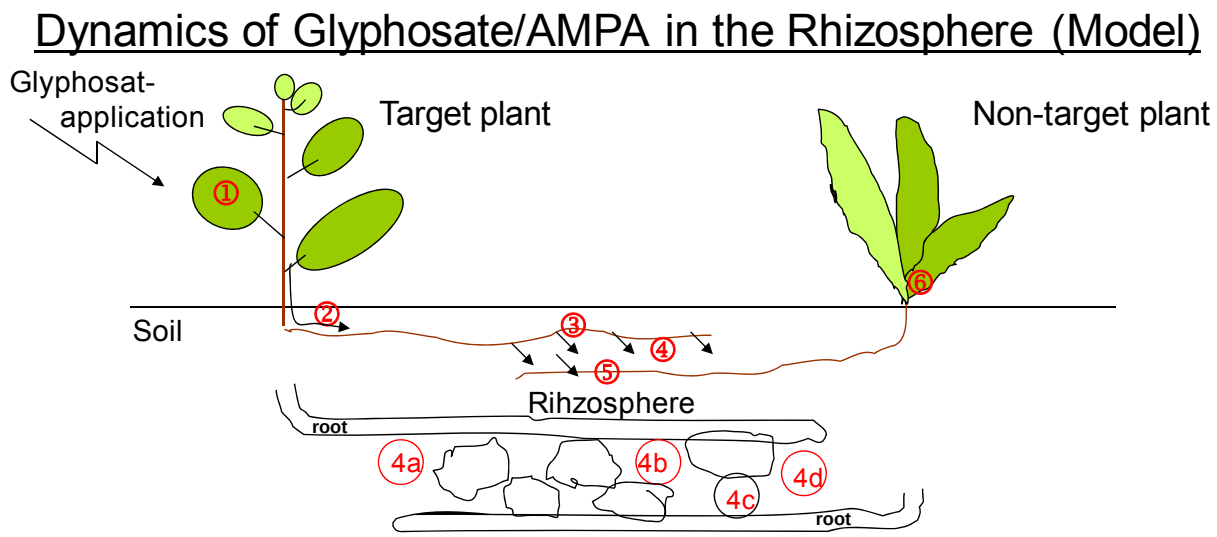
Extent of glyphosate drift contamination to neighboring non-target plants is a notorious variable that strongly depends on the method, rate and timing of glyphosate application (Atkinson, 1985) and external weather condition. Most commonly used spraying methods in glyphosate application include: hand spraying, ground application using tractor-mounted sprayer, helicopter application and fixed-wing aircraft application. Hence, the spraying method may play an important role in determining the buffer zone to protect neighboring field from drift contamination. A model to predict spray drift from evaporating herbicide droplet was developed by Thompson & Ley (1982) and based on this model it was reported that some native species would be damaged at a distance of 80 meters (Breeze *et al*., 1992). Glyphosate's manufacturer reported that drift from a ground application in Minnesota damaged 25 acres of corn (Monsanto Co., 1992), and the Washington Department of Agriculture reported damage to 30 acres of onions from a ground application of a glyphosate herbicide (Washington State Dept. of Health, 1993). Studies on forest sites conducted by Agriculture Canada (The Canadian Agricultural Ministry) calculated that buffer zones of between 75 and 1200 meters would be required to protect non-target vegetation (Payne, 1992).

1.9.2. Rhizosphere transfer of glyphosate from target to non-target organisms

Glyphosate is a systemic herbicide that is first absorbed by foliage and translocated throughout the plant via the phloem and further transported to metabolic sinks such as

meristems and roots. Laitinen *et al*. (2007) reported a significant contribution of glyphosate translocation from plant roots to the rhizosphere soil as glyphosate soil residue, which can be a potential reserve for subsequent crop intoxication. Results from a pot experiment undergone to study glyphosate release by roots of *Brachiaria decumbens* and its effects on eucalypt plants co-cultivated in the same pot have shown radicular release of glyphosate by *B. decumbens* and its absorption by eucalypt plants through roots (Tuffi Santos *et al*., 2008). Similarly, importance of rhizosphere glyphosate transfer from target to non-target plants in hydroponic and soil culture systems with detrimental effect on nutrient acquisition of co-cultivated crop plants have been demonstrated by Neumann *et al*. (2006). These reports demonstrate the release of glyphosate from treated plant roots and uptake by co-cultivated untreated intermingled neighbor roots.

Considering the general understanding that glyphosate as readily bound to soil matrix and therefore immobilized in most soils, there is a phenomenon of remobilization by chemical changes in the rhizosphere that needs more consideration. As expected, recent experimental research with four European soils found that glyphosate bound readily to the four soils studied could be also readily remobilized. In one of the soils studied, 80 percent of the added glyphosate was desorbed in a two hour period. The study concluded that glyphosate adsorption in soils is far from being permanent (Piccolo *et al*., 1994).



Where:
- ① foliar uptake of glyphosate
- ② transfer of glyphosate into apical root zones
- ③ release of glyphosate and possible metabolites (AMPA) into the rhizosphere of target plants
- ④ glyphosate dynamics in the rhizosphere
- ⑤ uptake of glyphosate by non-target plants
- ⑥ translocation of glyphosate/AMPA into the shoot of non-target plants and disorders

④ glyphosate/ AMPA dynamics in the rhizosphere
- a) extent of interactions between root system of target and non-target plants (intermingled roots)
- b) glyphosate immobilization in the rhizosphere
- c) glyphosate remobilization by root-induced changes in the rhizosphere of non-target plants
- d) interaction of glyphosate with Mn-reducing/oxidizing rhizosphere microorganisms
- e) effect of glyphosate on mycorrhizae and microbial diversity

Fig.1. 4. Schematic presentation of the dynamics of glyphosate (or its metabolite AMPA) in the rhizosphere.

1.9.3. Glyphosate toxicity on soil microbial community

When herbicides are applied in agricultural systems, the possibility exists that the chemical may exert certain side effect on the soil microflora. These may involve influence on soil processes such as energy flow and nutrient cycling, influencing the fertility of the soil system (Goring and Laskowski, 1981; Rosas and de Storani, 1987). Other effects may include shifts in microbial (including fungal species) community

structure. Wardle and Parkinson (1990) reported an increase in the frequency of three fungal species on organic particles in soils incubated with glyphosate (200µg g^{-1} soil) while one species was suppressed. Widenfalk *et al*. (2008) also reported that exposure to glyphosate caused significant shift in bacterial community composition at an environmentally relevant concentration. Similarly, a report by Araujo *et al*. (2003) showed an increased most probable number (MPN) count after 32 days incubation in the number of fungi and actinomycetes but reduction in the number of the rest community of bacteria. Other reports also demonstrated alteration of functional structure and reduced functional diversity of soil bacteria but increased microbial biomass by glyphosate pre-seed application. This two way alteration indicates luck of evenness in microbial diversity but also dominance by few functional groups (Lupwayi *et al*., 2008).

Glyphosate is also known to inhibit biological nitrogen fixation as a result of glyphosate toxicity to the beneficial microorganisms. Dvoranen *et al*. (2008) reported a decreased number and dry weight of nodules in glyphosate-resistant G*lycine max* (BRS 245 RR and BRS 247 RR) after a single glyphosate application.

Furthermore, glyphosate can have inhibitory or stimulatory effect to plant pathogens. Greenhouse and field trials with genetically modified glyphosate-resistant wheat (*Triticum aestivum*) showed low infection rate of the plant by leaf rust caused by P*uccinia triticina* when treated with a labeled rate of glyphosate prior to inoculation with the leaf rust (Anderson and Kolmer, 2005). The authors also reported infection type reduction on wheat caused by the stem rust fungus, *Puccinia graminis* f. sp. *tritici*. On the other hand, greenhouse studies of glyphosate-resistant sugar beet, showed increased disease severity following glyphosate application and inoculation with certain isolates of *Rhizoctonia solani* Kuhn and *Fusarium oxysporum* Schlecht. f. sp. *betae* Snyd. & Hans (Larson *et al*., 2006).

1.9.4. Interaction between glyphosate and fungal disease

Glyphosate´s primary site of action is the inhibition of the enzyme 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS), and prevents the production of essential aromatic amino acids which are used by plants in protein synthesis and to

produce many secondary plant products such as growth promoters, growth inhibitors and lignin (Franz *et al*., 1997). This may weaken the plant´s resistance to disease and expose it to be susceptible to pathogens. Therefore, soil borne fungi can act as synergistic in the herbicidal action of glyphosate, after glyphosate blocks the production of phenolics involved in disease resistance of plants to these pathogens (Levesque and Rahe, 1992). Many reports indicate strong correlation between glyphosate use and fungal disease prevalence. Glyphosate has been found to stimulate *Fusarium* spp. in greenhouse studies. Sanogo *et al*. (2000) observed greater disease severity and increased isolation frequency of *Fusarium solani* f.sp. *glycines* on glyphosate-treated GR soybean relative to untreated GR soybean. Kawate *et al*. (1997) also observed an increased level of Fusarium f.sp. pisi in the rhizosphere of glyphosate-treated *Lamium amplexicaule* L. (henbit dead-nettle) relative to untreated henbit. In Chehalis sandy loam soil, injury and death of Italian ryegrass (*Lolium multiflorum*) after glyphosate treatment were attributed to *Pytium spp*., whereas in Crooked sandy loam the damage appeared to be caused solely by the chemical (Kawate and Appleby, 1987). Glyphosate pretreatment to Sekiguchi lesion (*sl*) mutant rice suppressed Sekiguchi lesion formation and tryptamine accumulation after *Magnaporthe grisea* infection even under light, a favorable growth condition the mutant is known to have enhanced resistance to *M. grisea* infection responsible for Sekiguchi lesion formation and tryptamine accumulation (Imaoka *et al*., 2008). Sublethal doses of glyphosate inhibited the expression of resistance in soybean to *Phytophthora megasperma f.sp. glycinae*, in beans to *Colletotrichum lindemuthianum* and in tomato to *Fusarium* spp. (Brammal and Higgins, 1988; Johal and Rahe, 1988; Keen *et al*., 1982).

Field survey data from Saskatchewan suggested that glyphosate can promote Fusarium head blight (FHB) of wheat and barley. In each of the four years field trials, increased FHB in spring wheat was positively correlated to glyphosate application of the previous 18 months (Fernandez *et al*., 2005). Similarly, under zero tillage condition, previous glyphosate applications were reported to correlate positively with *F. avenaceum* and negatively with *F. equiseti* and *C. sativus* (Fernandez *et al.,* 2008). Levesque *et al.*, (1987) reposted that glyphosate application increased root colonization of various

treated weeds by *Fusarium avenaceum* and *Fusarium oxysporum*, and it also increased the propagule density of these *Fusarium* spp. in the soil. It is generally accepted that herbicide-induced weakening of the plant can predispose the plant to infection by facultative-type pathogens (Levesque and Rahe, 1992). Rhizomes of glyphosate treated quackgrass (*Elymus repens*, L. Gould) were exposed to heavy colonization by *Fusarium culmorum* and that an increase of this pathogen caused synergistic effect that led to the death of barley plants subsequently planted in the same pots (Lynch and Penn, 1980). Glyphosate application to kill volunteer cereals and weeds prior to planting spring burley under short interval between application and direct seeding, resulted to increased severity of Rhizoctonia root rot and reduced barley yield by as much as 50% (Smiley *et al.,* 1992).

1.10. Objectives:

Following the indicated problematics in the literature above, the main objectives of the present study were:

(i). to identify key factors essential for a better risk assessment of glyphosate intoxication of non-target organisms under controlled greenhouse conditions and field farmer´s practice. Considering the chemical structure and behavior of glyphosate in soil, the following key factors were hypothesized for investigation under two contrasting soil conditions:

- Relevance of waiting time between weed desiccation by glyphosate and subsequent crop planting under two contrasting soil conditions, i.e., less buffered acidic Ap of an Arenosol and highly buffered C horizon of a Luvisol.
- Remobilization risk of soil matrix fixed glyphosate by root-induced changes in the rhizosphere with detrimental effect in intoxicating the subsequent crop. Two main driving forces for root-induced rhizosphere changes were investigated i.e., supply of different N forms (NO_3^- or NH_4^+) as key players of root-induced change via differential anion/cation uptake; secondly by application of artificial carboxylates (Citric acid and Na-Citrate) to mitigate the potential release of citrate as main component of root exudates during different environmental stress conditions.

(ii) To gain a further insight into additional factors involved in determining the above mentioned risk factors and validation of the controlled greenhouse experiment results in typical farmer´s practice field condition. For this, more greenhouse and field experiments were conducted:

- Investigation of the role of waiting time and contribution of different binding forms of glyphosate in soils, employing different modes of glyphosate application i.e., direct incorporation of glyphosate into soil and indirectly via phloem transport of target plants (model weed plants) on the two contrasting soils that considered the role of localized hot spot formation by localized release of glyphosate from decaying roots.
- To further evaluate the involvement of decaying organic matter, soil incorporation of glyphosate treated shoot or root matter was considered under the two contrasting soils. This was set to check whether the release of glyphosate from a decaying root or shoot residue is an important factor for intoxication of the subsequent crop and whether soil type also plays a role in alleviating such risk.
- In validating the results obtained under controlled greenhouse conditions, a field experiment with a typical minimal tillage farmer´s practice was set to investigate the relevance of waiting time between weed desiccation by glyphosate and subsequent crop planting.

2.0. General Materials and Methods

In this chapter, a general description of the plant material used and cultivation approaches of the plants used in the model experiments, as well as growth conditions of the experiments routinely used throughout the study period are presented. In addition generally used analytical methods are described in detail. Special methodologies or growth conditions employed only in specific chapters are described in their respective chapters.

2.1. Plant material

Throughout the course of this study, two plant species were used as model plants for the study: (i) sunflower (*Helianthus annuus* L. cv. Frankasol) and (ii) rye grass (*Lolium perenne* L. cv. Kelvin). Sunflower was used as non-target crop plant prone to risk of intoxication by glyphosate residues applied to rye grass as weed grown prior to the sunflower plants. Sunflower was selected for its sensitivity to glyphosate which is reflected by a sensitive accumulation of shikimate in shoot and root tissues and is known as a biological indicator of glyphosate toxicity. While rye grass was selected as it is a fast growing plant and often also is a problematic weed in cropping systems. In addition, there are also some indications that some cultivars of rye grass are developing resistance to glyphosate pressuring farmer to apply more than the recommended dosage of glyphosate without considering the residual effect for intoxication of the non-target crops.

In evaluating the greenhouse model experiment results in the field farmer´s practice, wheat plant has also been used as non-target plant (*Triticum aestivum*, L. cv. Türkis) (chapter 3).

2.2. Used soils

Two contrasting soils, i.e. a sandy acidic Ap horizon of an Arenosol and calcareous loess sub soil (Luvisol) were used for all the experiments. The two soils were chosen for their contrasting properties. The Arenosol soil had a low pH and buffering capacity while the Luvisol had a high pH and buffering capacity. Main soil characteristics as well as mineral nutrients are given in table 2.1.

Table 2. 1. Chemical characteristics of the Arenosol and Luvisol soils used in the research work.

Soil Property	Arenosol soil	Luvisol soil	Reference/Remark
pH ($CaCl_2$)	4.5	7.6	
C_{org}	0.16%	<0.3%	
$CaCO_3$		30%	
Ca^{2+} [mg kg^{-1}]	0.4	59.9	a)
Mg^{2+} [mg kg^{-1}]	0.4	11.3	a)
Mn [mg kg^{-1}]	7.4	15.0	b)
Fe [mg kg^{-1}]	369.0	7.8	b)
Zn [mg kg^{-1}]	0.8	0.6	b)
B [mg kg^{-1}]	0.9	0.2	b)
Cu [mg kg^{-1}]	0.5	0.7	b)
Texture horizon	Sandy to loamy sand of an Arenosol	Loam C of a Luvisol	

a) Water extractable fraction (Beck *et al*., 2000)
b) Calcium chloride - diethylenetriamine penta-acetic acid (CAT)-extractable micronutrient concentrations (VDLUFA, 2004)

2.3. Conditions for plant growth

Experiments were conducted under greenhouse conditions with 16/8 hour day/night regime, temperature range of 17ºC to 35ºC, light intensity of up to 200µmol m^2 s^1 and humidity range of 30% to 70%, using the two contrasting model soils described in section 2.2.

Soils were always sieved to pass though a 2 mm mesh size and fertilized 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) before sowing. In addition, the calcareous subsoil was supplied with Fe as FeEDTA (20 µmoles kg^{-1} soil). Plant culture was performed in pots containing 500 g of fertilized soil with soil density of about 1.2 –

1.3 kg m^{-3} 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 sub-soil). Water losses were determined gravimetrically and replaced by daily applications of de-ionized water.

2.4. Glyphosate application

Glyphosate as Roundup Ultramax® formulation (Monsanto Agrar, Düsseldorf, Germany) was used for all the experiments. The spray solution was prepared following the producer guideline, i.e., 2L Roundup solution in 200L distilled water, finally resulting to an active ingredient glyphosate concentration of 28.4mM solution. Depending on the aim of the experiment or treatments, this solution (or at a lower or higher rate) was applied either directly to the soil or sprayed to rye grass as model weed with a hand sprayer (for details see individual chapters).

2.4.1. Rye grass pre-culture and glyphosate application

To investigate the effects of glyphosate residues in the plant tissue of target weeds on subsequently cultivated non-target plants, rye grass (*Lolium perenne* L.) was pre-cultivated as model weed in pots filled with the fertilized soils. To stimulate high weed coverage of the soil, a sowing density of 1 g rye grass seeds (germination rate 70%) per 42 cm^2 was used. After good coverage of soil by the weed seedlings, the young rye grass seedlings were sprayed with the glyphosate spray solution described above considering the leaf area and appropriate volume applied by translating from the amount recommended per hectare (Monsanto, pers. commun., 2007).

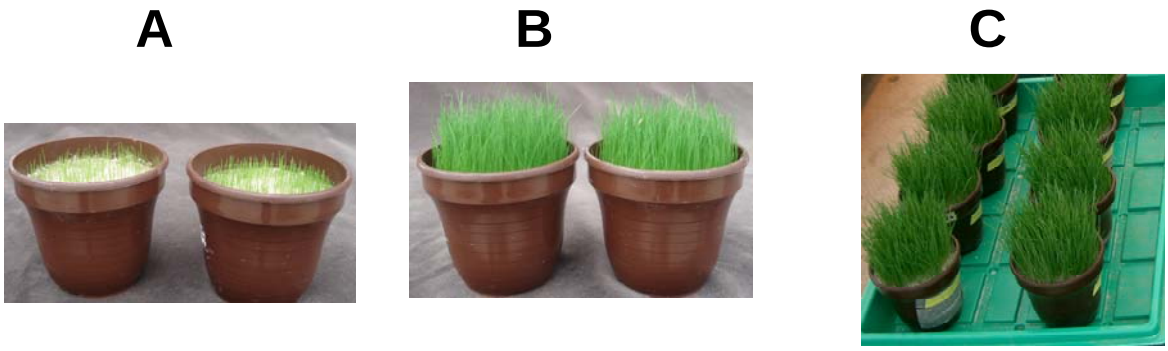


Photo 2. 1. Different growth stages (A= 1 day after germination; B=5 days after germination and C= 8 days after germination and growth stage for desiccation by glyphosate) of rye grass (*Lolium perenne* L. cv. kelvin) used as model weed.

2.4.2. Glyphosate soil application

To assess the effects of glyphosate fixed in the soil on following non-target plants, glyphosate spray solution was applied directly to the soil and homogenously mixed to the whole soil volume. Depending on the aim and approach of the experiment, a waiting time have been given for the glyphosate to be stabilized before sowing of the sunflower plants.

2.5. Sunflower sowing

Subsequently, after glyphosate applied directly to the soil or rye grass desiccation, sunflower seeds (*Helianthus annuus* L. cv. Frankasol) (germination rate of about 95%) were sown into the pots at different "waiting times". Seven seeds of sunflower were directly sown and after 7 days seedlings were tilled leaving two plants for further cultivation. The seedlings collected at tilling have been used for shikimate analysis as physiological indicator of glyphosate toxicity (see below).

2.6. "Waiting times"

Throughout this thesis, the term "waiting time" is used in reference to indicate the time gap between desiccating a weed (rye grass) by glyphosate and next crop planting (sunflower).

2.7. Plant harvest

At the end of each experiment, plants were removed from the pots by washing out the root systems with water. Then, roots and shoots separated, stored at appropriate environment depending planed analysis. Samples intended for shikimate analysis, were frozen in liquid nitrogen and stored at -20^{o}C. Samples intended for mineral analysis were dried at 60^{o}C and stored at room temperature till grinding.

2.8. Shikimate analysis

The frozen plant tissue was homogenized 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 (Singh and Shaner, 1998; Neumann, 2006).

Separation of shikimate as carboxylates was conducted on an Aminex 87H ion exclusion column (Bio-Rad, Richmond, CA, USA). 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 carboxylates 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.

2.9. Analysis of mineral nutrients

Dried leaves (60° C) were grinded and ashed in a muffle furnace at 500° C for 4 hours. After cooling, the samples were extracted twice with 2 mL of 3.4 M HNO_3 (v/v) and subsequently evaporated to dryness. The ash was dissolved in 2 mL of 4 M HCl, subsequently diluted 10 fold with hot de-ionized water, and boiled for 2 min. After addition of 0.1 mL Cs/La buffer to 4.9 mL ash solution (for Fe and Mn), while for P, colour reagent (molybdate-vandate-solution), was added according to methods of Gericke and Kurmies (1952). Mineral elements were determined by atomic absorption spectrometry (UNICAM 939, Offenbach / Main, Germany) for Mn, Fe, Zn, Cu and Mg; flam photometry for Ca and K and spectrophotometry for P.

2.10. Statistics

All treatments comprised 4 replicates and pots were arranged in the greenhouse in a completely randomized block design. Analysis of variance was performed with SPSS statistics software package by comparing means through one-way-ANOVA (SPSS Inc. Illinois, U.S.A).

Relevance of Waiting Time Between Weed Desiccation by Glyphosate and Subsequent Crop Planting.

3.1. Introduction

Glyphosate [(N-phosphonomethy)glycine] is a highly effective broad-spectrum herbicide, routinely employed to control weeds in no-till agriculture, orchards, forestry and genetically modified glyphosate resistant crops such as cotton, soybean, maize and canola. After foliar application, glyphosate is fast translocated to actively growing meristematic tissues of shoots and roots (Sprankle et al., 1975c; Gougler and Geiger, 1981). Its inhibitory effect is based on binding to the enzyme 5-enolpyruvylshikimik acid-3-phosphate synthase in the biosynthetic pathway of aromatic amino acids (Steinrucken and Amrhen, 1980) and leads to accumulation of shikimate on plant tissues that can be used as physiological indicator for residual injury by glyphosate.

Glyphosate is transported in both the xylem and phloem of treated plants in a similar pattern of distribution like photoassimilates (Sprankle *et al*, 1975c; Gougler and Geiger, 1981) and finally released to the rhizosphere from intact or decaying roots.

There is a general understanding that once glyphosate comes in contact with the soil, it is either biologically degraded by microorganisms as long as it is still in soil solution or strongly bound to soil colloids without any further soil activity (Sprankle *et al*., 1975b). For this reason, glyphosate is advocated to be used on farms without consideration of any waiting time between weed desiccation with glyphosate and subsequent crop planting (Monsanto Agrar Deutschland, Düsseldorf, Germany). However hydroponic experiments clearly demonstrated that glyphosate applied to a target plant being released to commonly shared hydroponic sphere and taken up by a co-cultivated non-target plant with detrimental effect on plant growth and nutrient uptake (Neumann *et al*., 2006). In the same research work Neumann *et al.* (2006) clearly demonstrated glyphosate rhizosphere transfer from target to non-target plants in soil undergone in greenhouse model experiments. Similarly, results from a pot experiment designed to study glyphosate release by roots of *Brachiaria decumbens* and its effects on co-cultivated eucalypt plants under greenhouse condition using pots filled with two types of

soils (clayey and sandy) have shown radicular release of glyphosate by *B. decumbens* and subsequent uptake by eucalypt plants through roots (Tuffi Santos *et al*., 2008). Furthermore, pot experiment results by Rodrigues *et al*. (1982) showed that increasing glyphosate dose and wheat plant density resulted to strong inhibition of plant height and biomass production of co-cultivated soybean. The authors were able to detect ^{14}C-glyphosate released into the soil from treated wheat plants with thin-layer chromatography. In another experiment, the authors were also able to detect trace amounts of the radio-labeled glyphosate on thin-layer plates of leaf and stem extracts of corn plants which were grown in the same pots with the treated wheat plants.

Similar situation can hold true to field grown plants as well, i.e., glyphosate released by senescing or intact roots of treated weeds. Thus, such released glyphosate can be taken up by seedlings of subsequently sown non-target crop before it is fixed to soil colloids or degraded by microorganisms if not enough waiting time is given.

The objective of the present study was to evaluate the relevance of waiting time between weed desiccation by glyphosate and subsequent crop planting under controlled greenhouse and field conditions. For this purpose, one greenhouse experiment using two contrasting soils and sunflower as model non-target plant was cultivated at different waiting times after desiccation by glyphosate of rye grass as model weed plant. To further confirm the model greenhouse results, a field experiment under farmer´s practice was set out considering different waiting times and application rates. It was expected that short waiting time of less than 3 weeks may result in a residual phytotoxicity effects by glyphosate. These toxicity effects should be reflected in hindered plant growth, reduced nutrient acquisition and increased intracellular shikimate accumulation in roots as primary victims of glyphosate toxicity.

3.2. Materials and Methods

3.2.1. Greenhouse model experiment

3.2.1.1. Conditions for plant growth

Controlled green house experiment was conducted under hot summer condition (temperature during noon up to 35°C) using two contrasting soils: a sandy acidic Ap horizon of an Arenosol with low buffering capacity (pH ($CaCl_2$) =4.5) and with a well-buffered calcareous Luvisol subsoil (pH ($CaCl_2$) 7.6).

Soils were sieved to pass through a 2 mm mesh size and fertilized with mineral nutrients (for details see chapter two). Plant culture was performed in pots containing 500 g of fertilized 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 sub-soil). Water losses were determined gravimetrically and replaced by daily applications of de-ionized water.

3.2.1.2. Weed (rye grass) desiccation by glyphosate and cultivation of sunflower

To investigate the relevance of waiting time between weed desiccation by glyphosate and subsequent crop planting, rye grass *(Lolium perenne* L. cv. Kelvin) was pre-cultivated as model weed in plastic pots filled with 500 g fertilized 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. 8 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. Each pot received 3.56 ml of glyphosate spray solution on the leaves, based on determination of the rye grass leaf area coverage (approx. 1782 cm^2 per pot). The plants died within 7 days. Subsequently, sunflower seedlings (*Helianthus annuus* L. cv. Frankasol) were sown into the same pots (10 seeds per pot) at -1(one day before glyphosate application) and 0, 1, 3, 6, 14 and 21 days after rye grass desiccation by glyphosate. Control treatments without glyphosate application were considered at -1 and 21 days waiting time, where rye grass shoots were removed by cutting at the soil

surface level with a sharp knife. An additional control was considered at -1 day waiting time by preparing pots filled with fertilized soil but without rye grass (bare soil, BS). 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 four replicates.

3.2.1.3. Plant harvest

Eight days after sowing (DAS), a first set of eight sunflower seedlings were carefully removed from the pots, leaving 2 for a longer cultivation. Roots were gently washed from soil and shoots were separated, frozen in liquid nitrogen and stored at -20°C for shikimate analysis. The two left behind sunflower seedlings were further cultivated until 27 DAS. At final harvest, the roots were washed out from soil and shoot and root parts were separated for biomass determination. Youngest fully expanded leaves were selected for analysis of micronutrients.

3.2.1.4. Shikimate analysis

The frozen plant root tissue was homogenized 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 (see chapter 2 for details).

3.2.2. Field Experiment

3.2.2.1. Conditions for plant growth

Field experiment was conducted on farmer's field in Hirrlingen, Tübingen, Germany under supervision of K. Weiss (2008) to confirm the model experiment results. Two levels of glyphosate dosage were used: 2 L ha^{-1} which is the minimal recommended rate by the producer (Monsanto Agrar Deutschland GmbH, Düsseldorf, Germany) and 6 L ha^{-1} which is employed under extreme cases by farmers. Two waiting times, i.e. 2 and 14 days after spring cover crop, winter wheat, desiccation by glyphosate and subsequent winter wheat (*Triticum aestivum* L. cv Türkis) sowing were given. An additional control was considered for the 14 day waiting time by using Agil-S

(Feinchemie Schwebda GmbH) and Basta (Bayer Crop Science Inc.) as herbicides mixture for removing the cover crop. Each treatment was replicated 6 times under randomized block design. The whole field experiment was divided into three blocks (A, B and C), based on the amount of straw existed and each treatment was made to occur twice in each block.

3.2.2..2. Cover crop desiccation by glyphosate

For the 2 L ha^{-1} treatment, winter wheat cover crop was 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 while for the 6 L ha^{-1} treatment a concentration of 85.2 mM concentration of the same glyphosate formulation was used.

For the 14 day waiting time treatments, either the 28.4 mM or 85.2 mM glyphosate concentration solution was sprayed at 12.09.08 on each respective block. 12 days later (24.09.08), the final 2 days waiting time treatments were sprayed with either of the respective glyphosate spray solutions. Two days after the last spray as the short waiting time application, winter wheat seeds were directly sown all over the blocks allowing similar external growth conditions for all the treatments.

3.2.2.3. Data collection

Seven weeks after sowing of winter wheat as the non-target plant, shoot samples were collected from each plot for SPAD value measurement and mineral nutrient analysis. Root samples were also collected for shikimate analysis. In addition, digital photo images of each plot were recorded. Moreover, visual percentage damage of the non-target winter wheat culture was documented at 2.5 and 6 months after sowing.

3.2.3. SPAD value measurement

SPAD value of wheat leaves collected from field was measured using a SPAD meter.

3.2.4. Analysis of mineral nutrients

Gericke and Kurmies (1952) method was followed for analysis of shoot mineral nutrient concentrations. Dried leaves were ground and ashed in a muffle furnace at 500° C for 5 hours placing them on crucibles. After cooling, around 100 mg of the samples were extracted twice with 1 mL of 3.4 M HNO_3 (v/v) and subsequently evaporated to dryness. The ash was dissolved in 1 mL of 4 M HCl, subsequently diluted 10 fold with hot de-ionized water, and boiled for 2 min. After addition of 0.1 mL Cs/La buffer to 4.9 mL ash solution, Fe, Mn, Zn, Cu and Mg concentrations were measured by atomic absorption spectrometry (UNICAM 939, Offenbach / Main, Germany) while Ca and K by flam photometry. For P measurement, instead of Cs/La buffer, a colour reagent (molybdate-vanadate solution) was added and analysed by spectrophotometer.

3.2.5 Statistics

All treatments of the greenhouse pot experiment comprised 4 replicates and pots were arranged in the greenhouse in a completely randomized block design. In the field experiment, each treatment had 6 replicates assigned to three blocks in a way that two replicates of each treatment fall on each block. Analysis of variance was performed with SPSS statistics software package by comparing means through one-way-ANOVA (SPSS Inc. Illinois, U.S.A).

3.3. Results

3.3.1. Greenhouse model experiment

3.3.1.1. Visual plant growth

In the model greenhouse experiment, waiting time between rye grass desiccation by glyphosate and sunflower sowing of less than 21 days, resulted to a hindered development of sunflower seedlings, particularly the root part was heavily damaged (Photo 3.1).

Photo 3.1. Seedling development of sunflower plants grown on the acidic Arenosol after rye grass desiccation by glyphosate at 14 days waiting time. Comparable phytotoxicity of glyphosate was also observed on the Luvisol. The photo was taken 8 days after sowing of sunflower.

3.3.1.2. Shoot and root biomass

Corresponding to the visual observation, at treatments with less than 21 days waiting time after rye grass treatment with glyphosate, shoot fresh weights were severely inhibited on both the acidic Arenosol and calcareous Luvisol compared with both controls, without a glyphosate treatment of rye grass (C-MW) and the bare soil (C-BS) (Fig. 3.1 A and B). Sunflower plants grown on Arenosol at 14 day waiting time were not healthy due to other factors than glyphosate toxicity and produced the lowest biomass.

3.3.1.3. Intracellular shikimate accumulation

On both the acidic Arenosol and calcareous Luvisol, plant growth inhibition at all waiting times less than 21 days were in like manner accompanied by intracellular shikimate accumulation in roots which is known as a physiological bio-indicator of glyphosate phytotoxicity (Fig. 3.2 A and B). During first phase seedling harvest for shikimate analysis, plants at 14 day waiting time treatment of the Arenosol soil were not healthy and it was not possible to get enough root material for shikimate analysis, therefore shikimate results for this date are not shown.

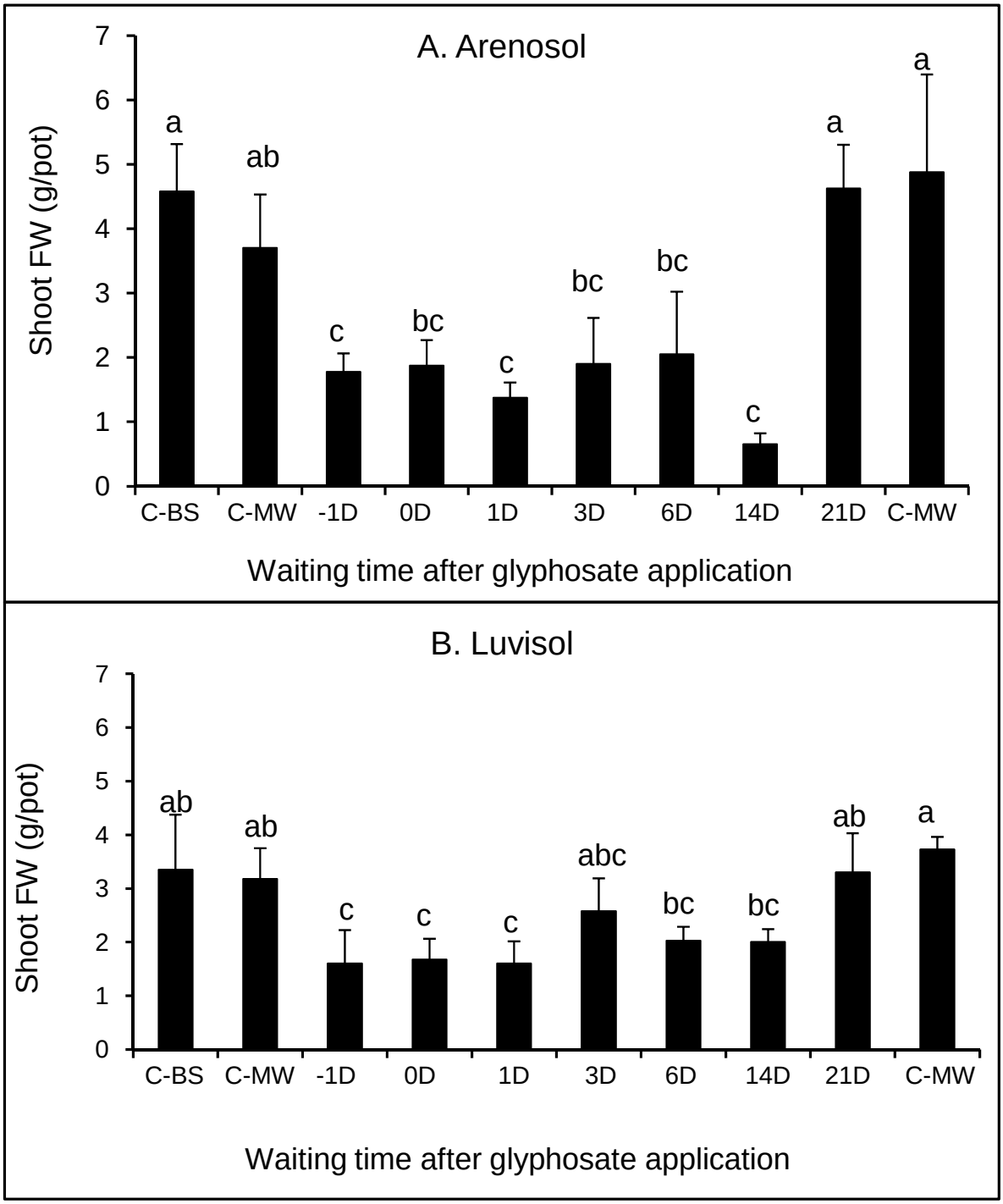


Fig.3.1. Shoot fresh weight of sunflower plants grown on: A. the acidic Arenosol and B. the calcareous Luvisol, at different waiting times (-1, 0, 1, 3, 6, 14, and 21 days) between rye grass desiccation by glyphosate and sunflower sowing. Two different controls were included i.e. bare soil (C-BS) at -1 d and mechanical weeding (C-MW) at -1 and 21 d waiting time. Plants were harvested at 27 day after sowing. Given data represent an average of 4 replicates with SD as bars, $p<0.05$.

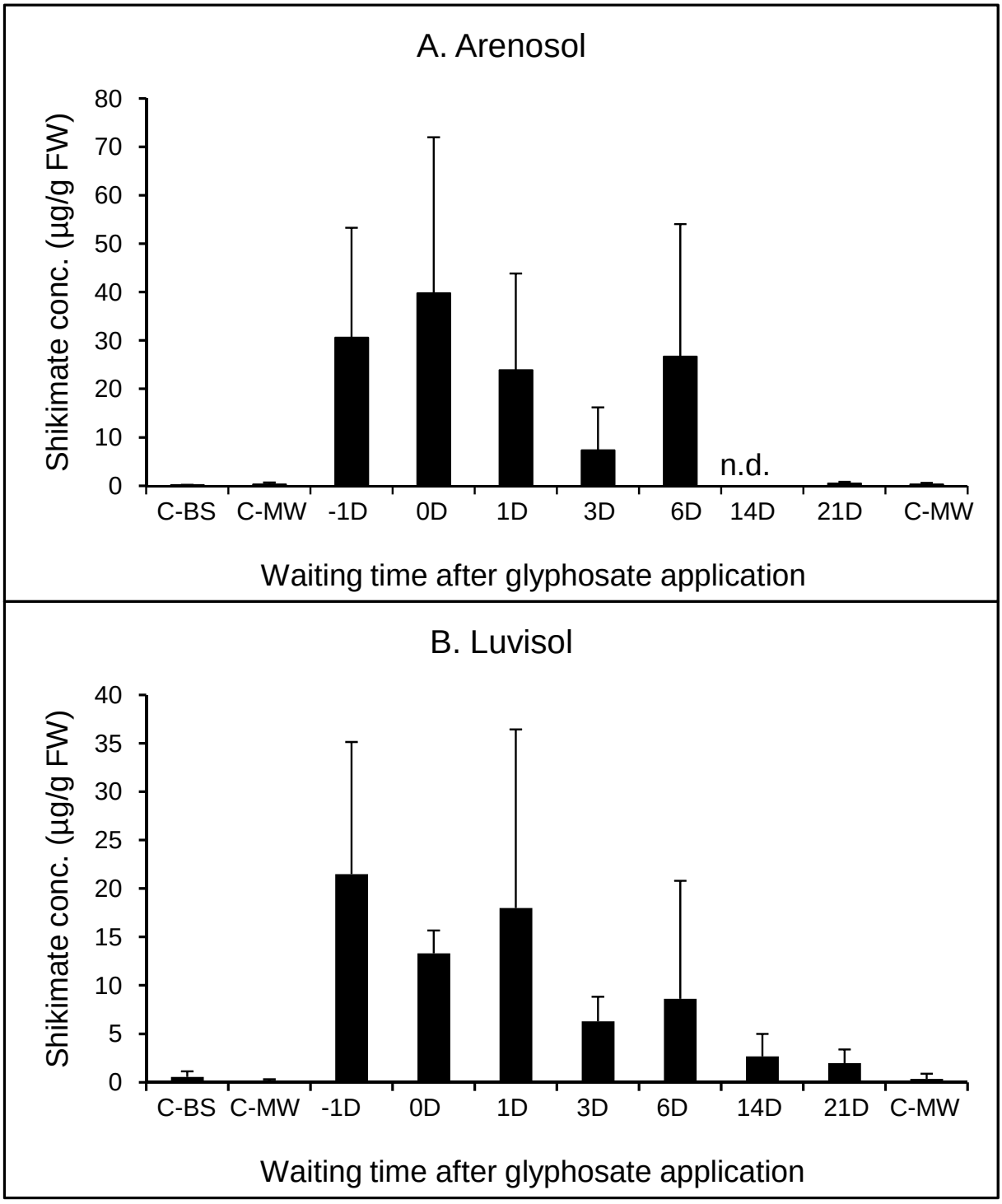


Fig.3.2. Intracellular shikimate concentration in roots of sunflower plants grown on the acidic Arenosol (A) or on the calcareous Luvisol (B) at different waiting times (-1, 0, 1, 3, 6, 14 and 21 d) between rye grass desiccation by glyphosate and sunflower sowing. Mechanical weeding (MW) at -1 and 21 d, plus bare soil without glyphosate application at -1 d waiting time represents controls. Root samples were taken of from the 6 sampled sunflower seedlings eight days after sowing (8DAS). Given data represent an average of 4 replicates with SD as bars, $p<0.05$.

3.3.1.4. Micronutrient acquisition

Micronutrient (Mn, Fe and Zn) analysis of the youngest fully expanded leaves however showed no significant difference between all the different treatments (Table 1 and 2). However, plants grown on the calcareous Luvisol subsoil showed lower Zn levels compared to the acidic Arenosol with worsened reduction tendencies closer to critical level at shorter waiting times. This absence of mineral nutrient concentration difference between the different treatments is probably attributed to a dilution factor as the plants on the short waiting time treatments were very stunted and the analyzed shoot parts were emerging cotyledons carrying the nutrient contents of the seeds.

Table 3.1. Micronutrient (Mn, Fe and Zn) concentration in the youngest fully expanded leaf (YFEL) of sunflower plants grown on the Arenosol at different waiting times (-1, 0, 1, 3, 6, 14, and 21 d) after pre-culture rye grass desiccation by glyphosate. Mechanical weeding (MW) at -1 and 21 d plus bare soil without glyphosate application at -1 d waiting time represents control treatments. Plants were harvested 28 days after sowing. Given data represents the average of 4 replicates ±SD.

Waiting time	Micronutrient concentration (µg/g DM)		
	Mn	Fe	Zn
Control(bare soil)	78.4±5.1	91.6±8.6	40.5±5.0
Control(mechanical weeding-MW)	114.2±12.9	98.1±13.6	53.3±6.0
-1D	107.4±20.9	97.3±18.1	48.9±7.8
0D	118.6±18.7	86.2±18.2	40.7±3.5
1D	103.5±53.2	106.1±37.4	43.7±15.4
3D	123.0±27.4	92.2±12.7	38.1±5.2
6D	119.8±15.9	100.1±11.8	44.2±2.2
14D	NA*	NA*	36.7±8.6
21D	139.0±12.3	97.7±17.9	41.6±7.3
Control at 21D (MW)	113.5±5.1	87.1±14.1	40.0±3.1

*NA= data not available due to loss of the samples.

Table 3.2. Micronutrient (Mn, Fe and Zn) concentration in the youngest fully expanded leaf (YFEL) of sunflower plants grown on the Luvisol at different waiting times (-1, 0, 1, 3, 6, 14, and 21 d) after pre-culture rye grass desiccation by glyphosate. Mechanical weeding (MW) at -1 and 21 d plus bare soil without glyphosate application at -1 d waiting time represents control treatments. Plants were harvested 28 days after sowing. Given data represents the average of 4 replicates ±SD.

Waiting time	Micronutrient concentration (µg/g DM)		
	Mn	Fe	Zn
Control(bare soil)	59.4±10.3	74.6±2.9	17.7±1.6
Control(mechanical weeding-MW)	67.9±25.4	98.1±18.7	34.0±13.7
-1D	61.6±31.8	85.9±26.3	13.1±4.9
0D	74.7±22.9	72.9±14.1	24.3±1.9
1D	101.5±12.1	88.2±7.4	32.8±7.6
3D	81.8±10.4	79.4±9.6	34.0±12.9
6D	93.0±19.7	83.5±7.5	19.8±5.0
14D	68.0±14.2	94.9±11.6	33.0±10.3
21D	91.6±11.8	118.2±20.6	26.5±8.3
Control at 21D (MW)	67.6±9.2	67.6±8.1	29.2±2.1

3.3.2 Field experiment

3.3.2.1. Visual plant growth

The field experiment conducted to confirm the controlled greenhouse experiment resulted similar negative effect on growth. Winter wheat plants cultivated at 2 days waiting time after cover crop desiccation by glyphosate, showed heterogeneous emergence and stunted growth compared to the plots with 14 day waiting time and control, irrespective of the applied glyphosate quantity (Photo 3.2). Similarly, visual scoring of the relative damage of wheat seedlings showed close to 50% damage when only 2 days waiting time was allowed while the control and 14 days waiting time showed only around 10% culture damage (Fig.3.3).

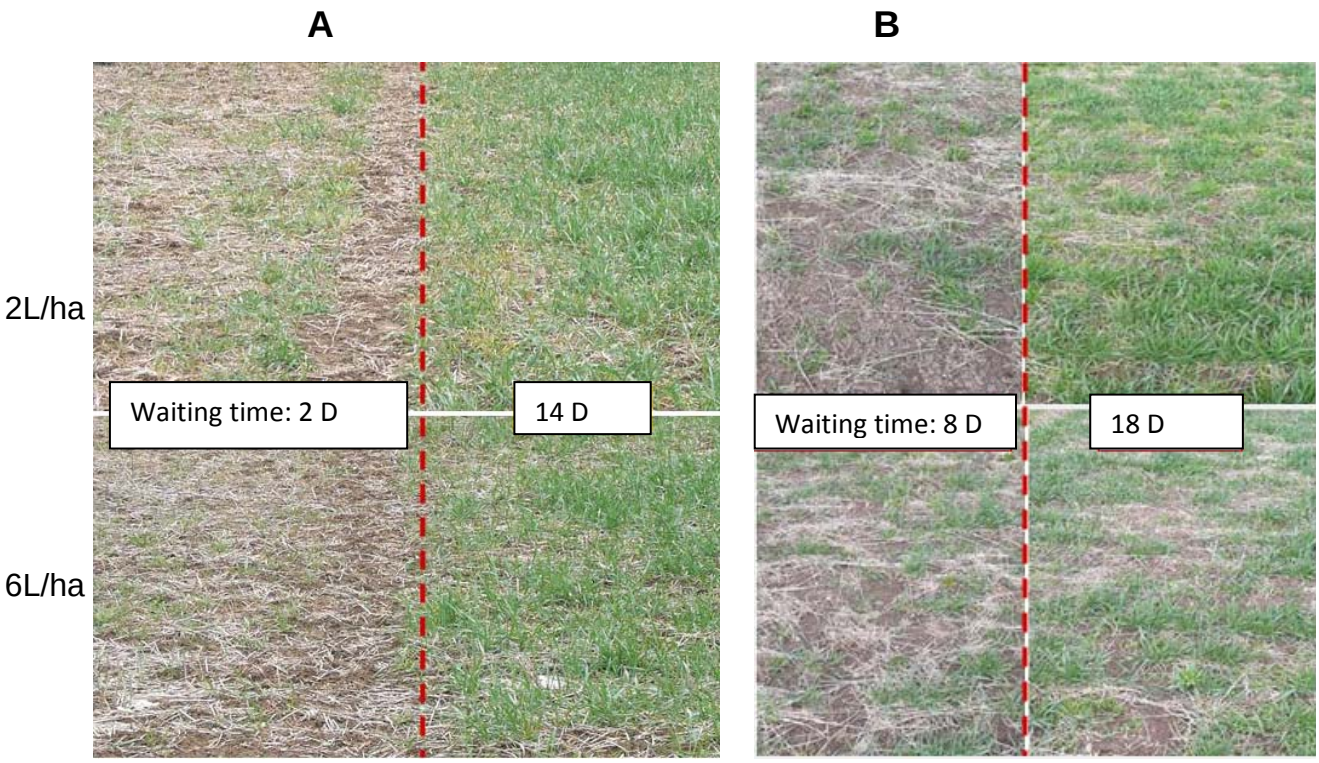


Photo 3.2. Effect of waiting time (2 days versus 2 weeks) and doses of Roundup UltraMax (2L versus 6L ha^{-1}) on damage of winter wheat establishment on farmers´ fields (A in Hirrlingen and B in Wendelsheim) with reduced tillage management practice. Photos were taken on 06.04.08, six months after winter wheat sowing.

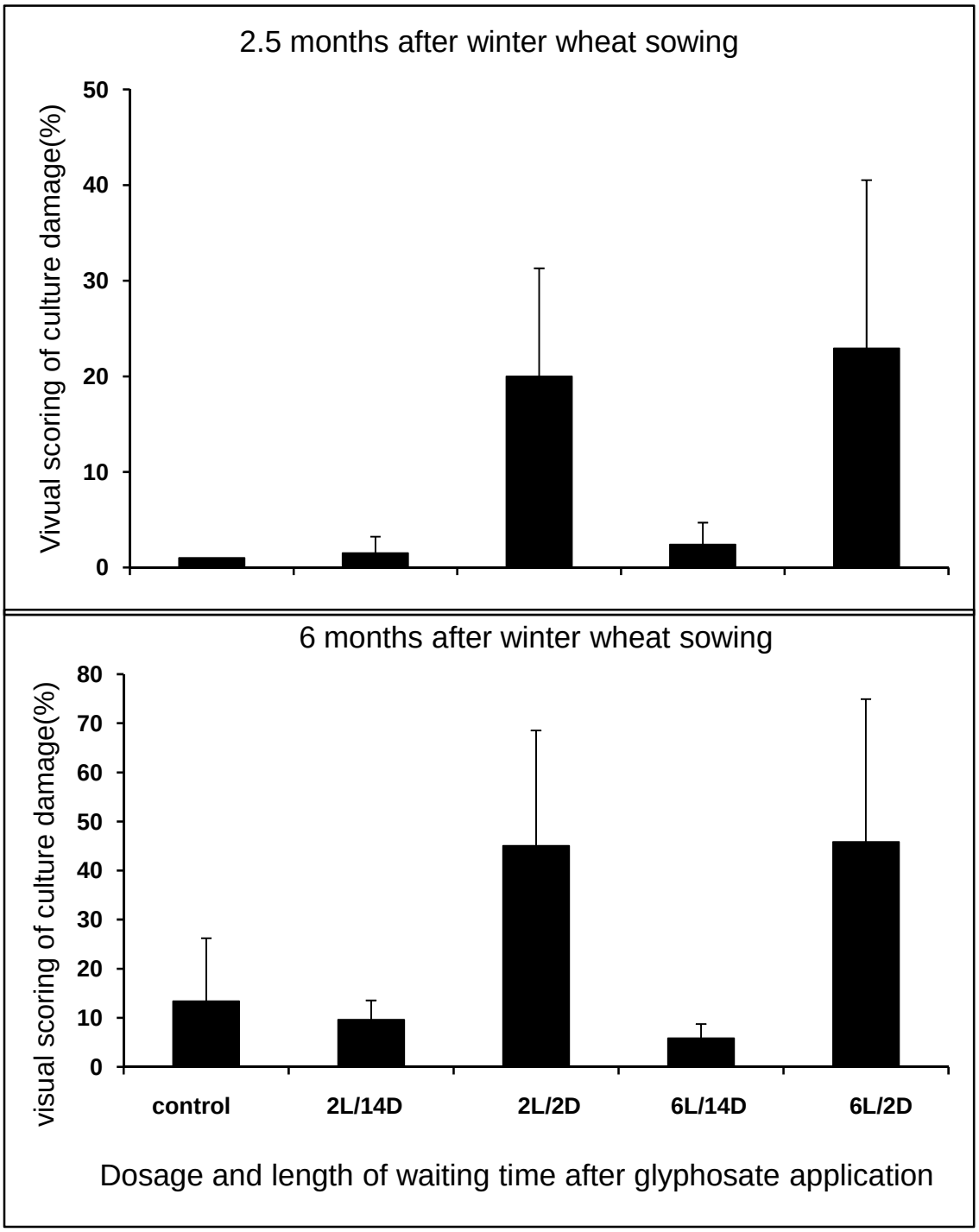


Fig.3.3. Visual scoring damage of wheat plants grown under minimum tillage farming practice sown at different waiting times (2 days versus 2 weeks) between glyphosate desiccation to weed and sowing of wheat and different dosage of glyphosate (2L versus 6L ha^{-1}). Agil-S and Basta mixture with 14 days waiting time represents the control. Scoring was done either on autumn 2007 (06.12.07) or on spring 2008 (31.03.08). The given data represents the average of 6 replicates ±SD.

3.3.2.2. SPAD value and mineral nutrient acquisition

Parallel to visually observed damage of winter wheat in plots with 2 days waiting time, SPAD values for chlorophyll content were also significantly lower compared to 14 day waiting time, irrespective of the amount of glyphosate applied (Fig. 3.4). Short waiting time (2 d) combined with a higher rate of glyphosate application reduced the concentration of divalent metal macronutrients (Ca^{2+} and Mg^{2+}) of winter wheat plants (Table 3.3). Surprisingly, despite the declined SPAD values of the short waiting time (2 d), Fe analysis did not show significant difference between the treatments (Table 3.4). Other micronutrients (Mn, Zn and Cu) however showed decreased concentrations at the combination of a short waiting time (2 d) and high application rate of glyphosate(6 L ha^{-1}) compared to a long waiting time (14 d) and low application rate of glyphosate (Table 3.4). Independent of waiting time (2 d versus 14 d) and amount of glyphosate applied (2 L versus 6 L ha^{-1}), concentrations of Mn, Zn and Cu in leaves were generally low, close to critical deficiency levels according to Bergmann (1992) (Table 4). Shikimate analysis of the winter wheat roots however did not show any difference between treatments and for all the treatments the values were below the detection limit (data not shown).

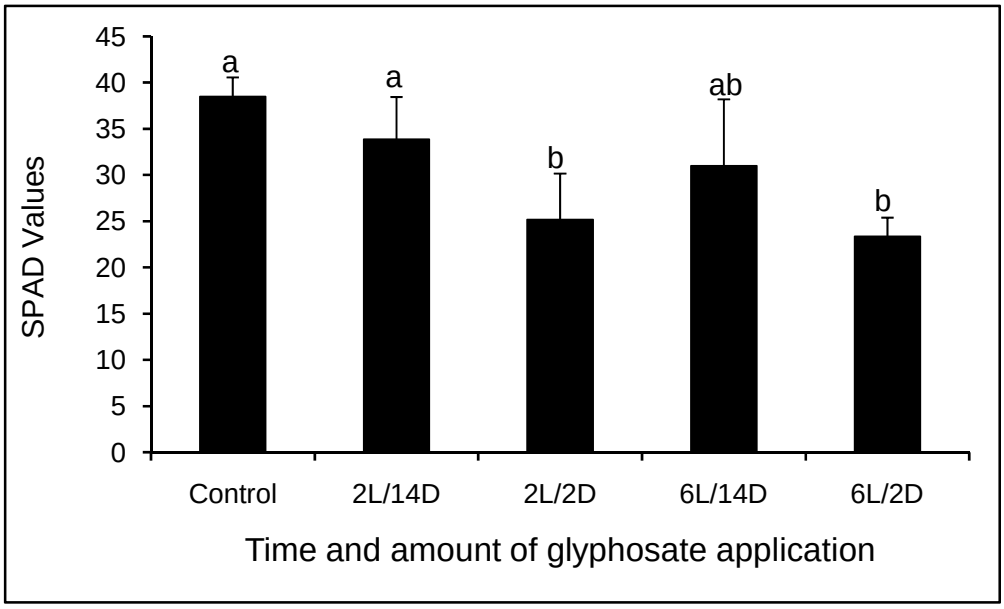


Fig.3.4. SPAD value measurement of winter wheat plants grown under field conditions under reduced tillage using high (6L ha^{-1}) and low (2L ha^{-1}) glyphosate rates to control weeds and considering short (2 days) and long (14 days) waiting time between weed desiccation by glyphosate and wheat sowing. Agil-S and Basta mixture with 14 days waiting time functions as control. Measurements were conducted on autumn 2007. Data given presents an average of 6 replicates with ±SD, $p \leq 0.05$.

Table 3.3. Shoot macronutrient concentrations of winter wheat plants cultivated under field conditions on farmer´s field with reduced tillage after desiccating pre-culture cover crop with high (6L ha^{-1}) and low (2L ha^{-1}) glyphosate levels and different waiting times (2d versus 2 weeks). Agil-S and Basta mixture with 14 days waiting time was used as control. Leave for mineral nutrient analysis was collected at autumn 2007. Similar letters along the column are not significantly different from each other at P<0.05 Tukey test.

Treatment (rate of gly/waiting time)	Macronutrient concentration (mg/g DM)			
	P	Mg	K	Ca
Control	1.9±0.3^{a}	1.5±0.1^{ab}	25.0±4.0^{a}	3.8±0.2^{ab}
2L/14D	2.1±0.4^{a}	1.5±0.2^{a}	24.9±5.0^{a}	3.9±0.2^{ab}
2L/2D	1.7±0.4^{a}	1.3±0.2^{ab}	19.2±4.8^{a}	3.7±0.6^{a}
6L/14D	2.0±0.2^{a}	1.5±0.1^{ab}	23.3±4.3^{a}	4.1±0.2^{ab}
6L/2D	1.6±0.4^{a}	1.2±0.2^{b}	18.2±4.9^{a}	3.4±0.3^{b}

Table 3.4. Shoot micronutrient concentrations of winter wheat plants cultivated under field conditions on farmer´s field with reduced tillage after desiccating pre-culture cover crop with high (6L ha^{-1}) and low (2L ha^{-1}) glyphosate levels and different waiting times (2d versus 2 weeks). Agil-S and Basta mixture with 14 days waiting time was used as control. Leaves for mineral nutrient analysis was collected at autumn 2007. Similar letters along the column are not significantly different from each other at P<0.05 Duncan test.

Treatment (rate of gly/waiting time)	Micronutrient concentration (µg/g DM)			
	Fe	Mn	Zn	Cu
Control	376.1±26.7^{a}	52.7±19.4^{a}	19.5±2.4^{ab}	7.1±2.5^{ab}
2L/14D	401.2±160.1^{a}	42.5±9.9^{ab}	19.9±2.2^{a}	13.7±12.3^{a}
2L/2D	340.2±118.5^{a}	30.0±10.1^{b}	17.1±0.8^{ab}	6.7±3.3^{ab}
6L/14D	314.0±87.6^{a}	44.0±10.9^{ab}	19.1±2.1^{ab}	5.9±1.6^{b}
6L/2D	295.8±64.6^{a}	37.7±17.1^{ab}	16.5±3.6^{b}	5.6±2.8^{b}

3.4 Discussion

3.4.1. Controlled greenhouse experiment

The question posed in this study was whether waiting time between weed desiccation by glyphosate and subsequent crop planting is required to alleviate the unintended residual phytotoxicity of glyphosate to the subsequent crop plants. The findings of this experiment indicated a clear requirement of waiting times contrary to the common belief of the farmers and continuously advertized by the producers in farmer journals that glyphosate for weed desiccation can even be applied after sowing before emergence of the following crop (Monsanto, Roundup Ultramax® product information). When sunflower plants as model crop plants were sown at less than 21 days after rye grass desiccation by glyphosate, on both the acidic Arenosol and the calcareous Luvisol under controlled greenhouse condition, biomass production of the sunflower seedlings was significantly reduced (Fig. 3.1 A and B). Under similar greenhouse conditions with loamy sandy soil, Cornish (1992) reported a 57% reduction of dry weight of tomato seedlings transplanted even 15 days after glyphosate soil spray at 4 L product per hectare rate. Even a greater reduction in dry weight occurred when superphosphate was mixed into the soil before glyphosate application. The same report records a dry weight reduction of tomato plants transplanted 16 days after glyphosate spraying under field conditions and concluded that three weeks of waiting time between glyphosate use and subsequent crop planting could save unwanted phytotoxicy effects of glyphosate residues.

The inhibition of sunflower biomass at less than 21 day waiting time were in close correspondence with an intracellular shikimate accumulation as physiological indicator for glyphosate toxicity on both experimental soils (Figs. 3.2 A and B). As 5-enolpyruvylshikimate-3-phosphate synthase (EPSP synthase) enzyme is the target for glyphosate, this herbicide kills plants by blocking the shikimatic acid pathway, resulting in intracellular accumulation of shikimate (Becerril *et al*., 1989; Della-Cioppa *et al*., 1986). Hence, the close correspondence of plant growth inhibition and intracellular shikimate accumulation in sunflower seedlings at waiting times less than 21 days proved the glyphosate residual phytotoxicity as the main cause than others.

Glyphosate residues in a soil or contaminations by drift can cause adverse effect on plant nutritional status. Many reports documented such inhibitory effect of simulated glyphosate drift contamination on nutrient uptake and translocation. Report by Eker *et al*. (2006) shows a strong inhibition of micronutrient (Mn and Fe) uptake and root-to-shoot translocation in sunflower plants grown under hydroponic culture after simulated sublethal drift glyphosate application. Similarly, rhizosphere root-to-root transfer from target to non-target plant under soil culture caused inhibited micronutrient acquisition by non-target plants (Neumann *et al.*, 2006). Interestingly, a report by Bott *et al*. (2008) revealed a glyphosate-induced impairment of plant growth and micronutrient status in glyphosate-resistant soybean (*Glycine max* L.). In the present controlled greenhouse study, however, there was no clear difference in micronutrient concentrations of the youngest fully expanded leaves of the sunflower seedlings between the different waiting times and controls (Tables 3.1 and 3.2). This is most probably attributed to dilution effect as the biomass of the sunflower seedlings in the controls (bare soil without rye grass and mechanical weeding) and 21 days waiting time was much higher than the other treatments at less than 21 days waiting time. Plants harvested from the short waiting times were strongly stunted and the shoot collected was only the emerging cotyledon filled with the nutrient contents of the seed.

3.4.2 Field Experiment

Similar to the results of the controlled greenhouse experiment, field trials confirmed the phytotoxicity of glyphosate residues to the subsequent non-target crop if not enough waiting time is given for detoxification of glyphosate in the soil. Stunted development and heterogeneous emergence of winter wheat plants occurred at field plots where the wheat sowing was done 2 days after cover crop desiccation by glyphosate, irrespective of the glyphosate amount applied (2 L ha^{-1} versus 6 L ha^{-1}) compared to the plants sown 14 days after glyphosate application. Similar residual glyphosate phytotoxicity on corn development sown 2 days after glyphosate application to B*rachiaria decumbens* was also previously reported (Constantin *et al*., 2008).

Visual scoring of culture damage showed up to 50% of the culture being injured by glyphosate residual toxicity that was visually persistent even after 6 months in case

winter wheat sowing was done already 2 days after cover crop desiccation by glyphosate (Fig. 3.3). When the waiting time increased to 14 days, the visual injury reduced to less than 10% of the culture (Fig. 3.3). Culture damage at vegetative stage can have detrimental effects for final grain yield. A report by Buehring *et al*. (2007) demonstrated a strong correlation between visual injury rating and potential yield losses of field corn exposed to sublethal doses of glyphosate.

In line with the greenhouse results and many previous reports, short waiting time (2 d) combined with higher glyphosate application rate (6 L ha^{-1}) resulted in reduction of both the macro and micronutrient concentration of wheat plants (Table 3.3 and 3.4). Glyphosate is known as a chelator of divalent cations (Glass, 1984; Schoenherr and Schreiber, 2004; Subramaniam and Hoggard, 1988). A research conducted to demonstrate the possible effects of glyphosate on uptake, translocation and intracellular localization of metal cations in soybean seedlings by Duke *et al*., (1985) clearly shows that root-fed or foliar applied glyphosate reduced uptake and translocation of Ca^{2+} and Mg^{2+} but not K^{+}. Glyphosate chelates Mg^{2+} and Ca^{2+} almost equally well, with the same stability constant and similar effects of pH on chelating properties (Madsen, 1978). Thus, the chelation of these ions by glyphosate may be related to glyphosate effects on Ca^{2+} and Mg^{2+} uptake and translocation. In agreement to these findings and possibly for the same reason, the Ca^{2+} and Mg^{2+} concentration of winter wheat shoots were reduced due to the combined effect of short waiting time (2 d) and elevated glyphosate application rate (6 L ha^{-1}) compared to the longer waiting time (14 d) and lower application rate (2 L ha^{-1}) in this study (Table 3.3). Similar to the divalent macronutrients, glyphosate also forms stable complexes with the divalent micronutrients, such as Fe, Mn, Zn and Cu, depending on their ionic state during the time of contact (Glass, 1984; Hall *et al*., 2000; Bernards *et al*., 2005). For the same reason, Eker *et al*. (2006) found a substantial decrease in leaf concentration of Fe and Mn by glyphosate drift application. Similarly, in the present study, a short waiting time (2 d) caused a decreased shoot Mn concentration irrespective of the application rate while Zn and Cu shoot concentrations declined by both short waiting times and higher glyphosate application rates (Table 3.4).

3.5. Conclusion

The commonly used glyphosate application direction in farmers´ fields seems inappropriate and needs consideration of an appropriate waiting time. Results of this work clearly demonstrated a consideration of not less than 21 days waiting time between weed desiccation with glyphosate and subsequent crop planting in order to avoid the frequently reported glyphosate residual toxicity on farmer´s fields. However further research is needed to determine site specific waiting times by studying factors that might influence the concentrations of glyphosate residue in a soil considering different soil types, plant species and environmental conditions.

3.6. Prospects

Further research is needed to clarify and determine the necessity of waiting times after glyphosate weed desiccation considering different soil, plant and environmental factors such as:

- Different soil types with regard to, e.g. pH, texture, P fertilization levels and P fixation capacities as glyphosate fixation and degradation may depend on such soil properties.
- Different temperature conditions and soil water content as soil microbial activity is affected by soil temperature and water and thus rate of glyphosate degradation.
- Different cover crops might degrade differently and thereby release of glyphosate residues can be different between various plant species.

Glyphosate transfer via the rhizosphere from target to non-target plants: Possible remobilization of detoxified glyphosate by root -induced changes in the rhizosphere.

4.1. Introduction

Glyphosate (N-(phosphonomethyl) glycine) is a non-selective broad-spectrum herbicide used in agriculture. With the application of the new bio-engineering technology, the use of glyphosate is dramatically increasing parallel with the development of glyphosate resistant crops such as cotton, soybean, maize and rape varieties. Glyphosate is a polar compound with three distinct groups (amine, carboxylate and phosphonate) which cause specific sorption reaction like hydrogen bonding (Piccolo and Celano, 1994; Piccolo *et al*., 1996) and stable co-ordination bonding to free and surface Fe^{3+} and Al^{3+} (McBride and Kung, 1989; Gimsing and Borggaard, 2007). These sorption behaviors make glyphosate unique as compared to most other herbicides and have elicited a general belief that it is rapidly adsorbed to the soil without any residual toxic effect to crop plants.

Sorption of glyphosate to soil matrix is dependent on many soil factors including soil pH, ionic strength, ortho-phosphate concentration and dominant cations (Ca^{2+}, K^{+}, NH_4^{+}) in soil solution (de Jonge and de Jonge, 1999). However, adsorption of glyphosate is shown to be a reversible process. Thus, glyphosate adsorbed to soils was reported to have a residual activity to some plant species (Salazar and Appleby, 1982).

In addition, phosphate levels in the soil can have a confounding effect on glyphosate adsorption or desorption as both compete for the same sorption sites. For instance on goethite and gibbsite, a preferential strong adsorption of phosphate over glyphosate has led to remobilization of presorbed glyphosate after phosphate addition (Veiga *et al*., 2001; Gimsing and Borggaard, 2002). Hence, it is apparent that phosphorus fertilization management might play a crucial role in determining the fate of glyphosate in the rhizosphere as already indicated by the work of Cornish (1992).

On the other hand there are various root-induced chemical changes in the rhizosphere as adaptation strategy for nutrient mobilization by distinct plant species, especially for P

and some micronutrients. Such chemical changes include changes in rhizosphere pH and redox potential, enhanced localized release of H^+ and release of organic carboxylates (Bertrand *et al*., 1999; Neumann and Römheld, 1999; Neumann and Roemheld, 2002). The main change of these processes is the rhizosphere pH, which is strongly dependent on many soil and plant factors like soil buffering capacity, plant genotype, plant nutritional status and root exudation (Jones, 1998; Neumann and Roemheld, 1999). Despite of the soil buffering capacity, the form of N supplied (NO_3^- or NH_4^+), i.e. anionic or cationic form of N supply is a main driving force for pH changes in the rhizosphere (Marschner *et al*., 1986). Although root exudates such as organic carboxylates may alter the rhizosphere pH in some instances, the most prominent pH change is caused by differences in the cation/anion uptake ratio, especially dependent on nitrate and ammonium supply. Usually, ammonium supply is correlated with a preferential cation uptake and thus with a higher net excretion rates of H^+ over HCO_3^- or OH^-, and nitrate supply causes the reverse (Marschner, 1995).

One of the most documented plant adaptation strategy to nutrient deficiency condition is the release of carboxylates, particularly citrate into the rhizosphere under low P status by various plant species, thereby mobilizing sparingly available nutrients such as phosphate by a mechanism of ligand exchange, dissolution or occupation of sorption sites (Gardner *et al*., 1983; Fox *et al*., 1990; Dinkelaker *et al.,* 1995; Gerke, 1995; Neumann and Roemheld, 1999).

The objective of the present study was to manipulate the rhizosphere environment with the aim to induce chemical changes for glyphosate re-mobilization in different culture media. For this purpose, two experiments were conducted using two contrasting soil types, a highly buffered calcareous subsoil (loess, Luvisol) with hardly shown root-induced rhizosphere acidification and a weakly buffered acidic surface soil (Arenosol) with easily shown root-induced rhizosphere acidification. In the first experiment, different forms of N supply (NO_3^- or NH_4^+) should result either in a rhizosphere acidification under NH_4^+ -N supply or in an alkalinization under NO_3^- supply. It was expected that the NH_4^+-induced rhizosphere acidification might result in a re-mobilization of adsorbed glyphosate with a subsequent uptake by planted non-target sunflower seedlings and an

accumulation of shikimate in sunflower roots as a bio-indicator. An additional experiment was conducted with the same soils but with supplementation of artificial carboxylates after planting sunflower seedlings on glyphosate pre-loaded soils to elucidate a ligand exchange as another possible mechanism involved in phosphate or glyphosate remobilization as an alternative approach.

4.2. Materials and Methods

4.2.1. Experiment one: Rhizobox experiment

4.2.1.1. Conditions for plant growth

Experiments were conducted under greenhouse conditions, using two contrasting soils: a sandy acidic top soil of an Arenosol with low buffering capacity and a well-buffered calcareous Luvisol (for detailed soil characteristics see chapter 2). Soils were sieved to pass through a 2 mm mesh size and fertilized with: 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 µmoles kg^{-1} soil). N fertilizer was supplied either as $Ca(NO_3)_2$ for the nitrate treatments or as $(NH_4)_2SO_4$ for the ammonium treatments at a rate of 100mg N kg^{-1} soil. The ammonium was stabilized with DMPP (a nitrification inhibitor from the company BASF) at a recommended rate of 1% of applied NH_4-N (1 mg DMPP kg^{-1} soil or 4µl of a 25% DMPP solution kg^{-1} soil). Glyphosate was added at four different rates: 0, 50, 100 and 500% of the recommended rate (2L RoundUp in 200L water) together with above-mentioned nutrients in solution and homogenously mixed and incubated for 21 days under room temperature to allow a sufficient adsorption of glyphosate on the soil matrix. For conversion of regular field application rates to small scale greenhouse experiments, a surface area of 290 cm^2 and 5 cm rhizosphere depth for 1 kg rhizobox soil was considered following a recommendation by Monsanto (pers. commun., 2007). This conversion resulted in a final application rate of 0, 0.29, 0.58 and 2.89 ml of the RoundUp spray solution (2L RoundUp in 200L water, i.e., 28.4mM active ingredient) per kg soil.

After 21 days incubation, soils were re-adjusted to optimum moisture level (15% v/w for the Arenosol and 18% v/w for the Luvisol) and filled into rhizoboxes (500g soil on dry

weight basis per rhizobox). Two pre-germinated sunflower (*Helianthus annuus* L. cv. TR6149SA) seedlings (6 days old) were transplanted in each rhizobox. Sunflower plants were grown for 10 days under hot summer conditions (maximum 35°C midday and about 25°C in the night) in a greenhouse and water losses were determined gravimetrically and recharged daily by applications of de-ionized water.

4.2.1.2. Rhizosphere pH measurement

Rhizosphere and bulk soil pH was determined by a pre-calibrated antimony microelectrode by carefully opening the root window of the rhizoboxes as millivolts and converted to pH values using corresponding measurements of pH standards from 3.0 to 9.0 pH.

4.2.2. Experiment two: *Pot experiment with supplementation of synthetic carboxylates*

4.2.2.1. Conditions for plant growth

Experiments were conducted under controlled conditions of a plant growth chamber, using the two contrasting soils as in the rhizobox experiment above under similar fertilization regimes and glyphosate preloading. In this experiment, N fertilization was supplied only as $Ca(NO_3)_2$ at a rate of 100mg N kg^{-1} soil for all treatments. Unlike experiment one, in this experiment, glyphosate treatment levels were reduced to three i.e., 0, 100 and 500% of the recommended rate and rhizosphere depth was modified to consider 25 cm depth. Hence, the volume of standard glyphosate solution applied per kg soil corresponding to each level was reduced by factor 5. Therefore, for 0, 100 and 500% levels, 0, 0.12 and 0.58ml of the standard glyphosate solution were added per kg soil and incubated for 21 days for a sufficient adsorption of glyphosate on the soil matrix.

After 21 days incubation, soils were re-adjusted to optimum moisture level (15% v/w for the Arenosol and 18% v/w for the Luvisol). Finally 500 g soil on dry weight basis was filled in each plastic pot. Seven sunflower (*Helianthus annuus* L. cv. TR6149SA) seeds were directly sown to each pot. After germination, two uniform sunflower seedlings were left for further growth after thinning (6 days after sowing). After thinning, synthetic carboxylates either sodium citrate or citric acid at a rate of 10µmole g^{-1} soil were added

per pot (supplemented as solution by titrating on top of the soil surface). Plants were left to grow for another 6 days after supplementation of synthetic carboxylates under growth chamber conditions. Water losses were determined gravimetrically and daily recharged with de-ionized water.

4.2.3. Plant harvest

Plants were harvested by separating shoots and roots for biomass determination. Youngest fully expanded leaves were selected for mineral analysis. Roots were washed free from soil with water and frozen in liquid nitrogen and stored at -20°C for shikimate analysis.

4.2.4. Shikimate analysis

The frozen root tissues were homogenized with 5% ortho-phosphoric acid (1 ml 100 mg^{-1} fresh weight) using mortar and pestle. Any 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 (see chapter 2 for details).

4.2.5 Statistical analysis

All treatments comprising 4 replicates as rhizoboxes/pots were arranged in the greenhouse/growth chamber in a complete randomized block design. Analysis of variance was performed with SPSS statistics software package (SPSS Inc. Illinois, U.S.A).

4.3. Results

4.3.1. Experiment one: *Rhizobox experiment*

4.3.1.1. Visual plant growth

In the rhizobox experiment no visible difference in sunflower growth either between the N forms (NO_3^- versus NH_4^+) or due to the different glyphosate application rates could be visually observed within each soil type (Photo 4.1). In general, sunflower seedlings in rhizoboxes filled with the Luvisol showed a lower shoot growth.

Photo 4.1. Sunflower seedlings grown on the Arenosol supplied with different forms of N and pre-incubated with glyphosate for 21 days at different levels (0, 50, 100 and 500% of the recommended rate). This is a representative photo to show the growth conditions taken from the rhizoboxes with the Arenosol. Plants on the Luvisol were grown under similar conditions. On both soils, there was no visually visible difference between the various treatments.

4.3.1.2. Shoot and root biomass

Corresponding with the visual observations (Photo 4.1), there were no significant differences between the various treatments with the two N forms and increasing glyphosate application rates in shoot and root fresh weight of sunflower seedlings in the

Arenosol (Fig. 4.1A). Only in tendency, shoot fresh weight of sunflower seedlings was slightly lower with supply of stabilized NH_4^+.

On the Luvisol, the fresh weight of the sunflower shoots was significantly lower with stabilized NH_4^+ compared with NO_3^- supply (Fig. 4.1B). Root fresh weight of sunflower seedlings on the Luvisol was found significantly lower only with stabilized NH_4^+ combined with the highest glyphosate pre-application (Fig. 4.1B).

4.3.1.3. Changes in rhizosphere pH

In Fig. 4.2 A and B, the measured rhizosphere pH values at harvest are presented for sunflower seedlings grown in the Arenosol and Luvisol, respectively. As expected, only on the weakly buffered Aresonol a pH decline of 1.5 pH unit could be observed with supply of stabilized NH_4^+, whereas in case of NO_3^- supply no difference or only a small insignificant pH increase between bulk and rhizosphere pH was visible. On the Luvisol with free $CaCO_3$ (high pH buffering) pH changes in the rhizosphere were insignificant, only a pH increase with NO_3^- - N could be measured (Fig. 4.2B).

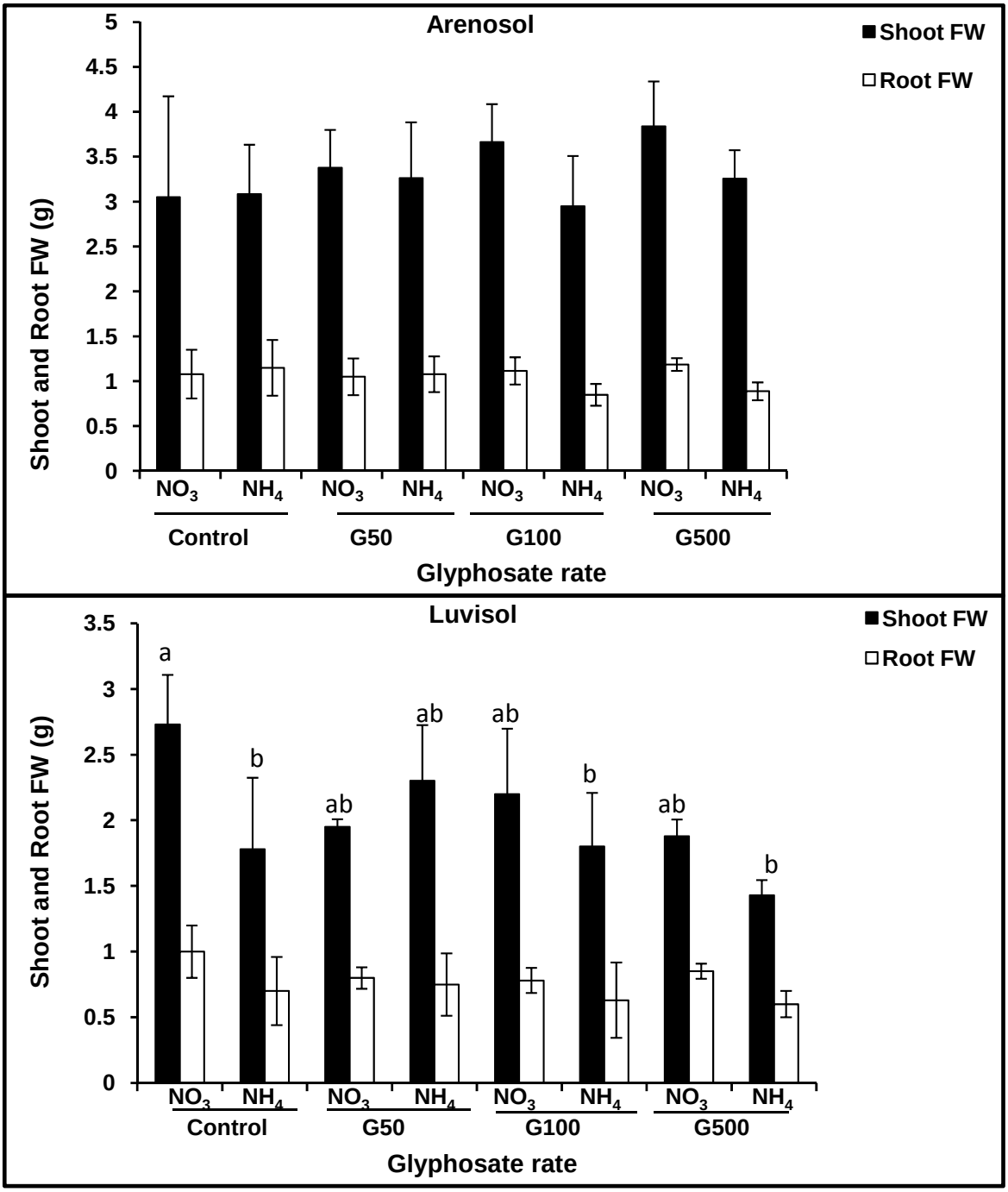


Fig.4.1. Shoot and root biomass of sunflower seedlings grown on Arenosol (A) or Luvisol (B) pre-incubated with glyphosate for 21 days at different levels (0, 50, 100 and 500% of the recommended rate) and supplied with different forms of N (NO_3^- or NH_4^+). Plants grown for 10 days in the pre-incubated soil before harvest. Given date present average of 4 replicates with SD as bars, $p \leq 0.05$.

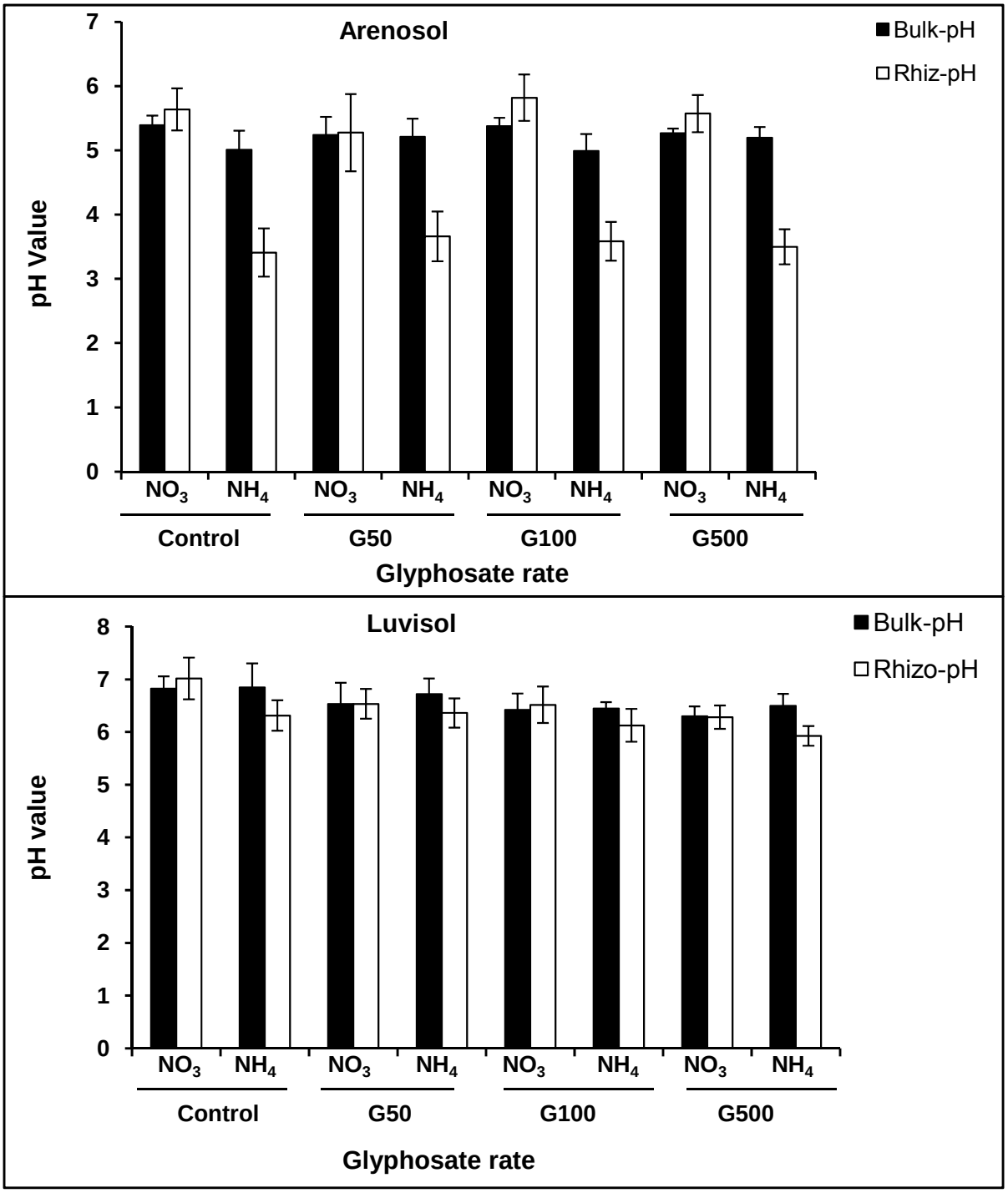


Fig. 4.2. pH change on the rhizosphere of sunflower plants grown on Arenosol (A) or Luvisol (B) pre-incubated with glyphosate for 21 days at different levels (0, 50, 100 and 500% of the recommended rate) and supplied with different forms of N (NO_3^- or NH_4^+) and pH measurement was done on 10 days after transplanting. Given date present averages of 4 replicates with SD as bars.

4.3.1.4. Shikimate concentration in roots

As shown in Fig.4.3A, despite of the detected decrease of the rhizosphere pH by stabilized NH_4^+ on the Arenosol, no increase of shikimate in the roots of sunflower seedlings could be found compared with the controls without a glyphosate pre-incubation. Thus, no re-mobilization of detoxified glyphosate by a decrease in the rhizosphere pH was observed. This was also the case on the well pH-buffered Luvisol; no difference in the shikimate concentration in root of the control (NO_3^- -N, no glyphosate) and the highest glyphosate pre-application combined with NH_4^+-N (Fig. 4.3B).

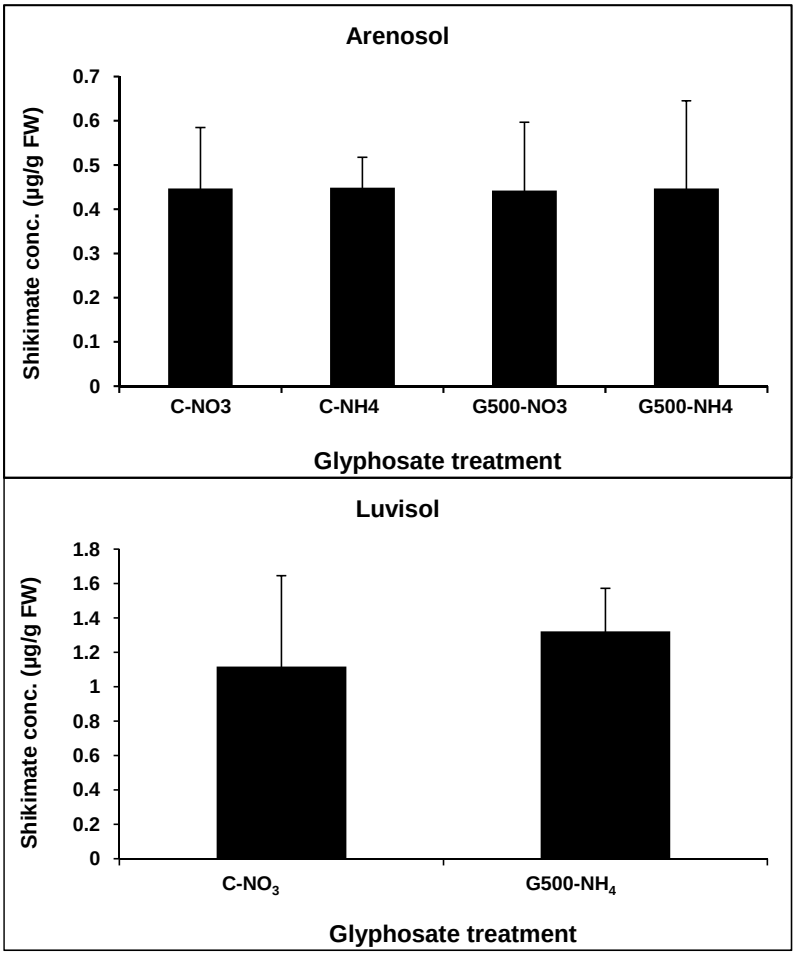


Fig 4.3. Root intracellular shikimate accumulation of sunflower plants grown on Arenosol (A) and Luvisol (B) pre-incubated with glyphosate for 21 days at 0 or 500% of the recommended rate and supplied with different forms of N (NO_3^- or NH_4^+). Only representative treatments expected for low (controls with out glyphosate) and high (500% glyphosate application) glyphosate remobilization potential were measure. Given data present average of 4 replicates with SD as bars.

4.3.2. Experiment two: *Pot experiment with supplementation of synthetic carboxylates*

4.3.2.1. Visual plant growth

During the 6 days-growth after supply of sodium citrate (NaC) or citric acid (CA), no visible difference in shoot growth could be observed on the Arenosol, even at the supply of citric acid where a double fold effect was expected, i.e., rhizosphere acidification and ligand exchange processes. Also on the Luvisol, no visible growth difference could be seen (Photo 4.2).

Photo 4.2. Sunflower seedlings grown on the Arenosol pre-loaded with different rates of glyphosate (0, 100 and 500% of the recommended rate) and supplemented with synthetic carboxylates as free citric acid (CA) or Na-Citrate (NaC) at 10µmol g^{-1} soil.

4.3.2.2. Shoot and root biomass

In agreement with the visual observations, there were no significant differences in the shoot and root fresh weights of the sunflower seedlings on the Arenosol induced by application of either sodium citrate (NC) or citric acid (CA) independent of glyphosate pre-incubation (Fig. 4.4 A and B).

In contrast, on the Luvisol the application of sodium citrate resulted in an inhibition of root growth at both glyphosate rates (Fig. 4.5 A and B). This root growth inhibition, however, could not be observed with addition of citric acid, possibly due to a fast reaction between citric acid and $CaCO_3$.

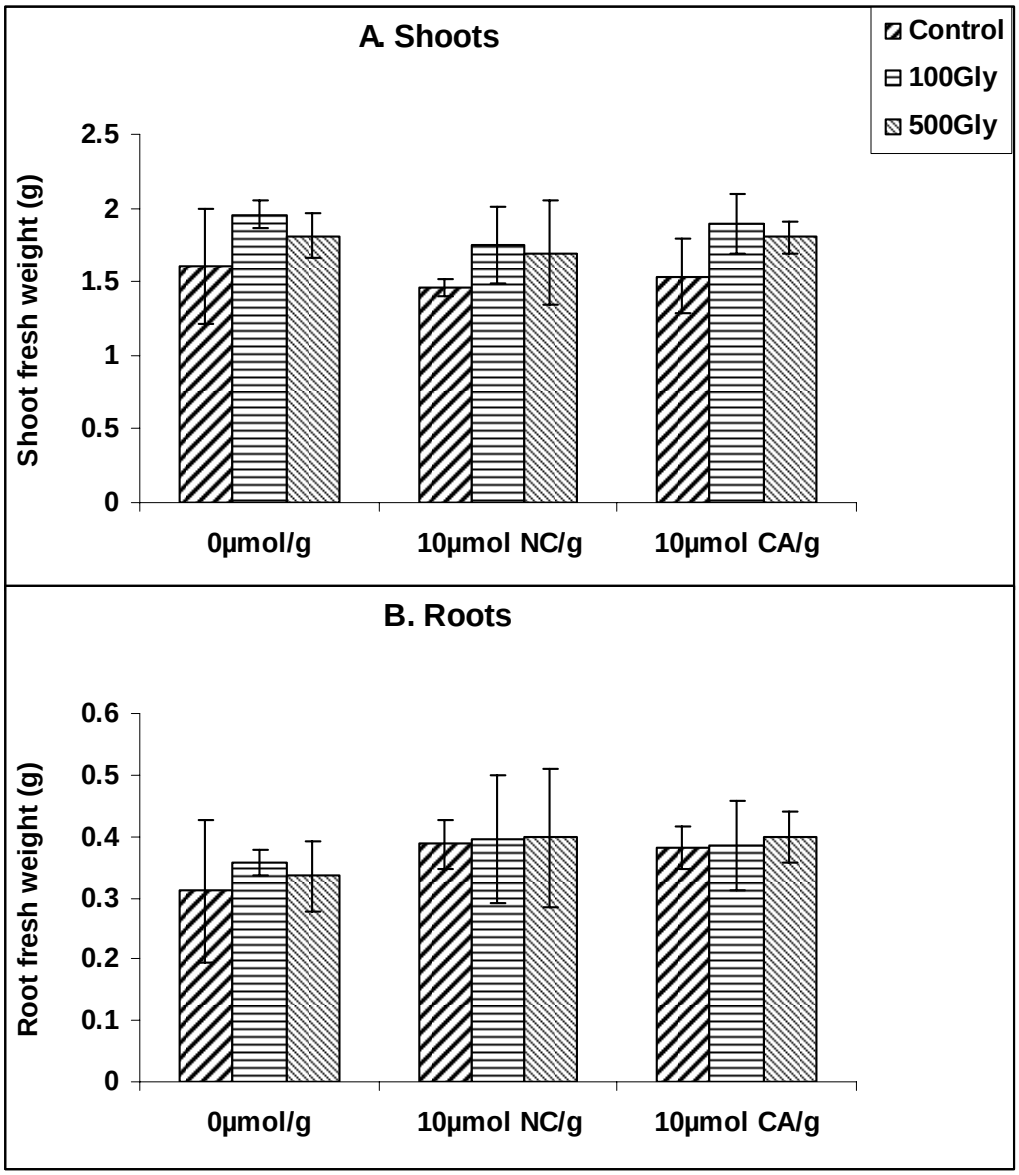


Fig.4.4 Shoot and root biomass of sunflower plants grown on the Arenosol pre-incunated with glyphosate at different levels (0, 100 and 500% of the recommended rate) for 21 days before sowing and suplementation of pots after 6 days growth of sunflower seedlings either sodium citrate (NC) or citric acid (CA) at 10µmole g^{-1} soil. Controls were soils incubated only with a complet fertilization but not glyphosate. Plants were harvested at 12 days after sowing. Given data present average of 4 replicates with SD as bars, $p \leq 0.05$.

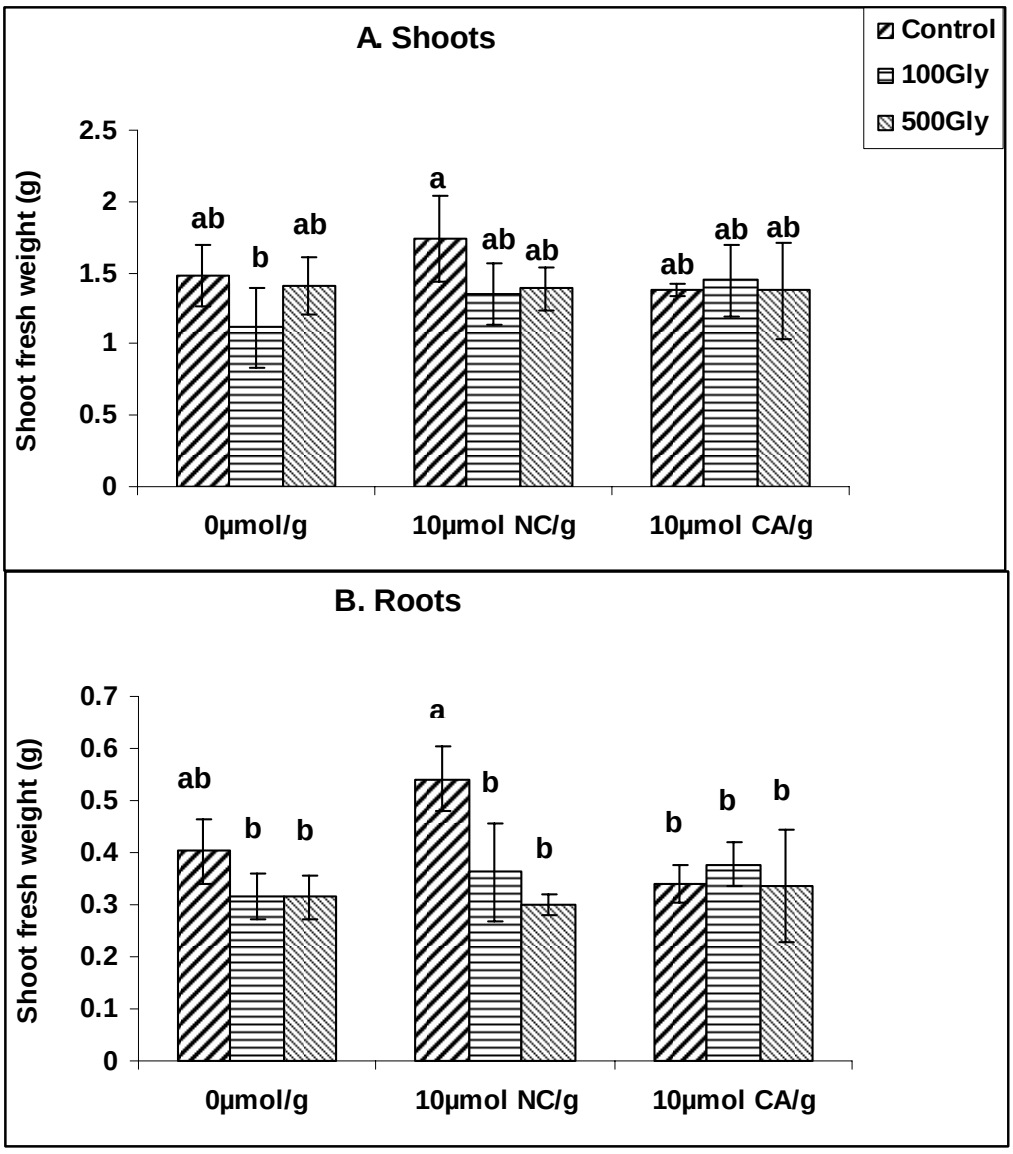


Fig.4.5 Shoot and root biomass of sunflower plants grown on the Luvisol pre-incunated with glyphosate at different levels (0, 100 and 500% of the recommended rate) for 21 days before sowing and suplementation of pots after 6 days growth of sunflower seedlings either sodium citrate (NC) or citric acid (CA) at 10µmole g^{-1} soil. Controls were soils incubated only with a complet fertilization but not glyphosate. Plants were harvested at 12 days after sowing. Given data present average of 4 replicates with SD as bars, $p \leq 0.05$.

4.3.2.3. Shikimate concentration in roots

The representative measured root samples for shikimate as bio-indicator for glyphosate phytotoxicity did not show significant difference between the controls (-glyphosate) and the glyphosate treatmens with the recommended rate (100%) on both soils (Fig. 4.6). From these findings again, no re-mobilization of glyphosate by ligand-exchange with citrate could be confirmed.

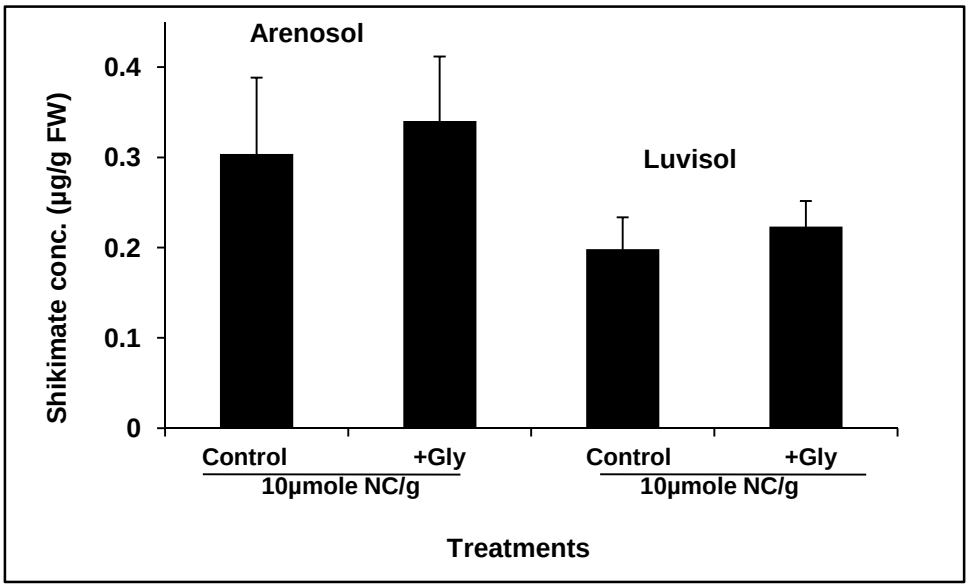


Fig.4.6. Root intracellular shikimate accumulation of sunflower plants grown on the Arenosol and Luvisol pre-incunated at 100% of the recommended rate of glyphosate for 21 days before sowing and suplementation of pots after 6 days growth of sunflower seedlings with sodium citrate (NC) at 10µmole g^{-1} soil. Controls were soils incubated only with complet fertilization but not glyphosate. Plants were harvested at 12 days after sowing. Given data present average of 4 replicates with SD as bars, $p \leq 0.05$.

4.4. Discussion

Nitrate and ammonium are the main forms of inorganic nitrogen supplied to plants. Plant growth response to different form of nitrogen has been well studied and many reports show that sole NO_3^- nutrition is associated to stimulated shoot growth accompanied by increased accumulation of zeatin and zeatin riboside in leaves and xylem exudates while sole NH_4^+ nutrition is associated with inhibited plant growth accompanied by reduced cytokinin(Walch-Liu *et al*., 2001; Rahayu *et al*., 2005; Lu *et al*., 2008). This is in agreement with the current results on the Luvisol (Fig. 4.1B) where control plants supplied with stabilized ammonium (NH_4^+) form of N had reduced shoot growth compared to control plants supplied with nitrate (NO_3^-) form of nitrogen. Though statistically insignificant, under high glyphosate level (100 and 500% of the recommended rate) and NH_4^+ nutrition tended to reduce shoot and root biomass production. In the Arenosol, however, plant growth (shoot or root) was not affected by different nitrogen form or increasing application rate of glyphosate (Fig. 4.1A).

As nitrogen comprise about 80% of the total cations or anions taken up by plants, the form of nitrogen supply has a strong impact on the uptake of other cations and anions through changes on the rhizosphere pH (Marschner, 1995). Rhizosphere acidification can be caused by an excess uptake of cations over anions and alkalization occurs when anion uptake exceeds cation uptake. Ammonium uptake is generally associated with acidification of the rhizosphere while nitrate nutrition induces an increase in rhizosphere pH (Roemheld *et al.,* 1984). Similarly, plants fed with stabilized ammonium strongly acidified their rhizosphere soil in the weakly buffered Arenosol by up to 1.7 pH units while plants fed with nitrate tended to alkalinize their rhizosphere to a lesser extent by up to 0.4 pH units (Fig. 4.2A). In the well buffered Luvisol, root-induced pH change in the rhizosphere was not strong. Plants fed with stabilized ammonium (NH_4^+) acidified their rhizosphere soil by around 0.5 pH units while the nitrate fed plants alkalinized their rhizosphere soil by only 0.2 pH units (Fig. 4.2B).

With the active phosphonate group at the end of the molecule, glyphosate shows a similar pattern of reaction like that of phosphate and both molecules compete for the same sorption sites in soil (Gimsing and Borggaard, 2001; 2002). Furthermore, many sorption-desorption experiments clearly demonstrate desorption of soil matrix fixed

glyphosate by addition of phosphate (de Jonge *et al*., 2001; Gimsing and Borggaard, 2001; 2002; Laitinen *et al*., 2008). Cornish (1992) reported a greater reduction in dry weight of tomato plants when superphosphate was mixed into the soil before glyphosate application. Interestingly, greenhouse experiment by Bott *et al*. (pers. commun.) shows soybean plant growth inhibition on glyphosate pre-incubated soil by an increasing rate of phosphate fertilization. This demonstrates a similar pattern of phosphate and glyphosate reaction in soil matrix. All chemical changes in the rhizosphere known to remobilize phosphate should also remobilize glyphosate.

In neutral or alkaline soils, rhizosphere acidification in plants fed with ammonium can enhance mobilization of sparingly soluble calcium phosphate and thereby favor the uptake of phosphate (Gahoonia *et al*., 1992). On acid soils, the pH increase induced by nitrate supply enhances phosphorus uptake, presumably by an exchange of phosphate adsorbed to iron and aluminum oxides by HCO_3^- (Gahoonia *et al*., 1992). Similar to phosphate, glyphosate forms sparingly soluble salts and/or complexes in the presence of divalent cations such as Ca^{2+} (Madsen, *et al*., 1978; Smith and Raymond, 1988; Sundaram and Sundaram, 1997). Previous reports demonstrated that in neutral to alkaline soils phosphate ions precipitate as Ca-phosphate (Lindsay *et al*., 1989) which probably holds true to glyphosate as well. Ca-phosphates have a decreasing solubility with increasing pH, except for pH values above 8 (Hinsinger, 2001). Hence, it is highly likely that root induced rhizosphere acidification of alkaline soils as a result of different form of N supply can solubilise sparingly soluble Ca-glyphosates precipitate. This raises the risk of a remobilization of soil matrix fixed glyphosate as a result of root-induced rhizosphere acidification, with the consequence of non-target plant intoxication. From the results of this experiment, however, it was not possible to confirm this assumption. There was no any glyphosate phytotoxicity due to remobilization of fixed glyphosate as the there was no accompanying intracellular shikimate accumulation (Fig. 4.3B) in accordance with the observed rhizosphere acidification and biomass reduction of the plants grown on the Luvisol fed with ammonium form of nitrogen. This may be attributed to the fact that the amount of glyphosate applied was very low in concentration since it was uniformly mixed with the whole volume of soil. But in reality when glyphosate is

applied to target plants, it is released by young root zones as hot spots in the soil with much higher concentrations. In addition, in the Arenosol where low precipitation was expected, the glyphosate molecules might have been already degraded by soil microorganisms during the 21 days pre-incubation. In the Luvisol, where higher Ca-glyphosate precipitation was expected, the root-induced rhizosphere pH reduction as a result of ammonium form of nitrogen nutrition might have been too small for glyphosate release as the change was only 0.5 pH unit (Fig. 4.2B).

Root exudation of organic carboxylates has also been considered as a source of root-induced rhizosphere acidification (Hoffland *et al*., 1989). Some plant species, such as white lupin, respond to P deficiency conditions by development of cluster roots characterized by localized fast rate of citrate and malate exudation (Johnson *et al*., 1994; Neumann *et al*., 1999; Neumann and Römheld, 1999). Therefore, scavenging of phosphate from extracellular sparingly soluble P source may be aided by exudation of carboxylates and root mediated pH change under a P-stressed environment (Gardner *et al*., 1983; Hoffland, 1992; Stroem, *et al*., 2005). Coupled to rhizosphere acidification, exuded organic carboxylates are also able to mobilize inorganic P into the soil solution via exchange chelation through competing with phosphate groups for the same binding/adsorption sites in soil and forming stronger complexes with Al^{3+}, Fe^{3+} and Ca^{2+} than phosphate does. Then phosphorus can be liberated from cation–P complex as an organic carboxylates complex with the cations or block the sorption of P to other charged sites or through the ligand exchange process (Geelhoed, *et al*., 1999; Hinsinger, 2001). Glyphosate as phosphated molecule faces the same fate like inorganic phosphate, i.e., root mediated change in the rhizosphere including excretion of organic acids (e.g. citrate) can remobilize glyphosate fixed on Al^{3+}, Fe^{3+} and Ca^{2+} cations by ligand exchange and rhizosphere acidification. In the present study with the Arenosol, however, no indication of glyphosate remobilization by synthetic carboxylate that caused plant damage could be shown (Fig. 4.4 A and B). Supplementation of the pots filled with soils pre-incubated with different levels of glyphosate for 21 days prior to planting with sodium citrate or citric acid even showed a tendency of better biomass production than the controls (especially shoot fresh weight) at both the 100 and 500%

glyphosate rate compared to no glyphosate application. Root growth, however, showed no significant differences in all treatments. This absence of glyphosate remobilization is likely to be attributed to the fact that this soil is less buffered (low Ca^{2+} availability) and might have had less inactivation of the applied glyphosate which might led to a faster degradation by microorganisms during the 21 days incubation period.

In the highly buffered calcareous Luvisol, supplementation with sodium citrate at 10µmole g^{-1} soil concentration but not with citric acid caused inhibition of root growth on glyphosate pre-incubated treatments (Fig. 4.5B). Shoot biomass production as well tended to be reduced by the addition of sodium citrate at a rate of 10µmole g^{-1} soil although the difference was not statistically significant. Analysis of intracellular shikimate accumulation as a bio-indicator for a possible glyphosate toxicity, did not show any shikimate accumulation after sodium citrate supplementation. Thus, the observed inhibited root (shoot) growth was due to another cause but not due to a glyphosate remobilization. The absence of a citric acid effect in the glyphosate pre-incubated treatments is hard to explain as in this highly buffered (high Ca^{2+} cation concentration) calcareous soil, a two fold effect of citric acid could be expected, firstly a soil acidification and secondly a citrate effect as ligand exchanger and thus a stronger dissolution of precipitated glyphosate. It is also possible that the plant growth inhibition observed by 10µmole g^{-1} soil Na-citrate addition was caused by Na toxicity rather than glyphosate. If that was the case, it can be hypothesized that there might have been an insufficient percolation of the supplemented artificial exudates to the rhizosphere soil to induce glyphosate remobilization as the exudates could remain absorbed on the top soil during addition. Again such a Na toxicity might be the fact that Na-citrate did not result in an inhibition of root growth on the non-glyphosate incubated soil but rather in an increase (4.5B).

4.5. Conclusion

Remobilization of phosphate fixed to the soil matrix by root-induced chemical changes remains well founded. As glyphosate has a phosphate group and show similar adsorption and desorption behavior in soils, the risk of glyphosate remobilization by

root-induced changes in the rhizosphere soil, including rhizosphere acidification/alkalinization remains a risk factor for non-target plant intoxication.

In the present study, possibly due to sublethal supply of glyphosate or insufficient induction of root-induced rhizosphere soil pH changes, the assumed glyphosate remobilization could not be confirmed in the conducted studies. The slight biomass reduction occurred under ammonium (in rhizobox experiment) and nitrate (in the pot experiment) were observed in glyphosate treatments without the expected relationship with an intracellular shikimate accumulation as physiological bio-indicator of glyphosate toxicity. Therefore, in further glyphosate risk assessments, a research in this direction is inevitably recommended.

4.6. Prospects

Considering the behavior of phosphonated glyphosate in soils, the risk of glyphosate remobilization by root-induced rhizosphere changes remains a treat to non-target plants. Therefore, this aspect requires further examination considering:

- Various plant genotypes with different responses towards phosphorus deficiency, such as white lupin with strong capacity of remobilizing sparingly soluble P by root-induced modification of its rhizosphere and other species like soybean known to have less influence on their rhizosphere in response to P deciciency.
- Different P levels for P fertilization that might induce desorption of fixed glyphosate as both phosphate and glyphosate compete for sorption sites.
- Considering more soil properties like different organic matter and clay content in soils.

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

5.1. Introduction

Glyphosate [(*N*-phosphonomethyl)glycine] 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 (v. 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-Mai *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 stabilization in soils is represented by the plant residues of glyphosate-treated weeds. Since in many plant species, glyphosate is not readily metabolized, 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 lyophilized 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 stabilization 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.

5.2. Materials and methods

5.2.1. 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 and with a well-buffered calcareous loess subsoil.

Soils were sieved by passing through a 2 mm mesh size and fertilized with mineral nutrients (for details see chapter two).

Plant culture was performed in pots containing 500 g of fertilized 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 sub-soil). Water losses were determined gravimetrically and replaced by daily applications of de-ionized water.

5.2.2. 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 *(Lolium perenne* L. cv. Kelvin) was pre-cultivated as model-weed in 500 g pots filled with the fertilized 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 (approx. 3300 cm^2 per pot) and the plants died within 7 d, 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 seedlings (*Helianthus 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 undertaken. This time period was defined as “waiting time”. 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 four replicates.

5.2.3. 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 fertilized 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.

5.2.4. 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.

5.2.5. Shikimate analysis

Shikimate in acidic tissue extracts was analyzed with modifications of the methods described by Singh and Shaner (1998) and Neumann (2006).

The frozen plant tissue was homogenized 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 m*M* 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.

5.2.6. 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 hours. After cooling, the samples were extracted twice with 2 mL of 3.4 M HNO_3 (v/v) and subsequently evaporated to dryness. The ash was dissolved in 2 mL of 4 M HCl, subsequently diluted 10 fold with hot de-ionized 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).

5.2.7. Statistics

All treatments comprised 4 replicates and pots were arranged in the greenhouse in a completely randomized block design. Analysis of variance was performed with SPSS statistics software package (SPSS Inc. Illinois, U.S.A).

5.3. Results

5.3.1. Visual plant growth

Glyphosate applications to pre-culture rye grass caused sever inhibition of shoot and root growth of following sunflower seedling compared to direct soil application of equivalent amount of glyphosate (Photo 5.1). Detrimental effect of glyphosate residues after rye grass treatment was comparable on both soils.

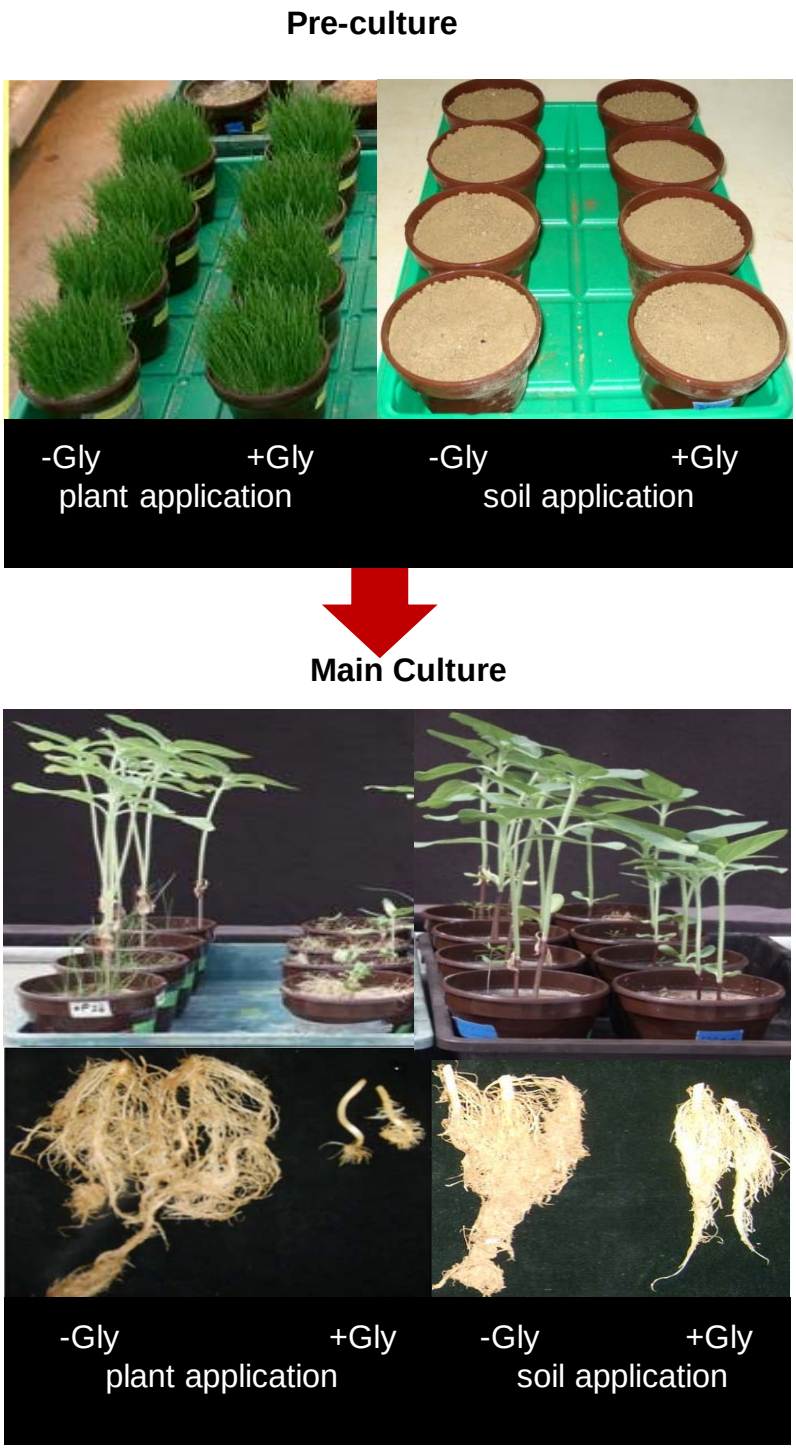


Photo 5.1. Shoot and root development of sunflower seedlings grown on an acidic Arenosol with (+Gly) or without (-Gly) pre-sowing glyphosate treatments on a pre-culture with *Lolium perenne* or direct glyphosate soil application. Photos were taken 25 days after sunflower sowing at harvest. Toxicity effect was also comparable in the Luvisol.

In contrast to direct soil application of glyphosate, the treatments with glyphosate application to the *Lolium* pre-culture were characterized by non-homogenous germination and large differences in seedling development of sunflower (Photo 5. 2). This was reflected in a high variability of biomass data (Tables 5.1 and 5.2) and intracellular shikimate accumulation in the respective treatments (Figs. 5.3 and 5.4).

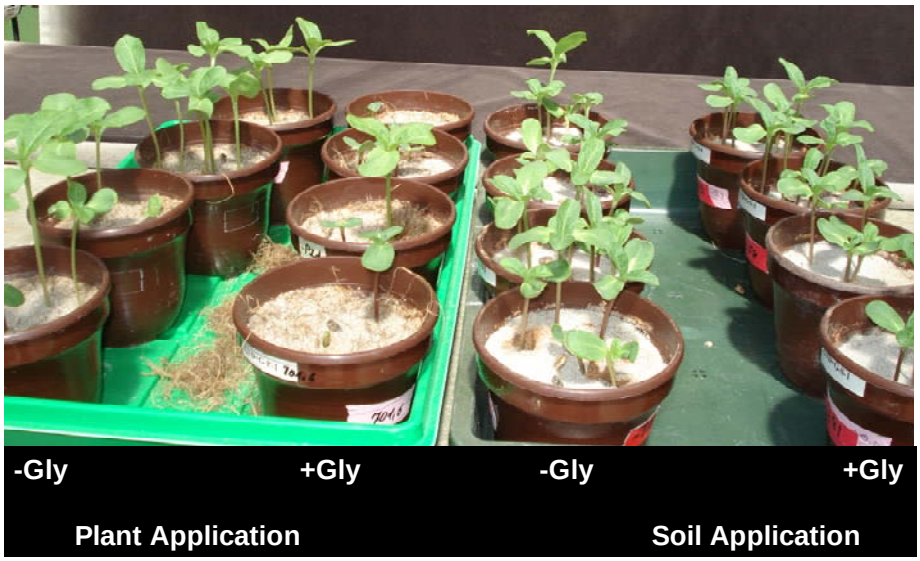


Photo 5.2. Germination and seedling development of sunflower plants grown on an acidic Arenosol soil at 21 d after desiccation of a ryegrass pre-culture by foliar glyphosate application (plant application) and after direct soil application of the same glyphosate dose (soil application).

5.3.2. Shoot and root biomass

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 zero days after glyphosate application for sowing of sunflower (Tables 5.1 and 5.2). 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% (Fig. 5.1; Tables 5.1 and 5.2). The inhibitory effects declined with increasing waiting times but still remained detectable even at 21 d after glyphosate application, although the differences were not significant in all cases.

Table 5.1. Shoot and root dry matter of sunflower plants (25 DAS) grown 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.

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}

Table 5.2. Shoot and root dry matter of sunflower plants (25 DAS) grown 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.

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}

5.3.3. Micronutrient acquisition

The pre-culture of rye grass without glyphosate application obviously increased Mn acquisition of sunflower on the Arenosol but not on the calcareous loess sub-soil (Figs. 5.1 and 5.2). On both soils, glyphosate pre-sowing treatments affected Mn concentrations in the youngest fully-expanded leaves in treatments with 0 d waiting time (Figs. 5.1 and 5.2). 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. 5.1).

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 d waiting time (Tables 5.3 and 5.4).

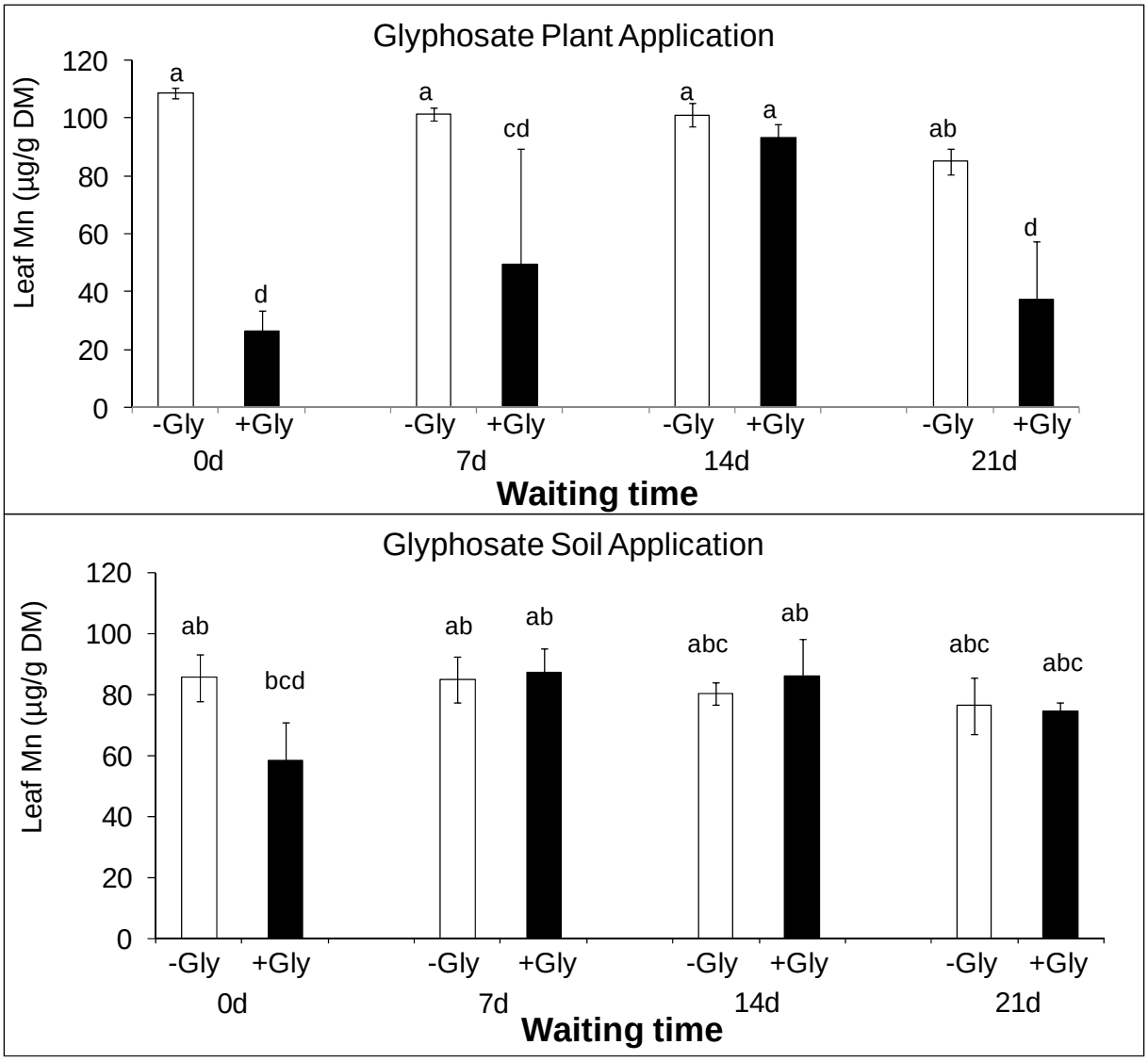


Fig. 5.1. Manganese concentration in the youngest fully expanded leaves of sunflower plants (25 DAS) grown on an acidic Arenosol with glyphosate application at 0, 7, 14 and 21 days before sowing to a pre-culture of rye grass or directly incorporated into the soil, respectively. Data represent means and standard deviations of 4 independent replicates. Significant differences between treatments are indicated by different characters.

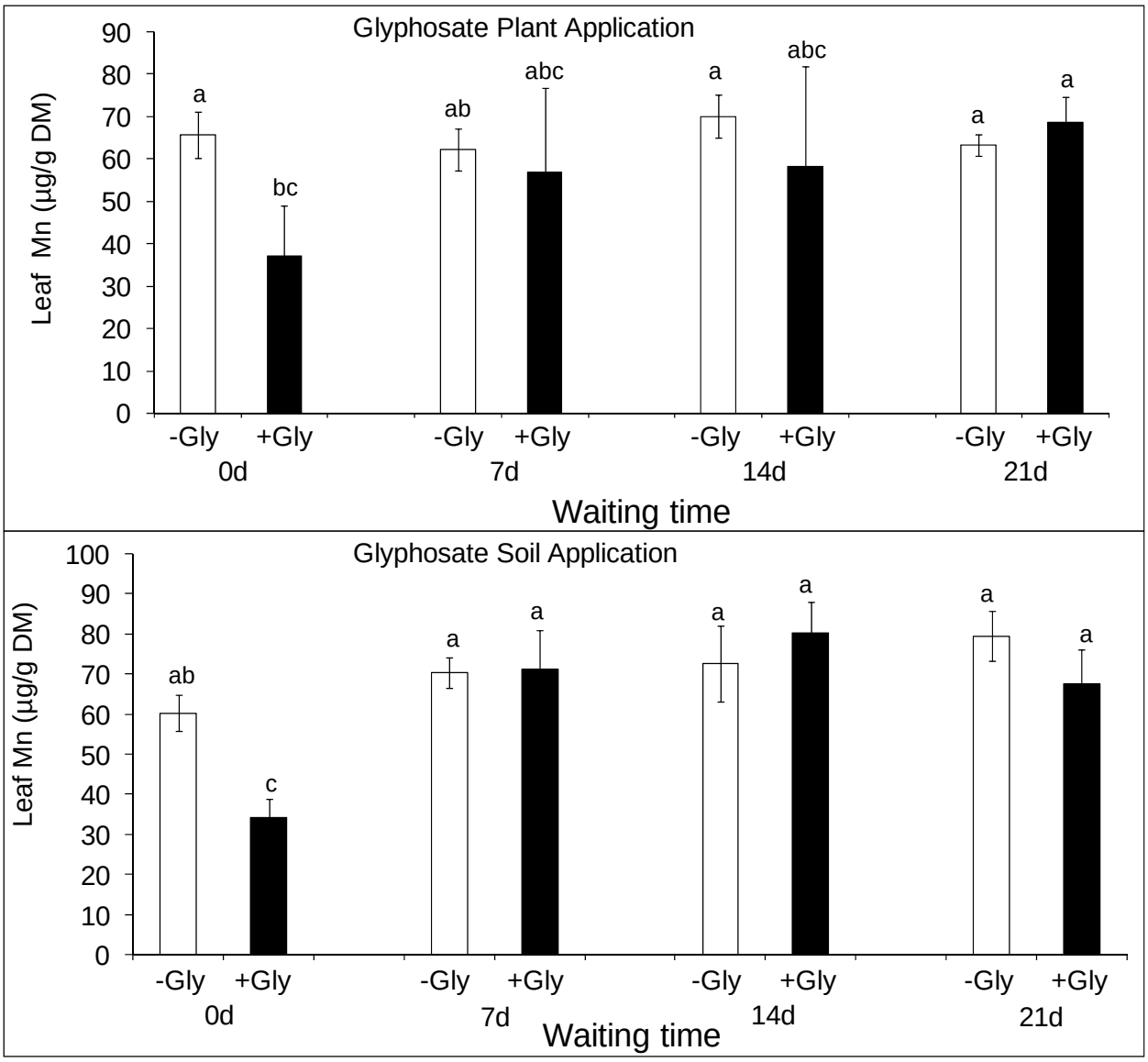


Fig. 5.2. Manganese concentration in the youngest fully expanded leaves of sunflower plants (25 DAS) grown on a calcareous loess sub-soil with glyphosate application at 0, 7, 14 and 21 days before sowing to a pre-culture of rye grass or directly incorporated into the soil, respectively. Data represent means and standard deviations of 4 independent replicates. Significant differences between treatments are indicated by different characters.

Table 5.3. Iron and Zinc concentration in the youngest fully expanded leaves of sunflower plants (25 DAS) grown on the 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.

Treatment	Fe concentration (µg/g DM)		Zn concentration (µg/g DM)	
	Plant application	Soil application	Plant application	Soil application
0d –Gly	79.7±9.9	120.1±25.8	37.3±1.7	41.3±3.5
0d +Gly	224.3±94.6	131.0±50.1	69.4±20.7	44.6±6.8
7d –Gly	59.1±7.7	88.8±16.4	40.7±3.3	47.1±10.7
7d +Gly	71.3±3.2	112.6±11.0	56.6±21.7	53.9±11.5
14d –Gly	74.3±14.8	98.4±8.9	40.4±2.5	45.6±2.8
14d +Gly	70.8±6.1	106.0±11.6	37.2±1.1	42.8±4.0
21d –Gly	100.0±7.5	107.3±8.6	37.7±3.8	36.6±1.6
21d +Gly	110.4±40.1	101.6±22.8	52.4±29.9	39.4±3.1

Table 5.4. Iron and Zinc concentration in the youngest fully expanded leaves of sunflower plants (25 DAS) grown on the Luvisol 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.

Treatment	Fe concentration (µg/g DM)		Zn concentration (µg/g DM)	
	Plant application	Soil application	Plant application	Soil application
0d –Gly	61.3±4.0	95.1±34.9	26.7±1.9	26.5±1.9
0d +Gly	138.5±11.1	64.2±11.9	60.8±10.1	29.1±8.2
7d –Gly	101.1±71.8	72.4±6.4	29.9±6.7	23.4±2.6
7d +Gly	60.5±16.5	71.7±11.0	24.0±7.3	25.2±2.6
14d –Gly	61.8±11.7	78.2±11.8	33.4±1.9	27.5±8.5
14d +Gly	119.3±59.0	87.2±7.6	34.7±21.7	29.3±2.1
21d –Gly	60.6±9.1	72.5±7.0	20.23±1.1	23.1±4.1
21d +Gly	60.6±8.9	63.5±6.7	22.8±2.3	23.7±2.1

5.3.4. Shikimate concentration in roots

The detrimental effects of glyphosate pre-sowing treatments on plant growth were reflected in a corresponding increase of shikimate concentrations in the root tissue as a physiological indicator for glyphosate toxicity (Fig. 5.3). In this case, the differences between the two glyphosate application modes already observed for inhibition of seedling growth (Tables 5.1 and 5.2) 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. 5.3).

5.3.5. General feature of measured parameters

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 (Tables 5.1 and 5.2; Figs. 5.1 –5.3).

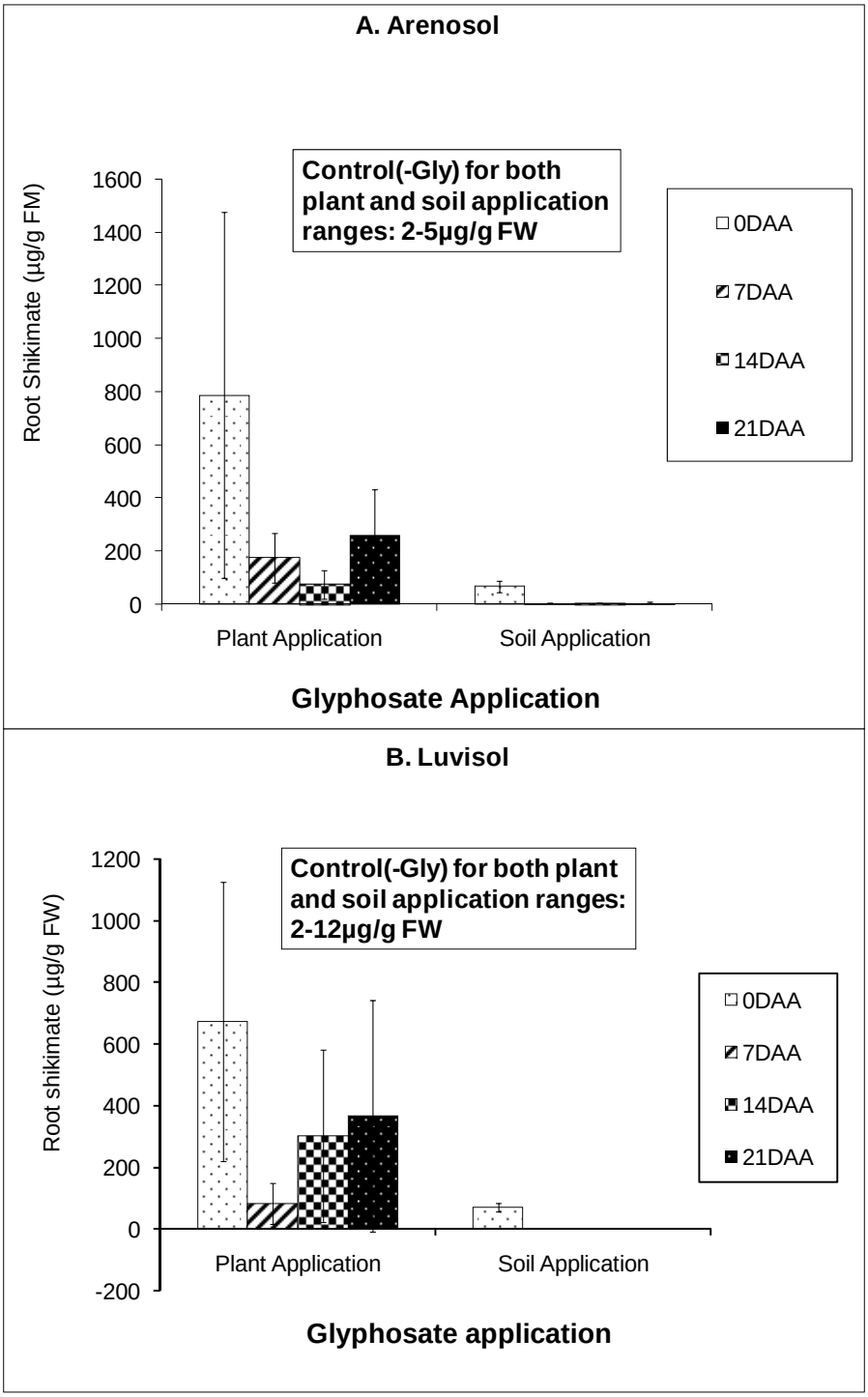


Fig. 5.3: Intracellular shikimate accumulation in the root tissue of sunflower seedlings (12 DAS) grown on an acidic Arenosol with glyphosate application at 0, 7, 14 and 21 days before sowing to a pre-culture of rye grass or directly incorporated into the soil, respectively. Data represent means and standard deviations of 4 independent replicates. The background levels of shikimate concentrations are shown as numeric values.

5.4. 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 minimize 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. 5.3), even when clearly visible effects on seedling growth and development are no more detectable by the first view (Fig. 5.2, Table 5.1 and 5.2). 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 increase 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 sub-soil (Figs. 5.1 and 5.2), 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 (Tables 5.1 and 5.2; Fig. 5.3), 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*., 1975b) and may thereby reduce the already low level of available Mn in the Arenosol. Also glyphosate-induced inhibition of root growth (Photo. 5.1; Tables 5.1 and 5.2) 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 co-factors for mechanisms of plant disease resistance (Cakmak, 2000; Datnoff *et al*., 2007; 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 *et al*., 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 d waiting time. In these cases, Fe and Zn concentrations even increased in the leaves of glyphosate-treated variants (Tables 5.3 and 5.4). 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 immobilization and inactivation in soils (Sprankle *et al*., 1975b). However, despite of much higher levels of $CaCO_3$ and of free water-extractable Ca^{2+} [59.9 mg kg^{-1} soil] and Mg^{2+} [11.3 mg kg^{-1} soil] in the calcareous sub-soil 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 (Tables 5.1 and 5.2) and intracellular shikimate accumulation (Figs. 5.3) 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 cmolc 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*., 1975a; Giesy, 2001; Monsanto, 2005a; Yamada, 2006). Accordingly, Cornish (1992) reported increased toxicity of glyphosate soil pre-treatments on tomato after simultaneous application of P fertilizers, 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 mobilization 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 metabolized and is preferentially translocated to young growing tissues of roots and shoots, where it can accumulate in millimolar concentrations (Reddy *et al*., 2004, Monsanto, pers. 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 immobilization 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 (Photo 5.2 but also Tables 5.1 and 5.2, Figs. 5.1 – 5.3) 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, while sunflower seedlings without contact to the hot spots remained unaffected. In contrast, direct soil application of glyphosate resulted in a homogenous 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 stabilization 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 lyophilized cell cultures of soybean but only the water-insoluble fraction was taken into account. Komoßa *et al*., (1992) characterized 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 (Roemheld *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 rage 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 fertilization 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).

5.5. Conclusion

Results of the present study underlines the importance of glyphosate-treated target plant roots as storage pools of glyphosate for intoxication of following crops. These findings also indicate the urgency of considering target plant roots for future risk assessments which have been yet uninvestigated in most assessments done in determining the risk of non-target plant intoxication.

The results also underline the relevance of “waiting time” after weed desiccation by glyphosate and subsequent crop planting to minimize the detrimental effect of glyphosate for non-target plant.

5.6. Prospects

Glyphosate bound to organic matter has never been considered in risk assessments so far, therefore this aspect requires further consideration in future risk assessments of residual toxicity of glyphosate considering:

- Different soil types with different binding forms for glyphosate.
- Different weed species, since decomposition rate of different root materials can be different and might take different time for determining the “waiting time”.

- Different growth conditions like, soil temperature, moisture and light, since these factor influence microbial activity in soil and thus decomposition rate of the root residues.

Dynamics of glyphosate in plant residues: Is the release of glyphosate by decaying root/crop residues an important process for intoxication of non-target plants?

6.1. Introduction

Glyphosate, a broad spectrum, non-selective herbicide, is the world´s most important and widely used herbicide. Initially the predominant use of glyphosate in agricultural production systems was for broad spectrum, non-selective weed control prior to crop sowing. This is, however, no longer the case because transgenic transformation has enabled a gene transfer for glyphosate resistance to a number of crop species, such as cotton, corn, soybean, and canola. Glyphosate is a systemic herbicide that is taken up by weed foliage and then translocated throughout the plant via the phloem and further transported to metabolic sinks such as meristems of shoots and roots (Feng *et al*., 1999). From roots, it can be released to the rhizosphere with detrimental effect on growth of following crops (Rodrique *et al*., 1982; Guldner *et al.,* 2005; Neumann *et al*., 2006).

The primary reason why glyphosate regarded as herbicide with negligible residual activities is its strong sorption characteristics on soil minerals such as clay minerals, iron and aluminum hydroxides (Piccolo *et al.*, 1994), and it is advocated that crops can be planted or seeded directly into treated areas following glyphosate application. In addition, it is believed to be easily degraded by soil microbes to natural products such as H_2O and CO_2. However, this rapid rate of glyphosate degradation by microbial metabolism represents the metabolism of the unbound glyphosate molecules in a free soil solution (Nomura and Hilton, 1977; Rueppel *et al*., 1977). The moderate persistence in soils, with reported half-lives ranging from 1 to 174 days (Wauchope et al., 1992), indicates that glyphosate degradation is a very heterogeneous process and depends on many soil and environmental factors. For instance, under low soil temperature or drought conditions following glyphosate application, glyphosate degradation in soil can be delayed as freezing and drought inhibit microbial activity, thereby the degradation of glyphosate (Stenrød *et al*., 2005).

Therefore, glyphosate stored in decaying roots and shoot of desiccated weeds can be one pool that primarily depends on the decomposition of the organic matter. The bioavailability of such plant-associated glyphosate residue is dominated by the type and strength of their bonding in the plant matrix (Wiren-Lehr *et al*., 1997). A study on

initial deposits and persistence of forest herbicide residues in sugar maple foliage shows leaf accumulation of glyphosate up to 1630 mg of acid equivalent per kg dry mass that took around 16 days to dissipate 90% of it (Thompson *et al*., 1994). From field experiments conducted to examine the fate of glyphosate in forest watershed, Feng and Thompson (1999) reported on glyphosate residues in leaf litter collected 15 days after application up to 12.5 µg g^{-1} dry mass for red alder (*Alnus oregona*, Nutt.) and 19.2 µg g^{-1} dry mass for salmonberry (*Rubus spectablis*, Pursh) that declined to less than 1µg g^{-1} within 45 days post application (DT <14 days). Further, the authors reported that in soil, glyphosate and AMPA residues were retained primarily in the upper organic layer of the profile, with >90% of the total glyphosate residue in the upper 0-15 cm layer. If such transient high accumulation of glyphosate on decaying weed holds true also to leaf litter residue under reduced tillage cropping systems, it deserves crucial attention to culminate residual phytotoxicity for a following crop.

The globally increasing adoption of no-till or reduced tillage systems like in Brazil, where most farmers practice direct drilling in which pre-sowing weed control is achieved with herbicides is also one factor pressuring farmers toward using more glyphosate in farming systems (Torresen *et al*., 1999). In such systems, glyphosate is applied pre-sowing for weed control in cereals or soybeans and glyphosate residue may remain in the straw and soil disturbance practice occurs usually only at crop sowing, which might lead to incorporation of the glyphosate contaminated straw to the upper soil layer where seed germination occurs. Hence roots of germinating seedlings may directly come in contact to the glyphosate contaminated weed residues. The following uptake of released glyphosate can cause sufficient phytotoxicity. For example, soil incorporation of glyphosate treated finely chopped water hyacinth (*Eichhornia crassipes* (Mart.) salms) shoot material led to reduced plant growth of tomato (*Lycopersicon esculentum* Mill.) seedlings (Stocker and Haller, 1999).

The objective of the present study was to investigate the potential phytotoxicity risk of glyphosate in reduced tillage systems where glyphosate is employed as means of weed control and minimal tillage is done during sowing without removing the glyphosate desiccated weed residues. Furthermore, the experiments intended to explore whether the decaying shoot straw or the root is an important reservoir of glyphosate for intoxication of the following non-target crop. In addition, the

decomposition rate of the plant residues and soil type factors were considered in these studies as important factors playing vital role in glyphosate dynamics in organic residues. For this purpose, an experiment was set up using two contrasting soils (weakly buffered Arenosol and highly buffered Luvisol) where a model weed (rye grass) pre-cultivated and desiccated by glyphosate was incorporated as shoot or root straw (chopped into 1 cm pieces) and homogenate (ground under liquid nitrogen) . It was expected that soil buffering capacity (available Ca^{2+} and Mg^{2+}) and organic matter decomposition rate play an important role in detoxifying the residual toxicity of glyphosate.

6.2. Materials and methods

6.2.1. Conditions for rye grass pre-culture

Rye grass weed was cultivated in continuously aerated nutrient solution. 2.26 g of seeds were grown directly in 2.5 L pots containing full strength nutrient solution and 12 pots were prepared for producing enough shoot and root material for the whole experiment. At the beginning, seeds were rolled in 10 pieces of wet glass wool and each glass wool inserted to each hole of the pot cover. The pots were made full so that the lower parts of the glass wool touches the nutrient solution to allow continues diffusion of nutrient solution to the seeds. Then pots covered with black plastic sheet to facilitate germination. Nutrient solution was replaced every three days until enough biomass of rye grass was produced.

After producing enough shoot and root material, pots were grouped into two, i.e., 6 pots for glyphosate treatment and 6 for without glyphosate treatment. By measuring leaf length and width, leaf area per pot was calculated. Two of the pots decided to be used for plus glyphosate treatment had smaller leaf area (6093 cm^2), while the rest four had 7802 cm^2. Hence, translating the recommended field application rate of glyphosate, i.e., 200L of 28.4mM glyphosate solution per hectare, the first two pots sprayed with 12.19 ml of 28.4mM glyphosate spray solution using a hand sprayer and the rest four received 15.60 ml of the same solution. Pots assigned for without glyphosate treatment were sprayed with distilled water.

Twelve hours after glyphosate treatment rye grass as model weed were harvested and separated into shoots and roots (as schematically depicted in Photo 6.1). Plant material from all plus glyphosate were bulked together and the same for the minus

glyphosate pots. Further, the glyphosate treated or untreated shoot and root separated into two groups to be used as straw or homogenate. Then, the shoot and root material intended to be applied as straw was chopped into 1 cm length using a scissor. The once intended to be applied as homogenate were rolled in an aluminium foil as plus or minus glyphosate for later homogenization. At this point all shoot and root materials were deep frozen under liquid nitrogen and stored at -20°C. Later, the shoot and root material decided to be applied as homogenate was ground under liquid nitrogen using mortar and pestle.

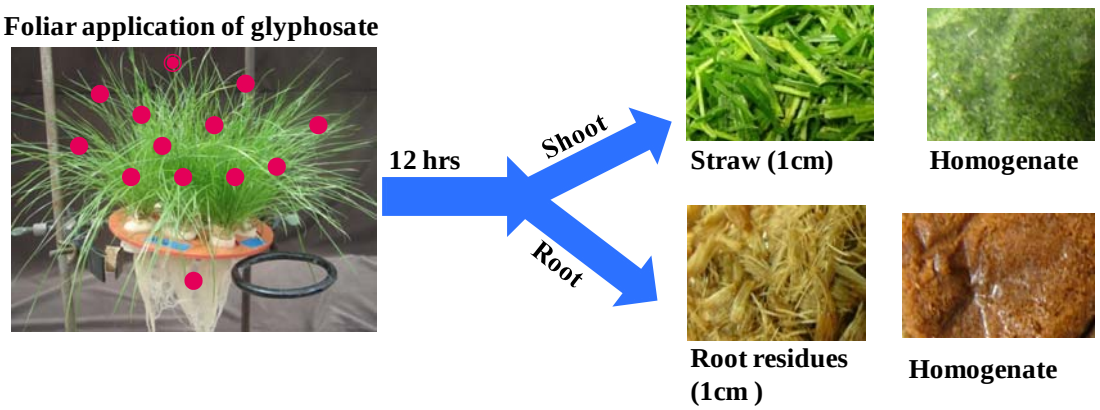


Photo 6.1. Schematic description of rye grass production, treatment with glyphosate and further preparation to be applied as shoot/root straw or homogenate.

6.2.2. *Conditions for sunflower plant growth*

Experiments were conducted under greenhouse conditions, using two contrasting soils: a sandy acidic Ap horizon of an Arenosol (pH ($CaCl_2$) =4.5; C_{org} 0.16%) with low buffering capacity and a well-buffered calcareous loess subsoil (pH ($CaCl_2$) 7.6; C_{org} <0.3%; $CaCO_3$ 30%). Soils were sieved to pass through a 2 mm mesh size and fertilized with essential nutrients (for details see chapter 2).

During fertilization, the glyphosate-treated or untreated rye grass shoot/root was mixed with the soil. They were applied as chopped residues (straw) or homogenates. In the case of straw, the shoot or root material from glyphosate-treated or untreated rye grass was cut into 1 cm pieces using a scissor, while the rest glyphosate treated or untreated shoot and root were homogenized under liquid nitrogen to be applied as homogenate. For treatments with shoot straw and homogenate application, 6 g fresh

weight (equivalent to 1200 mg dry matter kg^{-1} soil) shoot material was applied per pot of 500 g air dried soil. For the root application treatments, 3.5 g fresh weight (equivalent to 700 mg dry matter kg^{-1} soil) root material was applied as straw or homogenate for each pot with 500 g soil. These amounts for the shoot and root were decided based on preliminary experiments done to measure the shoot and root biomass production of rye grass grown on the same pots cultivated till good covering of the soil. Additional controls were considered by direct application of 2.36 ml of 28.4 mM glyphosate spray solution per 500 g soil and positive controls of bare soil without any glyphosate application.

Then soils were filled to 500 g pots and 7 seeds of sunflower (*Helianthus annuus* L. cv Frankasol) were directly sown. Ten days after sowing, five seedlings were thinned leaving only two plants for further growth and the shoot and root materials of the five thinned seedlings were deep frozen under liquid nitrogen for shikimate analysis. The remaining two sunflower plants were grown for a total of 26 days under hot summer conditions in a greenhouse and water losses were determined gravimetrically and replaced by daily applications of de-ionized water.

4.2.3. Plant harvest

Plants were harvested separating shoot and root for biomass determination. Shoot material fresh weight was recorded by direct weighing using gravimetric balance. Youngest fully expanded leaves were selected for mineral analysis. Root systems were washed out with water and carefully pressed between tissue paper for drying, then weighed for recording fresh weight.

4.2.4. Shikimate analysis

The frozen root tissue was homogenized 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 (for details see chapter 2).

4.2.5. Statistical analysis

All treatments comprised 4 replicates and pots were arranged in the greenhouse in a complete randomized block design. Analysis of variance was performed with SPSS statistics software package (SPSS Inc. Illinois, U.S.A).

6.3. Results

6.3.1. Visual plant growth

There was a striking difference between the two soils with respect to the inhibition of shoot and root growth by glyphosate residues from decaying glyphosate-treated rye grass organic matter. In the Arenosol, incorporation of glyphosate-treated rye grass shoot material induced a strong inhibition of sunflower shoot and root growth, while in the Luvisol there was no visible reduction in shoot or root growth induced by glyphosate application (Photo 6.1). Visually, sunflower plants grown on the Luvisol looked higher and stronger than plants grown on the Arenosol.

Photo 6.2. Depression of shoot and root growth of sunflower seedlings by glyphosate-treated rye grass residues incorporated either into a highly buffered Luvisol or a less buffered Arenosol. Residues were applied at a rate of 1200 mg dry matter kg^{-1} soil. Photos were taken 26 days after sowing of sunflower.

6.3.2. Plant height

Corresponding to visual observation, sunflower plants grown on the Arenosol supplied with glyphosate treated rye grass shoot straw or homogenate were stunted (Fig. 6.1A). Plant height was reduced by about 75% in the treatments with the glyphosate-treated residues or the corresponding homogenates. Glyphosate-treated root residues or homogenates incorporated into both soils, however, caused no significant effect on plant height (Fig. 6.1A). Direct soil application on the other hand resulted to similar plant height reduction as the glyphosate treated shoot straw or homogenate (Fig. 6.1A). In contrast, there was no plant height difference between all treatments in the Luvisol soil (Fig. 6.1B).

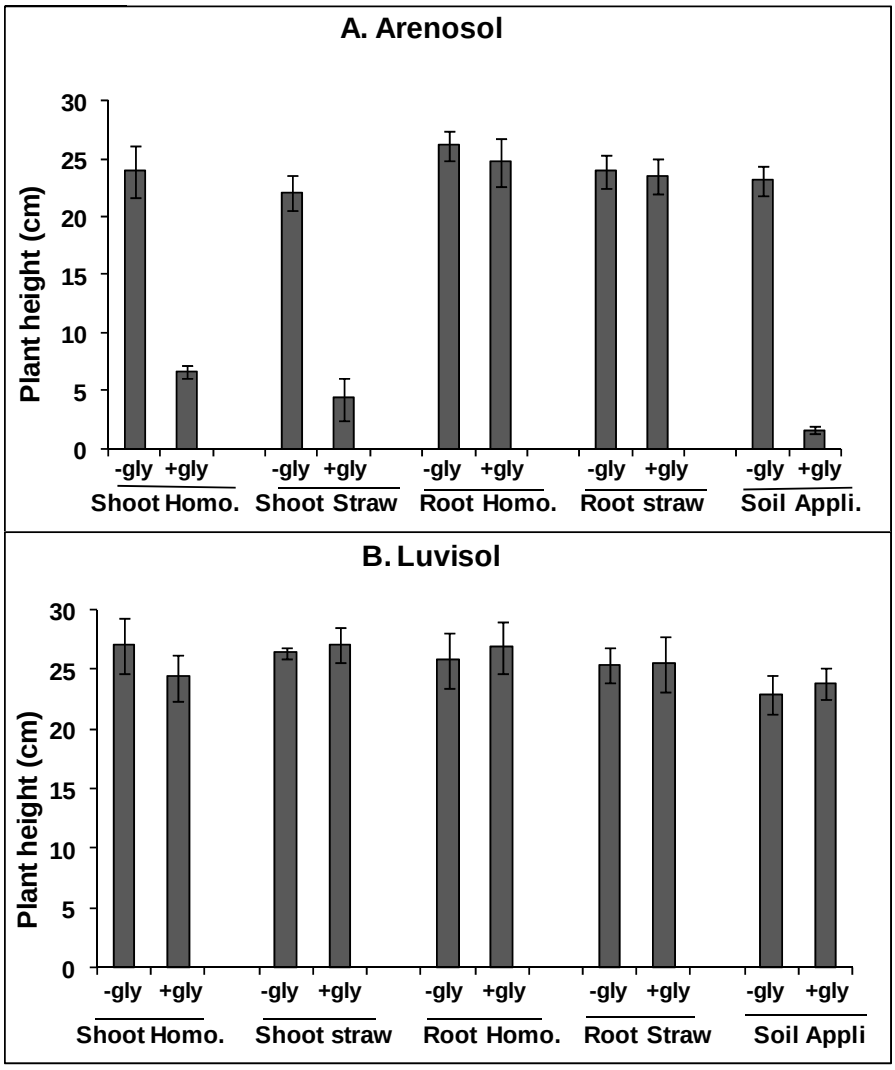


Fig.6.1. Plant height of sunflower seedlings grown on Arenosol and Luvisol supplied with glyphosate-treated rye grass shoots or roots as straw (cut to 1 cm piece) or homogenate (ground under liquid nitrogen). Shoot material was supplied on 1200 mg dry matter kg^{-1} soil and the root material on 700 mg dry matter kg^{-1} soil. Plant heights were measured at harvest, 26 days after sowing. The given data are the averages of four replicates ±SD.

6.3.3. Shoot and root biomass

Proceeding to the visual observation, there was a strong reduction in shoot and root biomass of sunflower seedlings grown in the Arenosol supplied with glyphosate-treated rye grass shoot straw or homogenate compared to controls (-glyphosate) and treated root (as straw or homogenate) supply (Fig. 6.2 A and B). Direct soil application also resulted to similar level of growth inhibition of shoots and roots as the glyphosate treated shoot straw or homogenate incorporation (Fig. 6.2 A and B). Shoot growth inhibition was stronger than root growth inhibition as can be seen in Fig.6.2. The observed relative inhibition by glyphosate-treated shoot residues of shoot growth was 88% compared to the non-glyphosate treated control and 81% inhibition of root growth (Fig. 6.2 A and B)

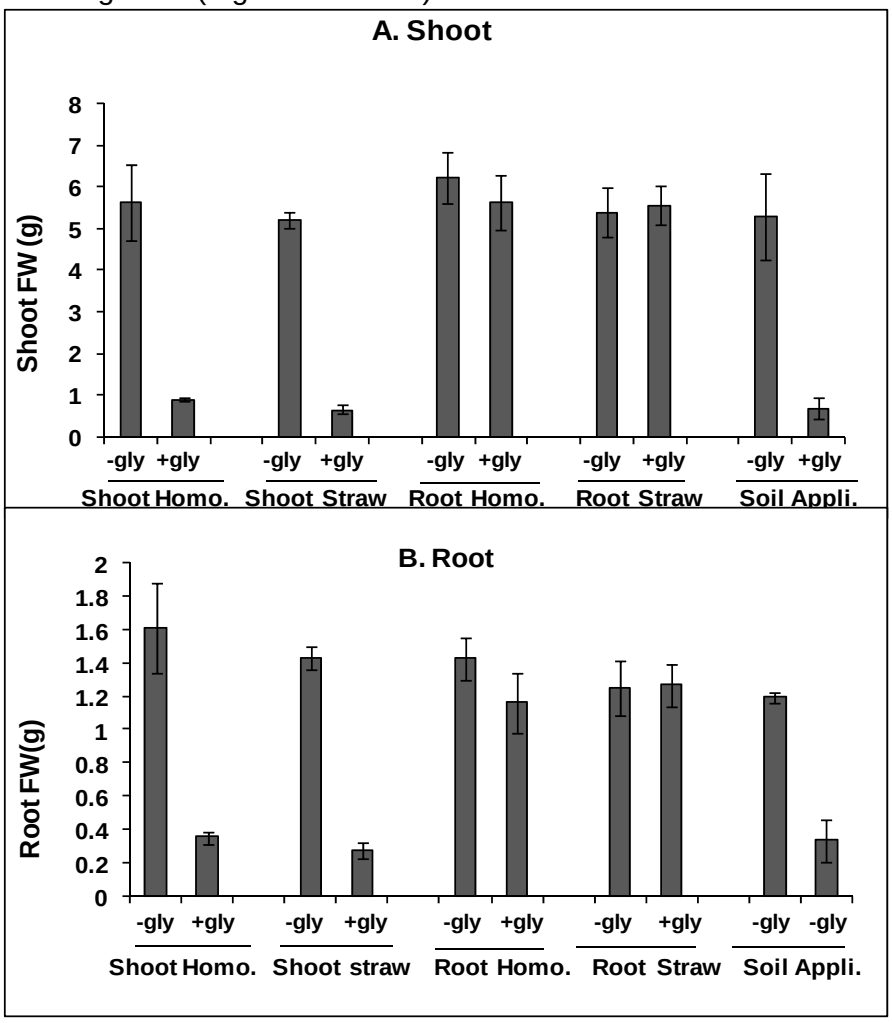


Fig.6.2. Shoot and root fresh weight of sunflower seedlings grown on the Arenosol supplied with glyphosate-treated rye grass shoots or roots incorporated either as straw (cut into 1 cm pieces) or homogenates (ground under liquid nitrogen). Shoot material was supplied on 1200 mg dry matter kg^{-1} soil and the root material on 700 mg dry matter kg^{-1} soil. The given data are the average of 4 replicates ±SD.

In contrast to the Arenosol, in the Luvisol incorporation of glyphosate-treated rye grass as shoot or root residues or homogenates had no effect on sunflower growth (Fig. 6.3 A and B). No significant difference in fresh weight of shoots or roots could be recorded. In some plus glyphosate treatments tended to show a higher fresh weight than the minus glyphosate treatments, but without a significant statistical difference (Fig. 6.3 A and B).

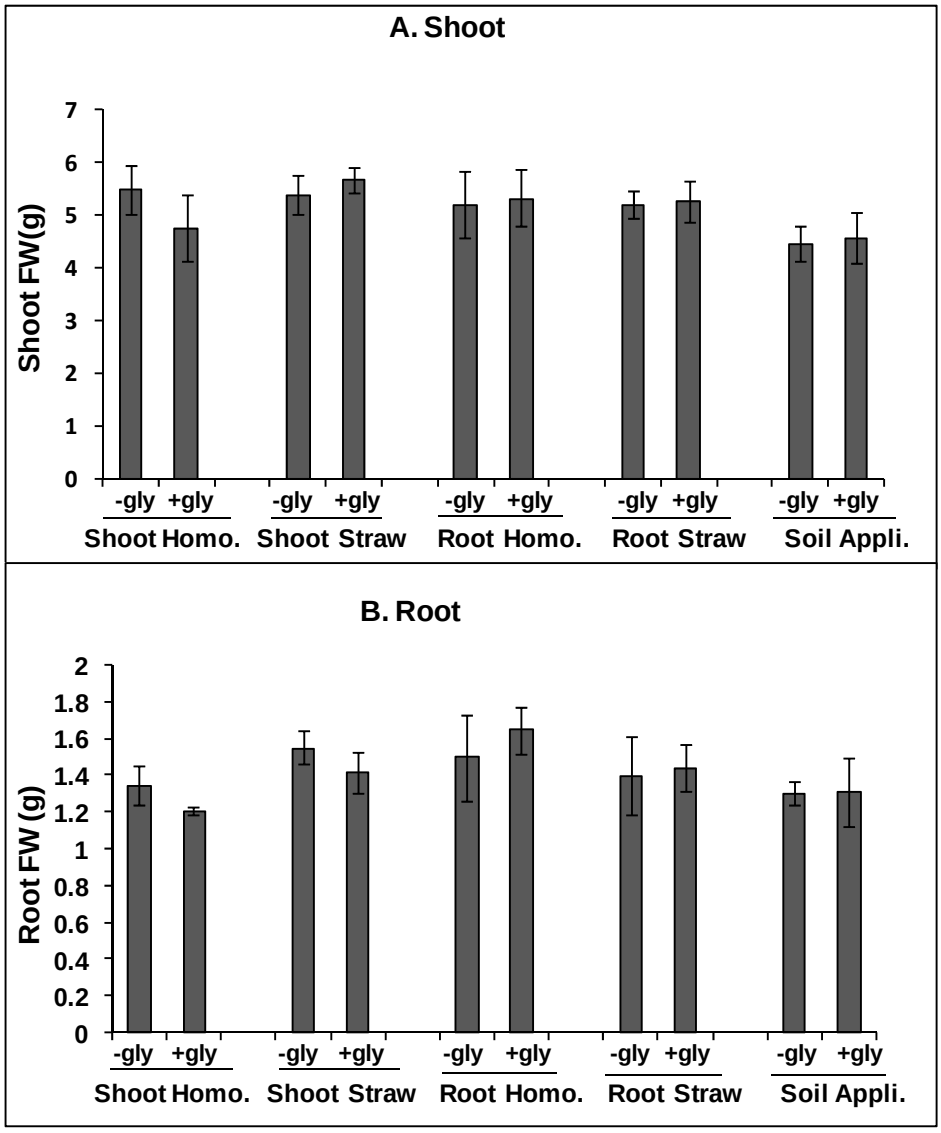


Fig.6.3. Shoot and root fresh weight of sunflower seedlings grown the Luvisol supplied with glyphosate-treated rye grass shoots or roots, incorporated either as straw (cut into 1 cm pieces) or homogenates (ground under liquid nitrogen). Shoot material was supplied on 1200mg dry matter kg^{-1} soil and the root material on 700 mg dry matter kg^{-1} soil. Plant fresh weight was determined at harvest, 26 days after sowing. The given data are the averages of 4 replicates ±SD.

6.3.4. Mineral nutrient acquisition

6.3.4.1. A. Micronutrient concentration in leaves

Parallel to inhibition of plant growth, micronutrient concentrations in leaves of sunflower seedlings grown on the Arenosol supplied with glyphosate-treated rye grass shoot straw or homogenates were significantly lower compared to controls (-glyphosate) and treatments with root residues or homogenates containing glyphosate (tables 6.1 and 6.2). Leaf concentration of Mn significantly decreased by application of glyphosate treated shoot straw (also in tendency by shoot homogenate) compared to controls (-glyphosate) (table 6.1). Similarly, Mn concentration decline due to a direct soil application of glyphosate in to the Arenosol (Table 6.1). In contrast to Mn concentration, Fe and Zn concentrations increased by application of glyphosate-treated rye grass shoot straw in to the Arenosol (Table 6.1). This increased Fe and Zn leaf concentrations could also be observed by a direct glyphosate application in to the soil (Table 6.1). But this increase in Fe and Zn concentration in leaves is possibly attributed to dilution effect as plant biomass production in the glyphosate treated shoot straw and homogenate were extremely stunted and this is also confirmed by the lower Fe and Zn content per plant on those treatments (Table 6.2).

In contrast, in the Luvisol there was no effect on leaf concentration of micronutrients associated to glyphosate phytotoxicity related to incorporation of glyphosate treated rye grass shoot/root residues and also direct soil application (tables 6.3). Shoot micronutrients (Fe, Mn, and Zn) analysis of the youngest fully expanded leaves showed no nutrient concentration difference between all the treatments, including the direct soil application (tables 6.3).

6.3.4.1. B. Micronutrient content

In line to declined leaf concentration, Mn contents also declined even at a stronger expression due to combined effect of inhibited Mn acquisition (Table 6.1) and growth inhibition (Fig. 6.2) by application of glyphosate-treated rye grass shoot material applied as either shoot straw or homogenate or by direct soil application of glyphosate in to the Arenosol (Table 6.2). Despite an increased Fe and Zn concentrations in leaves due to glyphosate-treated rye grass shoot residues or direct soil application in to the Arenosol, Fe and Zn contents were significantly lower

compared to the controls (-glyphosate) (Table 6.2), probably due to growth inhibition (Fig. 6.2).

In contrast, in the Luvisol there was no reduction in micronutrient contents of the sunflower seedlings associated to glyphosate phytotoxicity (Table 6.4).

These results indicate a soil type dependent differential phytotoxicity of glyphosate possibly related to a soil detoxification capacity, i.e., the organic matter associated glyphosate residual phytotoxicity is expressed on the weakly buffered acidic Arenosol but not in the highly buffered calcareous Luvisol.

Furthermore, treatment of the organic matter (homogenization under liquid nitrogen) to accelerate decoposition rate did not cause any differential phytotoxic effect since there was no difference in plant growth, nutrient acquisition and intracelular shikimate accumulation between straw and homogenate treatments on the two soils (Figs 6.1-6.6 and tables 6.1-6.5).

Generally, youngest fully expanded leaf Fe, Mn and Zn concentration of sunflower plants grown on the Arenosol was higher than the Luvisol (tables 6.1 -6.4).

Table 6.1. Micronutrient concentration (µg g^{-1} DM) of youngest fully expanded leaves of sunflower seedlings grown in the Arenosol supplied either with shoot or root residues or homogenates of glyphosate-treated rye grass. Shoot material was supplied at 1200 mg dry matter kg^{-1} soil and the root material at 700 mg dry matter kg^{-1} soil. Leaves for micronutrient analysis were collected after 26 days growth at harvest. Each given value presents the average of 4 replicates ±SD. Different letters along the same column denote significant difference from each other at P<0.05 Tukey test.

Treatment	Mincronutrient concentration (µg g^{-1} DM)		
	Fe	Mn	Zn
Shoot-H-gly	109.0±19.7^{d}	226.9±43.2^{bc}	66.5±2.7^{c}
Shoot-H+gly	238.6±52.7^{bcd}	71.0±23.7^{c}	72.7±2.2^{bc}
Shoot-S-gly	146.0±36.6^{bcd}	271.4±77.0^{b}	74.5±4.5^{bc}
Shoot-S+gly	254.2±29.7^{ab}	59.1±13.2^{c}	81.4±8.1^{ab}
Root-H-gly	132.8±13.1^{cd}	255.5±48.2^{b}	64.7±1.9^{c}
Root-H+gly	141.2±17.4^{bcd}	184.9±30.7^{bc}	65.0±3.5^{c}
Root-S-gly	262.5±51.1^{a}	274.6±61.9^{b}	64.6±1.7^{c}
Root-S+gly	182.3±17.5^{abcd}	280.8±53.5^{b}	62.9±5.7^{c}
Soil-gly	181.4±19.1^{abcd}	548.2±176.7^{a}	65.8±3.3^{c}
Soil+gly	253.2±113.7^{ab}	74.1±28.6^{c}	88.0±10.8^{a}

Table 6.2. Micronutrient content (µg per plant) of youngest fully expanded leaves of sunflower seedlings grown in the Arenosol supplied either with shoot or root residues or homogenates of glyphosate-treated rye grass. Shoot material was supplied at 1200 mg dry matter kg^{-1} soil and the root material at 700 mg dry matter kg^{-1} soil. Leaves for micronutrient analysis were collected after 26 days growth at harvest. Each given value presents the average of 4 replicates ±SD. Different letters along the same column denote significant difference from each other at P<0.05 Tukey test.

Treatment	Micronutrient content (µg per plant)		
	Fe	Mn	Zn
Shoot-H-gly	37.5±8.2^{bcd}	77.2±12.3^{b}	23.0±4.6^{a}
Shoot-H+gly	17.1±2.9^{cd}	5.2±1.8^{c}	5.3±0.4^{b}
Shoot-S-gly	42.5±10.1^{bc}	79.2±22.6^{b}	21.9±1.4^{a}
Shoot-S+gly	14.0±4.6^{d}	3.3±1.4^{c}	4.4±0.8^{b}
Root-H-gly	48.6±9.4^{b}	92.4±16.2^{b}	23.5±2.3^{a}
Root-H+gly	47.8±5.1^{b}	63.3±14.4^{b}	22.0±1.6^{a}
Root-S-gly	86.9±24.2^{a}	89.7±18.8^{b}	21.4±3.8^{a}
Root-S+gly	58.7±7.0^{b}	89.7±12.9^{b}	20.3±2.3^{a}
Soil-gly	60.0±13.6^{b}	174.2±35.0^{a}	21.6±3.0^{a}
Soil+gly	12.9±5.8^{d}	3.9±1.2^{c}	4.8±1.6^{b}

Table 6.3. Micronutrient concentration (µg g^{-1} DM) of youngest fully expanded leaves of sunflower seedlings grown in the Luvisol supplied either with shoot or root residues or homogenates of glyphosate-treated rye grass. Shoot material was supplied at 1200 mg dry matter kg^{-1} soil and the root material at 700 mg dry matter kg^{-1} soil. Leaves for micronutrient analysis were collected after 26 days growth at harvest. Each given value presents the average of 4 replicates ±SD. Different letters along the same column denote significant difference from each other at P<0.05 Tukey test.

Treatment	Mincronutrient concentration (µg g^{-1} DM)		
	Fe	Mn	Zn
Shoot-H-gly	102.1±8.6^{a}	137.8±7.2^{abcd}	31.0±3.8^{a}
Shoot-H+gly	108.6±20.0^{a}	145.8±3.4^{a}	30.7±5.9^{a}
Shoot-S-gly	98.2±8.8^{a}	139.2±3.1^{abc}	28.4±2.5^{a}
Shoot-S+gly	141.9±34.0^{a}	139.3±11.1^{abc}	28.2±0.7^{a}
Root-H-gly	103.0±14.0^{a}	141.2±8.2^{ab}	27.1±3.0^{a}
Root-H+gly	110.3±23.1^{a}	134.4±7.1^{abcd}	26.0±3.5^{a}
Root-S-gly	123.6±33.8^{a}	127.3±5.0^{bcd}	25.4±1.7^{a}
Root-S+gly	100.8±13.6^{a}	122.2±3.8^{d}	26.0±3.4^{a}
Soil-gly	112.3±17.2^{a}	124.1±3.6^{cd}	25.0±3.5^{a}
Soil+gly	110.9±14.3^{a}	124.6±7.8^{cd}	26.9±1.9^{a}

Table 6.4. Micronutrient content (µg per plant) of youngest fully expanded leaves of sunflower seedlings grown in the Luvisol supplied either with shoot or root residues or homogenates of glyphosate-treated rye grass. Shoot material was supplied at 1200 mg dry matter kg^{-1} soil and the root material at 700 mg dry matter kg^{-1} soil. Leaves for micronutrient analysis were collected after 26 days growth at harvest. Each given value presents the average of 4 replicates ±SD. Different letters along the same column denote significant difference from each other at P<0.05 Tukey test.

Treatment	Micronutrient content (µg per plant)		
	Fe	Mn	Zn
Shoot-H-gly	38.3±1.4^{ab}	52.0±5.7^{a}	11.8±2.2^{a}
Shoot-H+gly	34.5±5.9^{b}	46.6±5.8^{a}	9.8±1.7^{ab}
Shoot-S-gly	36.6±2.5^{ab}	52.0±3.1^{a}	10.6±1.5^{ab}
Shoot-S+gly	52.9±10.1^{a}	52.3±4.2^{a}	10.6±0.5^{ab}
Root-H-gly	36.0±5.4^{b}	50.0±9.9^{a}	9.4±0.7^{ab}
Root-H+gly	40.2±9.9^{ab}	48.8±5.2^{a}	9.5±2.0^{ab}
Root-S-gly	43.3±10.3^{ab}	45.0±3.8^{a}	9.0±0.6^{ab}
Root-S+gly	36.2±4.3^{b}	44.2±5.5^{a}	9.3±0.8^{ab}
Soil-gly	35.9±5.6^{b}	39.7±1.7^{a}	8.0±0.9^{b}
Soil+gly	35.9±6.0^{b}	40.5±6.1^{a}	8.7±1.2^{ab}

6.3.4.2. Macronutrients

Similar to Mn, concentrations and contents of Ca in youngest fully expanded leaves of sunflower seedlings was also strongly inhibited by incorporation of glyphosate-treated rye grass shoot straw and homogenate or a direct soil application of glyphosate on the Arenosol (Fig. 6.4 A and B). Interestingly, Ca concentration in leaves of sunflower plants grown on treatments supplied with glyphosate-treated rye grass shoot straw or homogenate were significantly lower than in plants grown on treatments supplied by direct soil application of equivalent amount of glyphosate (Fig. 6.4A). Compared to controls (-glyphosate) however, direct soil application of glyphosate induced reduction of leaf Ca concentration (Fig. 6.4A). Similarly, Mg content was also significantly reduced by glyphosate-treated rye grass shoot straw or homogenate supply, which is not seen in shoot concentration due to dilution effect (table 6.5). Direct soil application of comparable amount of glyphosate also caused similar reduction in Mg content of sunflower seedlings grown on the Arenosol (table 6.5).

In contrast to the Arenosol, in the Luvisol however, sunflower leaf concentration and content of micro and macronutrients were not affected by incorporation of glyphosate-treated rye grass shoot/root or even direct soil application of comparable amount of glyphosate. Shoot micronutrients (Fe, Mn, and Zn) and Macronutrients (Ca and Mg) analysis of the youngest fully expanded leaf showed no nutrient concentration difference between all the treatments, including the direct soil application (Figs. 6.4; 6.5 and table 6.5). Exceptionally to all results in this experiment, sunflower shoot Mg concentration in glyphosate treated root straw application treatments was significantly lower than untreated root straw applications but possibly this is an artifact than treatment effect (table 6.5).

Generally youngest fully expanded leaf Ca and Mg concentration of sunflower seedlings grown on the Luvisol was higher than Arenosol (Fig. 6.4A and 6.5A; table 6.5).

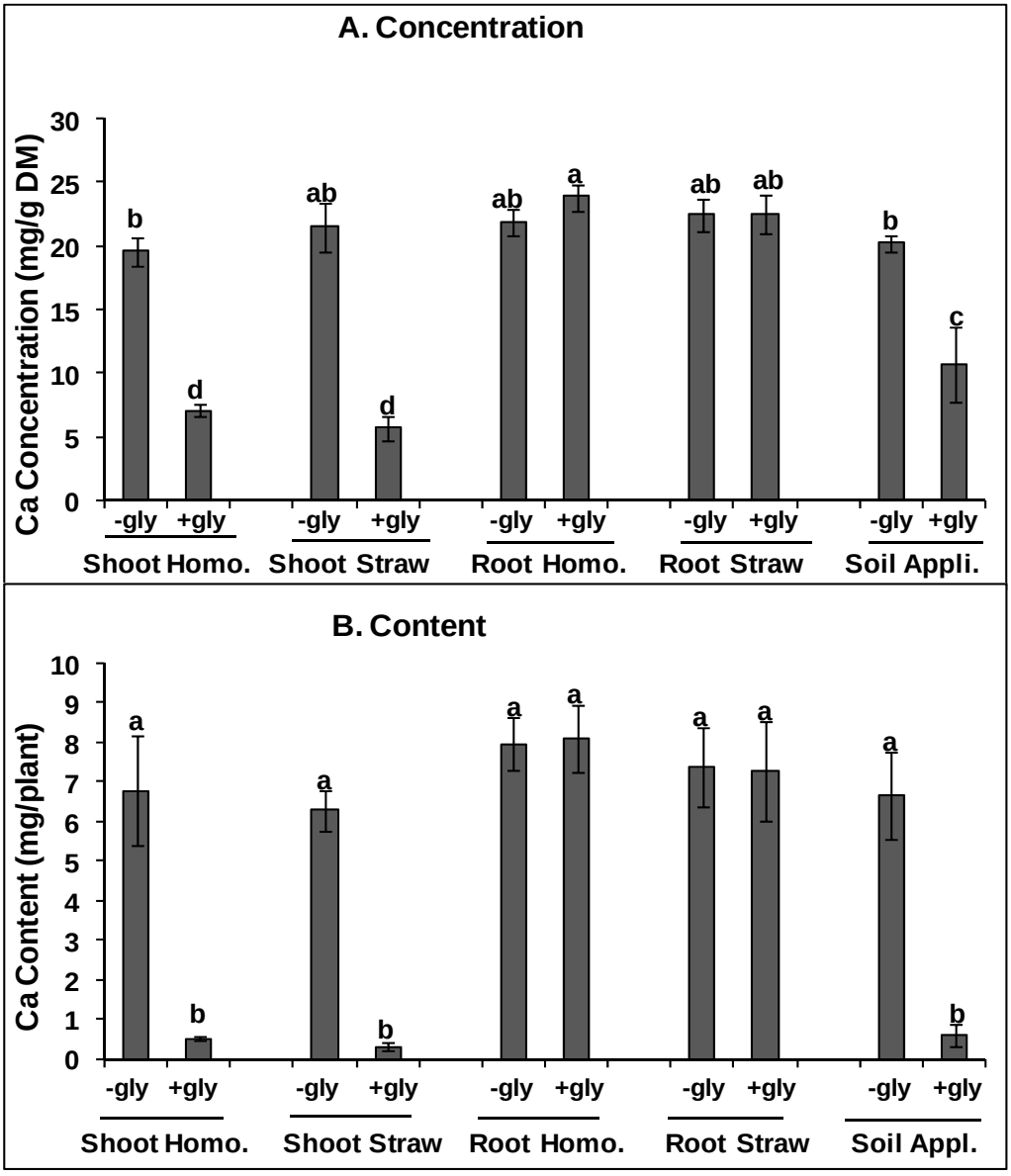


Fig.6.4. Ca concentration (mg g^{-1} DM) and content (µg per plant) of youngest fully expanded leaves of sunflower seedlings grown in the Arenosol supplied either with shoot or root residues or homogenates of glyphosate-treated rye grass. Shoot material was supplied at 1200 mg dry matter kg^{-1} soil and the root material at 700 mg dry matter kg^{-1} soil. Leaves for macronutrient analysis were collected after 26 days growth at harvest. Given data present average of 4 replicates with SD as bars, $P<0.05$.

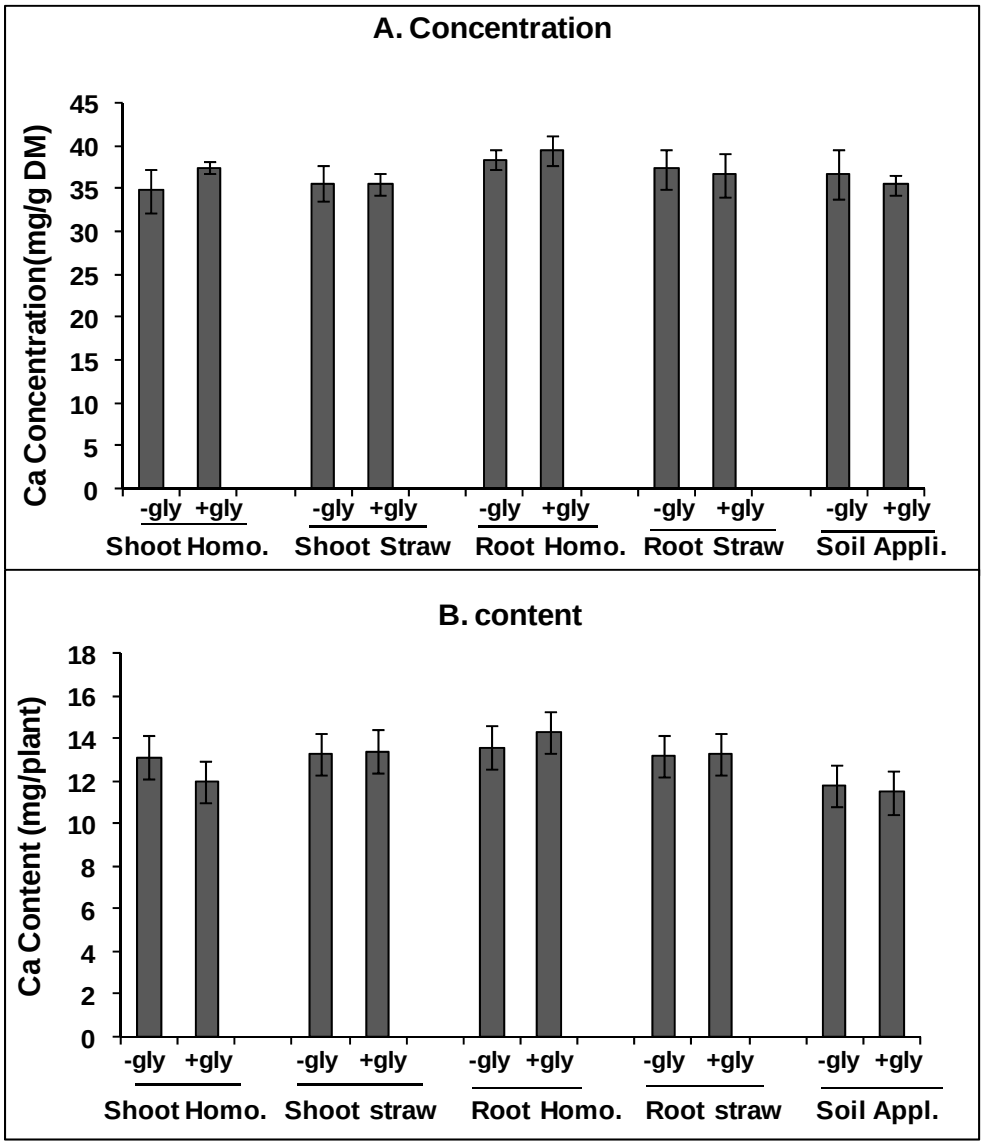


Fig.6.5. Ca concentration (mg g^{-1} DM) and content (µg per plant) of youngest fully expanded leaves of sunflower seedlings grown in the Luvisol supplied either with shoot or root residues or homogenates of glyphosate-treated rye grass. Shoot material was supplied at 1200 mg dry matter kg^{-1} soil and the root material at 700 mg dry matter kg^{-1} soil. Leaves for macronutrient analysis were collected after 26 days growth at harvest. Given data present average of 4 replicates with SD as bars, $P<0.05$.

Table 6.5. Mg concentration (mg g^{-1} DM) and content (mg per plant) of youngest fully expanded leaves of sunflower seedlings grown in the Arenosol and Luvisol supplied either with shoot or root residues or homogenates of glyphosate-treated rye grass. Shoot material was supplied at 1200 mg dry matter kg^{-1} soil and the root material at 700 mg dry matter kg^{-1} soil. Leaves for mineral analysis were collected after 26 days growth at harvest. Each given value presents the average of 4 replicates ±SD. Different letters along the same column denote significant difference from each other at P<0.05 Tukey test.

Treatment	**Arenosol**		**Luvisol**	
	Mg Concentration (mg g^{-1} DM)	Mg Content (mg per plant)	Mg oncentration (mg g^{-1} DM)	Mg Content (mg per plant)
Shoot-H-gly	5.8±0.5^{abc}	2.0±0.6^{ab}	13.0±0.6^{d}	4.9±0.4^{ab}
Shoot-H+gly	5.4±0.2^{bc}	0.4±0.0^{c}	12.3±0.6^{d}	3.9±0.7^{b}
Shoot-S-gly	5.8±0.6^{abc}	1.7±0.2^{b}	12.9±0.7^{d}	4.8±0.4^{ab}
Shoot-S+gly	5.2±0.7^{c}	0.3±0.1^{c}	13.3±0.3^{cd}	5.0±0.4^{ab}
Root-H-gly	7.0±0.5^{a}	2.6±0.3^{a}	15.8±1.4^{ab}	5.6±1.4^{a}
Root-H+gly	7.1±0.5^{a}	2.4±0.4^{ab}	15.1±0.5^{abc}	5.5±0.6^{ab}
Root-S-gly	6.9±0.4^{a}	2.3±0.5^{ab}	15.2±0.9^{ab}	5.4±0.7^{ab}
Root-S+gly	6.7±0.6^{ab}	2.2±0.4^{ab}	15.0±0.9^{bc}	5.4±0.8^{ab}
Soil-gly	6.4±0.1^{abc}	2.1±0.3^{ab}	16.8±0.8^{a}	5.4±0.5^{ab}
Soil+gly	6.5±1.0^{abc}	0.4±0.1^{c}	16.0±0.3^{ab}	5.2±0.5^{ab}

6.3.5. Intracellular shikimate accumulation

Fig. 6.6 presents data on shikimate accumulation in roots of sunflower seedlings grown in the Arenosol and Luvisol for 10 days. In the Arenosol, all the treatments with inhibited plant development i.e., application of glyphosate-treated shoot straw and homogenate or a direct soil application showed also a strong intracellular shikimate accumulation while all other treatments supplied with non-contaminated rye grass shoot material (applied as straw or homogenate) and glyphosate-treated and untreated root material showed no shikimate accumulation (Fig. 6.6 A and B).

In contrast, there was no intracellular shikimate accumulation as physiological bio-indicator for a glyphosate injury in roots of sunflower seedlings grown in the Luvisol with a soil incorporation of glyphosate-treated rye grass residues (shoot and root). But there was a little accumulation of shikimate in the direct soil application (Fig. 6.6B). But compared to the plants grown in the comparable treatments of the

Arenosol (556 µg g^{-1} FW), in the Luvisol the intracellular shikimate accumulation was negligible (45 µg g^{-1} FW) and the small shikimate accumulation was not associated with inhibition of any physiological process of plant development.

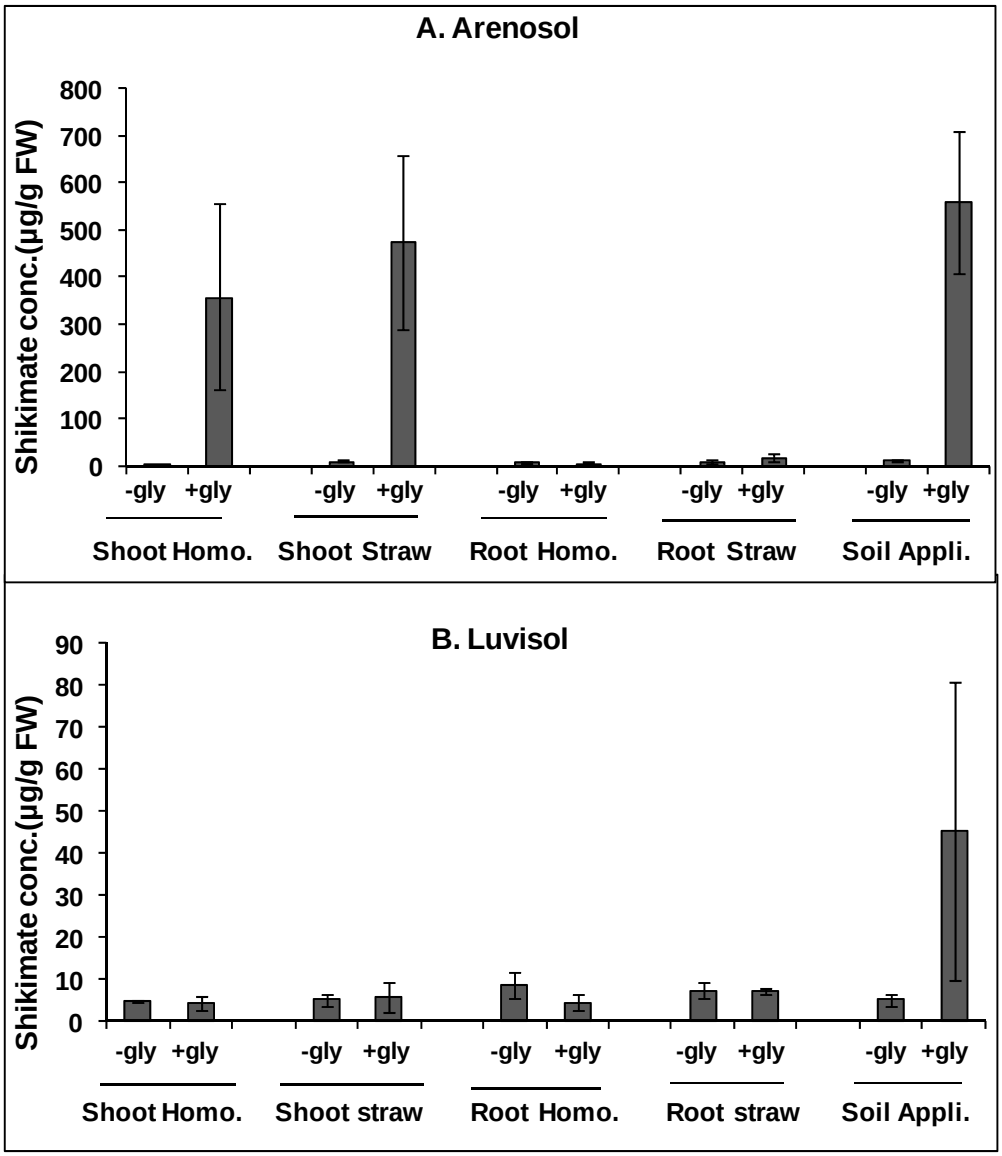


Fig.6.6. Intracellular shikimate accumulation in root of sunflower seedlings grown on the Arenosol and Luvisol supplied either with shoot or root residues or homogenates of glyphosate-treated rye grass. Shoot material was supplied at 1200 mg dry matter kg^{-1} soil and the root material at 700 mg dry matter kg^{-1} soil. Root materials for shikimate analysis were collected after 10 days growth at thinning. Each given value presents the average of 4 replicates ±SD.

6.4. Discussion

Glyphosate residues associated with plant matter caused detrimental effect on plant growth on the weakly buffered acidic Arenosol but not in the highly buffered calcareous Luvisol (Photo 6.2; Fig. 6.2 and 6.3). This soil type dependent residual phytotoxicity of glyphosate is associated to a chain of factors, starting from the decomposition rate of organic residues till to detoxification of glyphosate by adsorption to the soil matrix. Shoot and root growth of sunflower plants was inhibited by incorporation of glyphosate-treated rye grass shoot straw or homogenates into the Arenosol but not to the Luvisol (Photo 6.2; Fig. 6.2 and 6.3). This is most probably related to the difference in soil property between the two soils. At this level of glyphosate supply, the detoxification capacity of the highly buffered calcareous subsoil with high Ca and Mg concentrations as potential ligands mediating glyphosate immobilization and inactivation in soils (Sprankle *et al*., 1975b) might have played a primary role in preventing glyphosate toxicity, while this glyphosate supply level seems beyond the detoxification capacity of the less buffered acidic Arenosol soil with low level of Ca and Mg concentrations.

The observed difference may also be due to differences in the decomposition rate of the supplemented rye grass residues enriched with glyphosate. Many previous experiments on decomposition rate of glyphosate-treated straw at realistic application rates gave variable results. Discrepancies between these data might be therefore due to big differences between the experimental approaches and environmental conditions including differences in soil properties (Grossbard, 1985). Since different soils are characterized by a different microbial species composition responsible for the biological degradation of straw, and thus for the release of glyphosate stored in the plant residues. Grossbard (1985) showed that some known efficient cellulose-degrading fungi (eg. *Chaetomium globosum*) are tolerant to higher amounts of glyphosate while others, similarly known as efficient cellulose-degrading fungi (including some *Fusarium* spp.) are susceptible to glyphosate.

Furthermore, mineralization of soil organic matter is more rapid in coarse-textured than fine-textured soils (Ladd *et al*., 1985; Hassink, 1997). This is due to physical protection of the organic matter from decomposers by physico-chemical stabilization, which refers to the associations formed between the soil minerals and organic-

materials (eg. adsorption to clay minerals, formation of complexes) and pure physical stabilization, which refers to aggregate formation and consequently physical encapsulation and/or shielding of organic matter from microbial and enzymatic attacks (Krull *et al*., 2003). Hence the textural difference between the two soils (acidic sandy Arenosol and calcareous loam Luvisol) might have played a role in influencing the decomposition rate of plant residues and thus the release of glyphosate stored in the plant residues. In addition, supplementation of cations increases sorption of organics on clay surfaces (Sollins *et al*., 1996). Therefore, if such process played a primary role in the present study, then the differential phytotoxicity of glyphosate contaminated rye grass shoot incorporation to the two contrasting soils, i.e., weakly buffered acidic sandy Arenosol (water extractable Ca^{2+}: 0.4 mg kg^{-1} soil and Mg^{2+} =0.4 mg kg^{-1} soil) and highly buffered calcareous loam Luvisol (water extractable Ca^{2+}: 59.9 mg kg^{-1} soil; Mg^{2+}: 11.3 mg kg^{-1} soil) can be explained by a slow decomposition rate and release of glyphosate in the Luvisol might have helped the detoxification of glyphosate toxicity to sunflower plants. The Arenosol with higher concentration of other organic matter sorbents such as Al and Fe oxides (Sollins *et al.*, 1996) should have compensated the low Ca and Mg cations, however the compensation potential might have been insufficient.

On both soils, application of glyphosate-treated rye grass root caused no plant toxicity reflected by missing plant growth inhibition or intracellular shikimate accumulation (Figs. 6.2; 6.3 and 6.6). This is possibly caused by three reasons: (1) the amount of glyphosate supplied via the root could have been well below the range of the detoxification capacity of both soils as the amount of root supplied (700 mg dry matter kg^{-1} soil) was close to half the amount of shoot material supplied (1200 mg dry matter kg^{-1} soil), (2) another reason could also be related to differences in decomposition rate between the shoot and root material, thereby to the release of glyphosate stored in the plant residues, as root material with higher phenol and lignin content may decompose at slower rate than the shoot material. (3) During the short treatment of pre-cultured rye grass with glyphosate still a lower percentage of the glyphosate taken up by leaves was translocated into roots (no corresponding measurements of glyphosate partitioning were done).

Previous reports indicated an inhibition of micronutrient acquisition by frequent use of glyphosate as herbicide (Franzen *et al*., 2003; Eker *et al*., 2006; Neumann *et al*.,

2006; Bott *et al*., 2008). In agreement to these reports, in the present study, incorporation of glyphosate-treated rye grass straw or homogenate inhibited the acquisition of Mn by sunflower plants grown on the weakly buffered Arenosol (Tables 6.1 and 6.2). In the highly buffered Luvisol however, incorporation of equivalent amount of glyphosate-treated rye grass straw or homogenate caused no effect in Mn acquisition (Tables 6.3 and 6.4). Fe and Zn concentration seemed to increase at those treatments where growth inhibition by glyphosate observed i.e., on the Arenosol treatments received glyphosate-treated rye grass shoot straw or homogenate (table 6.1), but this is most probably attributed to dilution effect due to the extreme growth depression of the sunflower seedlings on these treatments. Moreover, higher plant Fe and Zn content (table 6.2) reveals that the increase in concentration on these treatments is due to dilution effect. Mn concentration and content however decreased by application of glyphosate enriched shoot straw or homogenate and direct soil application compared to root straw or homogenate application in addition to controls (-glyphosate) in the Arenosol and all treatments in the Luvisol. Previous report by Neumann *et al*. (2006) also found a soil type dependent differential phytotoxicity, preferentially expressed on the acidic Arenosol compared to the calcareous Luvisol used in the present study and it was proposed that amelioration effect comes from immediate precipitation of glyphosate in the rhizosphere by higher levels of Ca in the Luvisol.

Furthermore, macronutrient (particularly Ca) acquisition was also inhibited in treatments where glyphosate phytotoxicity were observed (Fig. 6.4 A and B). Ca concentration (mg g^{-1} DM) and content (mg per plant) strongly decreased in variants supplied with glyphosate-treated rye grass shoot straw or homogenate on the Arenosol compared to root straw or homogenate supplied and controls without glyphosate treatments (Fig. 6.4 A and B). On these treatments, Mg content was also significantly reduced though it was not clear from the concentration due to dilution effect (table 6.5). In the strongly buffered Luvisol however, there was no notable effect on macronutrient acquisition reflected on leaf concentration or content (Fig. 6.5 A and B; table 6.5).

This soil type dependent residual phytotoxicity of glyphosate is most likely associated to the difference in polyvalent cation content of the two soils as it is well established

that polyvalent cations can antagonize the herbicidal activity of glyphosate (Hall *et al*., 2000; Bernards *et al*., 2005).

Figure 6.6 presents intracellular shikimate accumulation in roots of sunflower plants grown on the Arenosol and the Luvisol. In correspondence to inhibition of plant growth at treatments with glyphosate-treated rye grass shoot straw or homogenate incorporation to Arenosol resulted to high intracellular shikimate accumulation in the roots of sunflower plants. In the treatments with glyphosate treated root straw or homogenate in the Arenosol and shoot/root straw or homogenate in the Luvisol, however, did not induce any shikimate accumulation. The primary target of glyphosate is inhibition of the shikimic acid pathway and results in the accumulation of high levels of shikimate in plant tissues (Duke, 1988; Steinrucken and Amrhein, 1980). Hence, measurement of shikimate accumulation in plant tissue can be used as an important physiological bio-indicator for glyphosate residual phytotoxicity on non-target plants (Mueller *et al*., 2003; Neumann *et al*., 2006).

Therefore, the coincidence of plant (shoot and root) growth inhibition and intracellular shikimate accumulation due to glyphosate treated rye grass shoot straw or homogenate supply treatments (Fig. 6.5) implies pure glyphosate phytotoxicity rather than any other cause. In the Arenosol soil, similar intracellular shikimate accumulation in sunflower roots caused by application of equivalent amount of glyphosate directly supplied to the soil and incorporated via glyphosate contaminated organic matter indicates that this level of glyphosate is beyond the detoxification capacity of the soil, and a residual toxicity from incorporation of glyphosate treated weed residues during plowing remains a treat for crop intoxication on such weakly buffered soils. Direct soil application of glyphosate in the Luvisol tended to cause low level of root intracellular shikimate accumulation without significant plant growth inhibition but this shikimate accumulation (45 µg g^{-1} FW) was not comparable to the root shikimate accumulation in plants grown in the comparable treatments of the Arenosol (557 µg g^{-1} FW). This implies that the soil type dependent differential phytotoxicity of glyphosate is due to difference in detoxification capacity of the two soils.

Generally there was no any difference between plants grown in shoot/root straw and homogenate treatments within each soil. This may indicate that treatment of the organic matter (homogenization under liquid nitrogen) did not influence the

decomposition rate of the plant material, thereby had no effect on the glyphosate release rate from decaying residues.

6.5. Conclusion

- There is striking residual phytotoxicity risk by glyphosate originating from decaying organic residues of glyphosate-desiccated weed. The glyphosate residual phytotoxicity is reflected in inhibited biomass production and hindered mineral nutrient acquisition (Mn, Ca) and confirmed by intracellular shikimate accumulation as physiological bio-indicator for glyphosate injury.
- The glyphosate residual phytotoxicity was expressed only on the weakly buffered acidic Arenosol but not on the highly buffered calcareous Luvisol. This implies, the phytotoxicity effect is soil type dependent and is associated to glyphosate detoxification capacity of the two soils.
- Facilitation of organic matter decomposition rate by grinding under liquid nitrogen seems not to have any effect in this particular case since there was no difference between the shoot straw and homogenate applications. But this could be noted as site specific outcome till further exploration is undergone.
- Pending results of future studies, these presented data support special care being given during plowing of glyphosate-treated fields to minimize an incorporation of weed residues because of a possible damage of following crops.

6.6. Prospects

- The soil type dependent differential detoxification potential needs further consideration. Fresh soil directly collected from field where strong microbial activity exists might have a faster decomposition and thus a faster release of glyphosate from treated residues.
- Under consideration of different waiting times between incorporation of weed residues and crop planting, a practical recommendation for farmers are urgently needed.
- Screening a wide range of soils with different properties for drawing conclusive guidelines is required.
- Using a wide range of weed species for their decaying rate should be considered for a better risk assessment.

- Different growth conditions (soil temperature, moisture etc.) will influence decomposition rate and thus to get evaluated for an appropriate recommendation for farmers.

7.0. General Discussion

Glyphosate (N-[phosphonomethyl]glycine) is a broad-spectrum, water soluble, non-selective post-emergence systemic herbicide sold under the trade names of RoundUp Ultramax®, RoundUp-Pro®, Rodeo®, GlyPro®, Accord®, Glyphomax®, Touchdown® and Vision®. It effectively controls most annual and perennial plants and it is the world´s biggest-selling chemical used for weed control in agricultural, silvicultural and urban environments (Baylis, 2000). One reason for the popularity of glyphosate is its effect on roots and rhizome systems of weed following foliar application. As a broad-spectrum and systemic post-emergence herbicide applied through the leaf, glyphosate is phloem mobile and readily translocated throughout the plant within a few days after treatment (Sprankle *et al*., 1975c; Sandberg *et al*., 1980; Franz *et al*., 1997). On reaching the soil, glyphosate will be fixed on clay minerals, soil oxides and hydroxides and soil organic matter, through mechanisms of H-bonding and ion-exchange (Sprankle *et al*., 1975b; Miles and Moye, 1988). And the sorption of glyphosate in soils depends on many soil properties such as soil pH, concentration of cations (Ca^{2+}, Mn^{2+}, Zn^{2+}, Mg^{2+}, Fe^{3+} or Al^{3+}), levels of iron–humic acid complexes, soil inorganic phosphate levels etc. (Sprankle *et al*., 1975b; Nomura and Hilton, 1977; Piccolo *et al*., 1995). These sorption behaviors make glyphosate unique as compared to most other herbicides and have elicited a general belief that it is rapidly sorbed to the soil without any residual effect. However, glyphosate adsorption to the soil matrix is a reversible process and glyphosate adsorbed to soil was reported to have a residual activity towards some plant species (Salazar and Appleby, 1982). Hence, 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, such as following crops in various rotations and soil microorganisms including N_2 fixing bacteria and AM mycorrhiza. In addition, concern on possible residual phytotoxic effect of glyphosate on non-target plants (with regard to inhibited nutrient acquisition, effect on rhizosphere microbial community structure and increased disease prevalence) has increased with increasing glyphosate use driven by the introduction of RR-crops and reduced tillage systems and as a reflection of such concern, considerable amount of scientific reports are emerging in the literature (Smiley *et al*., 1992; Huber and McCay-Buys, 1993; King *et al*., 2001; Kremer *et al*., 2001; Charlson *et al*., 2004; Fernandez *et al*.,

2005; Huber *et al*., 2005; Eker *et al.,* 2006; Neumann *et al*., 2006; Ozturk *et al*., 2007; Bott *et al*., 2008).

The aim of this thesis was to identify possible risk factors associated with frequent use of glyphosate in agro-ecosystems to alleviate the continuously observed effects on non-target plants. For this purpose: (1) relevance of waiting time between weed desiccation by glyphosate and subsequent crop planting, (2) remobilization risk of soil matrix fixed glyphosate mediated by pH changes in the rhizosphere, (3) combined effect of waiting time and glyphosate binding forms in soil and (4) contribution of glyphosate released from decaying organic matter for intoxication of non-target plants were investigated under controlled greenhouse conditions using two contrasting soils: weakly buffered acidic Arenosol (top soil) and highly buffered calcareous Luvisol (subsoil). Furthermore, a field experiment was conducted to partially confirm the found results of controlled greenhouse experiments.

7.1. Relevance of waiting time in alleviating glyphosate toxicity to non-target plants.

Glyphosate is systemic within the plant, applied via the foliage, phloem mobile and is readily translocated into metabolic sinks including plant roots from where it can finally be released into the rhizosphere (Rodrique *et al*., 1982; Feng *et al*., 1999; Neumann *et al*., 2006). In the rhizosphere, it is either biologically degraded or strongly bound to soil colloids (Sprankle *et al*., 1975b). For this reason, producer instructions allow even pre-sowing glyphosate treatment until the first days after sowing (Monsanto, Roundup Ultramax® product information). However, the process of degradation and adsorption seems to require some period of time to proceed and to avoid intoxication of subsequent crop plants as already indicated by some scientific reports (Cornish, 1992; Smiley *et al.,* 1992; Constantin *et al*., 2008). The results of pot experiments conducted under controlled greenhouse conditions and using the two contrasting soils considering 0-21 days waiting times as well as field experiment on farmer´s field considering 2 and 14 days waiting times (chapter 3 and 5) underline the importance of waiting time interval between weed desiccation by glyphosate and subsequent crop planting, to avoid or at least minimize detrimental effects on the following culture. Analysis of physiological parameters, such as intracellular shikimate accumulation as metabolic indicator for glyphosate toxicity, biomass production and micronutrient status revealed, that the risk of toxic effects, induced by glyphosate pre-

sowing treatments, increased with declining waiting time and can persist up to three weeks (chapter 3: Figs. 3.1 and 3.2; chapter 5: Fig. 5.4). This is in agreement with 3 weeks waiting time recommended by Cornish (1992) for loamy sandy soil. Accordingly, local RoundUp guidelines in Israel recommend avoiding glyphosate use in sandy soils and during stress conditions such as drought and in Brazil allow extended "waiting times" depending on the type of soil and crop intended to cultivate (Monsanto co. guideline). At treatments where biomass production was inhibited, higher root tissue intracellular shikimate accumulation was measured (chapter 3: Figs. 3.3 and 3.4; chapter 5: Figs. 5.2 and 5.3) which confirms glyphosate toxicity since it is a known inhibitor of 5-Enolpyruvylshikimate-3-phosphate synthase (EPSP synthase) enzyme that leads to intracellular shikimate accumulation (Becerril *et al*., 1989; Della-Cioppa *et al*., 1986). Hence, the coincidence of plant growth inhibition and intracellular shikimate accumulation of the sunflower seedlings at waiting times less than 21 days proves to be pure glyphosate residual phytotoxicity rather than any other causes.

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). In agreement to this, Mn concentration in youngest fully developed leaves was reduced on short waiting time treatments of sunflower plants grown in both the Arenosol and Luvisol (Figs. 5.4 and 5.5). However, glyphosate-induced impairment of Mn nutrition was more strongly expressed on the sandy Arenosol with low buffering capacity compared with the well-buffered calcareous sub-soil (Figs. 5.4 and 5.5), indicating a possible role of different soil types in determining the expression of glyphosate toxicity. This was not however, associated with corresponding differences of intracellular shikimate accumulation or plant biomass production (Tables 5.1 and 5.2; Figs. 5.2 and 5.3), suggesting rather soil-specific differences in Mn availability than differential expression of glyphosate toxicity on the two investigated soils as possible causes, at least in this level of glyphosate application. 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 is known to form poorly soluble complexes with Mn (Sprankle *et al*., 1975b) and may thereby reduce the already low level of available Mn in the Arenosol. Also glyphosate-induced inhibition of root growth (Fig. 5.1; Tables 5.1 and 5.2) may counteract Mn acquisition with a strong consequence for Mn uptake on the Arenosol with low levels of plant-available Mn. Theses results are in line with recent field observations of Mn deficiency problems due to glyphosate use in low Mn (calcareous) soils (Y. Bayer, Pers, communication, 2009).

Similar to the controlled greenhouse experiments, results of the field trial at Hirrlingen/ Tübingen confirmed the glyphosate residual phytotoxicity on subsequently cultivated non-target crop if enough time is not given for dissipation (chapter 3). Stunted development and heterogeneous emergence of winter wheat plants occurred at field plots where the wheat sowing was done 2 days after cover crop desiccation by glyphosate, irrespective of the glyphosate application rate (2 L ha^{-1} vs 6 L ha^{-1}) compared to plants sown 14 days after glyphosate application. This heterogeneous emergence indicated a formation of “hot spots” with glyphosate containing and decaying roots of desiccated weed or cover crops. Depending on contact of roots with such “hot spots”, wheat plants as non-target plants got intoxicated or grow unaffected (Roemheld *et al*., 2008). At a short waiting time (2 d), visual scoring of wheat damage showed up to 50% of the plants injured by glyphosate residual toxicity. This observed damage was visually persistent still at harvest after 6 months (Fig. 3.5) and indeed previous culture damage at vegetative stage is reported to positively correlate to potential yield loss at harvest (Buehring *et al.,* 2007). Wheat plants grown on the short waiting time (2 d) plots also showed reduced macro and micronutrient (Ca, Mg, Zn and Cu) concentrations particularly expressed at an elevated glyphosate application rate (6 L ha^{-1}). This can be due to root injury by glyphosate toxicity as roots are the primary victim or chelating effect as glyphosate is known chelator of divalent cations (Glass, 1984; Schoenherr and Schreiber, 2004; Subramaniam and Hoggard, 1988). Previous reports also demonstrated restricted translocation and intracellular localization of metal divalent cations (eg. Ca^{2+} and Mg^{2+}) by root-fed or foliar applied glyphosate reflected by reduced uptake and translocation of Ca^{2+} and Mg^{2+} but not K^{+} (Candan, 2008; Duke *et al*., 1985).

Glyphosate chelates Mg^{2+} and Ca^{2+} almost equally well, with the same stability constant and similar effects of pH on chelating properties (Madsen *et al*., 1978). In agreement to these findings and possibly for the same reason, the Ca, Mg, Zn and Cu concentration of winter wheat shoot was reduced by combined effect of short waiting time (2 d) and elevated glyphosate application rate (6 L ha^{-1}) compared to the longer waiting time (14 d) and lower application rate (2 L ha^{-1}) but P, K, Fe and Mn were not affected (Table 3.3 and 3.4). Similar to the divalent macronutrients, glyphosate also forms stable complex with the divalent micronutrients (e.g. Fe, Mn, Zn and Cu) depending on their ionic state during the time of contact (Glass, 1984; Hall *et al*., 2000; Bernards *et al*., 2005).

7.2. Glyphosate remobilization by root-induced changes on the rhizosphere.

Nitrate and ammonium are the main forms of inorganic nitrogen supplied to plants. Chapter 4 presents remobilization risk of glyphosate by root-induced pH change in the rhizosphere associated to N form supplied and carboxylate supplementation on two contrasting soils pre-incubated with glyphosate. As nitrogen comprise about 80% of the total cations or anions taken up by plants, the form of nitrogen supply has a strong impact on the uptake of other cations and anions through changes of the rhizosphere pH (Marschner, 1995). Rhizosphere acidification can be caused by an excess uptake of cations over anions and alkalization occurs when anion uptake exceeds cation uptake. Ammonium uptake is generally associated with acidification of the rhizosphere while nitrate nutrition induces an increase in rhizosphere pH (Roemheld *et al.,* 1984). In agreement to this, plants fed with stabilized ammonium strongly acidified their rhizosphere soil in the weakly buffered Arenosol by up to 1.7 pH units while plants fed with nitrate tended to alkalinize their rhizosphere though to a lesser extent by up to 0.4 pH units (Fig. 4.3). In the well buffered Luvisol, root-induced pH change in the rhizosphere was not strong. Plants fed with stabilized ammonium (NH_4^+) acidified their rhizosphere soil by around 0.5 pH units while the nitrate fed plants alkalinized their rhizosphere soil by only 0.2 pH units (Fig. 4.4).

As previous reports indicate, glyphosate shows a similar pattern of reaction like that of phosphate in soil and both molecules compete for same sorption sites, as very often observed desorption of soil matrix fixed glyphosate by addition of phosphate (de Jonge *et al*., 2001; Gimsing and Borggaard, 2001; 2002; Laitinen *et al*., 2008). In

controlled greenhouse experiment, increasing rate of P fertilization to glyphosate pre-loaded soil resulted in a depression of soybean plant growth (Bott, per. Comm. 2008). This demonstrates a similar pattern of phosphate and glyphosate reaction in soil matrix and all chemical changes in the rhizosphere known to remobilize phosphate can co-mobilize glyphosate.

In neutral or alkaline soils, rhizosphere acidification in plants fed with ammonium can enhance mobilization of sparingly soluble calcium phosphate and thereby favor the uptake of phosphate (Gahoonia *et al*., 1992). On acid soils, the pH increase induced by nitrate supply enhances phosphorus uptake, presumably by exchanging with HCO_3^- for phosphate adsorbed to iron and aluminum oxides (Gahoonia *et al*., 1992). Similar to phosphate, glyphosate forms sparingly soluble salts and/or complexes in the presence of divalent cations such as Ca^{2+} (Madsen *et al*., 1978; Smith and Raymond, 1988; Sundaram and Sundaram, 1997). Hence, it is highly likely that root-induced rhizosphere acidification of alkaline soils as a result of different form of N supply to solubilise sparingly soluble Ca-glyphosate precipitates. This might increase the risk of remobilization of soil matrix fixed glyphosate as a result of root-induced rhizosphere acidification leading to non-target plant intoxication. To test this hypothesis, an experiment was conducted using two soils with contrasting properties pre-incubated with different rates of glyphosate and supplied with stabilized NH_4^+ or NO_3^- as N form to extrapolate the N supply form as driving force for rhizosphere pH change. From the results of this experiment, however, it was not possible to confirm this hypothesis. No glyphosate phytotoxicity due to glyphosate remobilization could be observed as there was no accompanying intracellular shikimate accumulation (Fig. 4.6) parallel to rhizosphere acidification and biomass reduction of sunflower plants grown on the Luvisol soil fed with ammonium form of nitrogen. This might be attributed to the fact that the amount of glyphosate applied was too low in concentration since it was uniformly mixed with the whole soil volume. However, in reality when glyphosate is applied to target plants, it is taken up by leaves and translocated to roots where it is released to a localized area, forming “hot spots” with a high glyphosate concentration. In addition, in the Arenosol where low precipitation of Ca-glyphosate is expected, the glyphosate molecules might have been already degraded by soil microorganisms during the 21 days incubation time or strongly

adsorbed to P binding sites of an acidic tropical soil. In the Luvisol, where higher Ca-glyphosate precipitation is expected, the root-induced rhizosphere pH decrease as a result of ammonium form of nitrogen nutrition which might have been minimal as the change was only 0.5 pH unit (Fig. 4.6).

Plant growth response to different forms of nitrogen has been well studied and many reports show that sole NO_3^- nutrition is associated with stimulated shoot growth while sole NH_4^+ nutrition is associated with inhibited plant growth (Walch-Liu *et al*., 2000; Rahayu *et al*., 2005; Lu *et al*., 2008). This is in agreement with the current results of the Luvisol (Fig. 4.2) where control plants supplied with stabilized ammonium (NH_4^+) form of N had reduced shoot growth compared to control plants supplied with sole nitrate (NO_3^-) form of nitrogen. Though statistically insignificant, under high glyphosate level (100 and 500% of the recommended rate) and NH_4^+ nutrition tended to reduce shoot and root biomass production. In the Arenosol, however, plant growth (shoot or root) was not affected by different nitrogen forms or increasing glyphosate application rate (Fig. 4.1).

Root exudation of carboxylates has also been considered as a source of root-induced rhizosphere acidification and to assist the release of phosphate from extracellular sparingly soluble P sources (Gardner *et al*., 1983; Hoffland *et al*., 1989; Hoffland, 1992; Stroem, *et al*., 2005). In addition to rhizosphere acidification, exuded organic acids are also able to mobilize inorganic P into the soil solution via exchange chelation through competing with phosphate groups for the same binding/adsorption sites in soil and forming stronger complexes with Al^{3+}, Fe^{3+} and Ca^{2+} than phosphate does. Thus phosphate can be liberated from cation–P complexes as an organic carboxylates complex with the cations or block the sorption of P to other charged sites or through the ligand exchange process (Geelhoed *et al*., 1999; Hinsinger, 2001). Glyphosate as phosphated molecule faces the same fate like mineral inorganic phosphate, i.e. root mediated change in the rhizosphere including excretion of organic acids (e.g. Citrate) can remobilize glyphosate fixed on Al^{3+}, Fe^{3+} and Ca^{2+} cations by ligand exchange and rhizosphere acidification. In the present study with Arenosol soil, however, showed no indication of glyphosate remobilization by synthetic carboxylates that could cause plant damage (Fig. 4.7). Na-citrate or citric acid supplementation of the pots filled with soils pre-incubated with different levels of

glyphosate for 21 days prior to planting, even showed a tendency of better biomass production than the controls (especially shoot fresh weight) on both the 100 and 500% glyphosate rate compared to no glyphosate application. Root growth, however, showed no difference on all treatments. This absence of glyphosate remobilization is likely to be attributed to the fact that this soil is less buffered (low Ca^{2+} ions) and might have had less precipitation of the applied glyphosate which might have led to faster degradation by microorganisms during the 21 days incubation period.

On the highly buffered Luvisol, supplementation with 10µmol g^{-1} soil Na-citrate but not citric acid caused inhibition of root biomass production on glyphosate pre-incubated treatments (Fig. 4.9). Shoot biomass production as well tended to be reduced by the addition of sodium citrate (10µmol g^{-1}), though the difference was not statistically significant. Intracellular shikimate analysis, which was performed to confirm the involvement of remobilized glyphosate by Na-citrate supplementation, did not show such a shikimate accumulation. Absence of citric acid effect on glyphosate pre-incubated treatments is hard to explain as in this highly buffered (high Ca^{2+} cation concentration) calcareous soil, the two fold effects of citric acid in soil acidification and citrate effect as ligand exchanger should have caused more dissolution of precipitated glyphosate. Or it is likely that the plant growth inhibition observed by Na-citrate addition (10µmole g^{-1} soil) was caused by Na toxicity rather than glyphosate. If that was the case, it can be hypothesized that there might have been insufficient percolation of the exudates into the rhizosphere soil to induce glyphosate remobilization as the carboxylates may have remained absorbed on the top soil layer during titration.

7.3. Glyphosate stabilization in target plant roots

To evaluate the potential role of target plant roots in stabilization and being a potential reservoir of glyphosate with intoxication of subsequent crop plants, model experiments were conducted with an application of glyphosate to a pre-cultured rye grass and with a direct soil application of equivalent amount of glyphosate prior to sunflower sowing at different waiting times (0-21 days). Toxicity of glyphosate pre-sowing treatments on sunflower seedlings was strongly dependent on the mode of glyphosate application. When glyphosate was sprayed on pre-cultured rye grass seedlings as model weed, detrimental effects on plant growth and the Mn nutritional

status, as well as increased intracellular shikimate accumulation in root tissue were more strongly expressed than at a direct soil application of the same amount of glyphosate (chapter 5). 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*., 1975b; Giesy, 2001; Monsanto, 2005a, Yamada, 2006).

The increased expression of toxicity effects after a glyphosate pre-sowing application to a rye grass pre-culture compared with a direct soil application might indicate that 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 many plant species, glyphosate is not readily metabolized and is preferentially translocated to young growing tissues of roots and shoots, where it can be accumulated in millimolar concentrations (Reddy *et al*., 2004, Monsanto, pers. communication). In soil-grown target plants, this inhomogeneous distribution of glyphosate within the root tissues may lead to the formation of “hot spots” of glyphosate as root residues in soils, containing high levels of glyphosate, which is subsequently released during microbial degradation of the plant material. Glyphosate intoxication of non-target plants might be induced by root contact with these “hot spots”. The inhomogeneous distribution of glyphosate-contaminated plant material in the soil could also explain the much higher variation of the data on the biomass of individual sunflower plants, shikimate accumulation and Mn-nutritional status after glyphosate application to the rye grass pre-culture as compared to a direct soil application (Tables 5.1 and 5.2, Figs. 5.2 – 5.4) 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, while sunflower seedlings without contact to the “hot spots” remained unaffected. In contrast, direct soil application of glyphosate resulted in a homogenous distribution and bio-availability of the herbicide over the investigated soil profile and much less expressed variation in inhibition of sunflower growth (Photo 5.2).

The potential role of plant residues as a pool for glyphosate stabilization 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; Komoßa *et al*., 1992; Thompson *et al*., 1994; von Wirén-Lehr *et al*., 1997; Reddy *et al*., 2004). 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 for intoxication of non-target organisms. To improve bio-safety in face of the global increase in agricultural use of glyphosate, the following open questions have been considered for the future avoidance of such negative effects under real field conditions. Those open questions include impact of external factors, such as soil properties, soil moisture levels, temperature, 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 fertilization management.

7.4. Effect of glyphosate from decaying weed straw.

The predominant use of glyphosate in agricultural production systems is for broad spectrum, non-selective weed control prior to crop sowing. The globally increasing adoption of no-till or reduced tillage systems are becoming a driving force for increasing glyphosate use (Torresen *et al*., 1999). In such systems, glyphosate is applied pre-sowing for weed control and glyphosate may remain in root and shoot residues. Usually in these reduced tillage systems soil disturbance occurs only at crop sowing, which might lead to incorporation of the glyphosate-contaminated straw to the upper soil layer where germination of following non-target crop takes place. Hence roots of germinating seedlings may directly come in contact with the glyphosate contaminated weed residues with a subsequent phytotoxicity. Some previous reports indicate possible detrimental implication from contaminated straw to crops (Stocker and Haller, 1999). To evaluate such risk, a pot experiment was conducted under controlled green house conditions with two contrasting soils. Glyphosate was supplied as glyphosate pre-treated rye grass plant material either as shoot or root residue in the form of straw or homogenate (chapter 6). Plant matter

associated glyphosate residue caused detrimental effect on plant growth depression on the weakly buffered acidic Arenosol, whereas in the highly buffered calcareous Luvisol, there was no visible effect (Photo 6.2; Fig. 6.2 and 6.3). Analysis of physiological parameters such as intracellular shikimate accumulation as metabolic indicator for glyphosate toxicity, biomass production and micronutrient status revealed, that detrimental effect linked to glyphosate toxicity originated from treated shoot residues of rye grass or homogenate incorporated into the Arenosol culture but not into the Luvisol (Photo 6.2; Fig. 6.2 and 6.3). This is most probably related to the difference in properties between the two soils. At this level of glyphosate supply, the detoxification capacity of the highly buffered calcareous subsoil with high Ca and Mg availability as potential ligands was high enough for an adequate immobilization or inactivation of glyphosate (Sprankle *et al*., 1975b). This might have played a primary role in preventing glyphosate toxicity. On the weakly buffered acidic Arenosol with a low level of Ca and Mg concentration, the level of glyphosate supply was higher than the detoxification capacity. Furthermore, the soil type dependent difference in toxicity by glyphosate enriched organic matter can also be related to differences in microbial community composition and soil texture between the two soils as these properties play also an important role in the decomposition rate of the shoot residues (Grossbard, 1985; Ladd *et al*., 1985; Hassink, 1997). The glyphosate-induced toxicity observed on the treatments with a direct soil application of the weakly buffered Arenosol but not of the well buffered Luvisol, confirms that differential toxicity is mainly related to the detoxification potential difference of the two contrasting soils.

In contrast to glyphosate treated shoots, application of glyphosate treated rye grass roots caused no plant toxicity as reflected by no plant growth depression or intracellular shikimate accumulation (Figs. 6.2; 6.3 and 6.6). This possibly was due to three reasons: (1) the amount of glyphosate supplied via the root could have been well below the range of the detoxification capacity of both soils as the amount of root residues supplied (700mg dry matter kg^{-1} soil) was close to half the amount of shoot residues supplied (1200mg dry matter kg^{-1} soil), (2) less glyphosate was accumulated in roots than shoot parts and (3) another reason could also be related to decomposition rate difference between the shoot and root material, thereby to the

release rate of glyphosate stored in the organic matter, as root material with higher phenol and lignin content may decompose at slower rate than the shoot material.

Previous reports indicated a micronutrient acquisition inhibition by frequent use of glyphosate herbicide (Franzen *et al*., 2003; Eker *et al*., 2006; Neumann *et al*., 2006; Bott *et al*., 2008). In agreement with these reports, in the present study, at treatments where detrimental effect on plant development observed, mineral nutritional status (Ca, Mg, Fe, Mn and Zn) of sunflower plants was also reduced (Tables 6.1, 6.2 and 6.5; Fig. 6.4). The difference between the two soils was obvious, nutrient acquisition effect was conspicuous only in the Arenosol but not in the Luvisol. In addition to the difference in detoxification capacity between both soils, differences in nutrient bio-availability of both soils might have also aggravated the observed inhibition of nutrient acquisition. Therefore only on the strongly weathered nutrient poor Arenosol such detrimental effects by glyphosate could be observed. Since micronutrients, such as Mn and Zn are important physiological co-factors 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 *et al*., 2004; Fernandez *et al*., 2005; Huber *et al*. 2005).

All together, the achieved results of the model pot experiments are in correspondence with that of the reported field experiments. Further, the results revealed the important role of glyphosate desiccated weed plants in a soil as a glyphosate pool for intoxication of following crops. More information on transformation of these glyphosate enriched crop residues and its glyphosate release during microbial decomposition are urgently needed for a better precaution and risk assessment of glyphosate for farmer´s practice.

Summary

Glyphosate ([N-phosphonomethyl] glycine) is a non-selective, post-emergence, organo-phosphorous, broad-spectrum herbicide used worldwide for controlling weeds in horticulture, agriculture, silviculture, and urban landscapes. It effectively controls most annual and perennial plants and it is the world´s biggest-selling herbicide. On top of its low price, the main reason for the popularity of glyphosate is its effect on roots and rhizome systems of weeds following foliar application. Glyphosate is a systemic herbicide easily translocated from the shoot to roots and released into the rhizosphere. Coming in contact with soil, glyphosate will be fixed on clay minerals, Al^{3+} and Fe^{3+} oxides and hydroxides and soil organic matter, through mechanisms of H-bonding and ion-exchange. This sorption of glyphosate in soils depends on many soil properties such as soil pH, concentration of divalent-cations, levels of iron–humic acid complexes, soil inorganic phosphate levels etc. These sorption behaviors make glyphosate unique as compared to most other herbicides and have elicited a general belief that it is rapidly sorbed to the soil without any residual effect. However, glyphosate adsorption to the soil matrix is a reversible process and glyphosate adsorbed to soil was reported to have a residual activity towards some plant species. Hence, 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, such as following crops in various rotations and soil microorganisms. In addition, concern on possible residual phytotoxic effects of glyphosate on non-target plants has increased with increasing glyphosate use driven by the introduction of RR-crops and reduced tillage systems. As a reflection of such concern, considerable amount of scientific reports are emerging in literature (Fernandez *et al*., 2005, Huber *et al*., 2005, Neumann *et al*., 2006, Bott *et al*., 2008).

In face of such increasing number of yet unexplained observations of negative side effects after glyphosate application (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), this thesis was initiated to identify possible risk factors associated with the frequent use of glyphosate in agro-ecosystems. For this purpose: (1) relevance of waiting times between weed desiccation by glyphosate and subsequent crop planting, (2) remobilization risk of soil matrix fixed glyphosate mediated by pH

changes in the rhizosphere, (3) glyphosate preservation in target plant roots (4) contribution of glyphosate released from decaying weed residue for intoxication of non-target plants were investigated in controlled greenhouse conditions using two contrasting soils: weakly buffered acidic Arenosol (top soil) and highly buffered calcareous Luvisol (subsoil). Furthermore, field experiments were conducted to partially confirm the found results of controlled green house experiments.

In **chapter 3 and 5**, results of model experiments conducted under controlled greenhouse conditions and using the two contrasting soils as well as field experiments on farmer´s fields are shown and discussed. All these conducted experiments revealed that the residual toxicity of glyphosate has increased with declining waiting time between glyphosate weed desiccation and subsequent crop planting. In the greenhouse experiments with the two soils, growth of sunflower seedlings as model crop plant and its biomass production were strongly impaired by glyphosate pre-sowing treatments in the variants with 0 d waiting time. With increasing waiting time from 7 to 21 days, the observed impairment got less expressed. The inhibitory effect on seedling growth was always associated with a corresponding increase of 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 manganes-nutritional status which was still detectable after a waiting time of up to 21 d, particularly on the Arenosol. The glyphosate-induced impairment of Mn nutrition was more strongly expressed on the sandy Arenosol with a low pH buffering and Ca availability compared with the well pH-buffered calcareous sub-soil, indicating a role of different soil types in determining the expression of glyphosate toxicity. This inhibition of Mn acquisition was not associated with the corresponding differences of intracellular shikimate accumulation or plant biomass production, suggesting rather soil-specific differences in Mn availability than differential expression of glyphosate toxicity on the two investigated soils as possible causes, at least at this level of glyphosate application. Also glyphosate-induced inhibition of root growth might interfere with Mn acquisition with a strong consequence for Mn uptake on the Arenosol with a low level of plant-available Mn.

Results of the field experiment at Hirrlingen/Tübingen confirm the relevance of waiting time. Stunted development and heterogeneous emergence of winter wheat plants

occurred at field plots where the wheat sowing was done 2 days after cover crop desiccation by glyphosate, compared to plants sown 14 days after glyphosate application. This heterogeneous emergence indicated a formation of “hot spots” with glyphosate containing and decaying roots of desiccated weed or cover crops. Depending on contact of root with such hot spots wheat plants as non-target plants got intoxicated or grew unaffected (Roemheld *et al*., 2008). At short waiting time (2 d), visual scoring of wheat damage showed up to 50% of plants injured by glyphosate residual toxicity. This observed damage was visually persistent still after 6 months and the damage at the vegetative stage was reported to be positively correlate with loss of grain yield at harvest at Hirrlingen/Tübingen in correspondence with literature (Buehring *et al*., 2007). Plant growth depression of wheat plants cultivated at short waiting time plots had also reduced nutritional status (Ca, Mg, Zn and Cu) compared to plants cultivated at long waiting time plots, particularly expressed when glyphosate application rate was elevated from 2L to 6 L ha^{-1}. Theses findings of the present field experiments are in agreement to previously recommended reports which recommend 3 weeks waiting time to be considered to alleviate or minimize glyphosate residual toxicity (Cornish, 1992).

Since glyphosate shows a similar pattern of reaction like that of phosphate in soil, it has been hypothesized that processes responsible for phosphate mobilization in the rhizosphere are likely to co-mobilize also glyphosate. Root-induced acidification of the rhizosphere, mainly driven by physiologically acidic NH_4^+-based fertilization might increase the bio-availability of soil matrix fixed glyphosate, in particular glyphosate precipitated as Ca-glyphosate. To test this hypothesis, an experiment was conducted using two soils with contrasting properties pre-incubated with different rates of glyphosate and supplied with stabilized NH_4^+ or NO_3^- as N mineral fertilizers (**chapter 4**). From the results of this experiment, however, it was not possible to confirm this hypothesis. No glyphosate phytotoxicity due to glyphosate remobilization could be observed since there was no accompanying intracellular shikimate accumulation parallel to the rhizosphere acidification or biomass reduction of sunflower plants grown on the Luvisol fed with ammonium form of nitrogen. This might be attributed to the fact that the amount of glyphosate applied was too low in concentration since it was uniformly mixed with the whole soil volume. However, in reality when glyphosate is applied to target

plants, it is taken up by leaves and translocated to roots where it is released to a localized area, forming hot spots with high glyphosate concentration. In the Luvisol, where higher Ca-glyphosate precipitation is expected, the root induced rhizosphere pH decrease as a result of ammonium form of nitrogen nutrition might have been minimal as the change was only 0.5 pH unit in the rhizosphere. In addition, in the Arenosol where low precipitation of Ca-glyphosate is expected, the glyphosate molecules might have been already degraded by soil microorganisms during the 21 days incubation time or strongly adsorbed to P binding sites of the acidic tropical soil.

Root exudation of organic carboxylates has also been considered to assist the release of phosphate from extracellular sparingly soluble P source via exchange chelation. A similar phenomenon was expected for glyphosate. In the present study, however, supplementation of Na-citrate or citric acid to the two contrasting soils pre-incubated with different levels of glyphosate have not shown a strong evidence of an adequate glyphosate remobilization to cause plant damage. On the acidic Arenosol, there was no difference in sunflower growth between all the treatments. On the Luvisol soil, supplementation with 10µmol g^{-1} soil Na-citrate but not citric acid caused inhibition of root biomass production on glyphosate pre-incubated treatments. But this was not accompanied by intracellular shikimate accumulation as physiological bio-indicator for glyphosate toxicity. This absence of glyphosate remobilization was likely attributed to either glyphosate degradation by microorganisms during the 21 days incubation period or to an insufficient percolation of the added artificial exudates into the rhizosphere of sunflower seedlings (**chapter 4**).

In many plant species, glyphosate is not readily metabolized but preferentially translocated to young growing tissues of roots and shoots, where it can get accumulated in millimolar concentrations. In soil-grown target plants, this inhomogeneous distribution of glyphosate within the root tissues may lead to the formation of “hot spots” of glyphosate containing root residues in soils. Subsequently this stored glyphosate as “hot spots” can be released during microbial degradation of the plant material. To evaluate the potential of a target plant roots in stabilization and subsequent release of glyphosate with intoxicating of subsequent crop plants, model experiments were conducted with an application of glyphosate to rye grass and with a direct soil application of equivalent amount of glyphosate prior to sunflower sowing at different waiting times (0-21 days).

Toxicity of glyphosate pre-sowing treatments on sunflower seedlings was strongly dependent on the mode of glyphosate application. When glyphosate was sprayed on pre-cultured rye grass seedlings as model weed, detrimental effects on plant growth and the Mn nutritional status, as well as increased intracellular shikimate accumulation in root tissue were more strongly expressed than at a direct soil application of the same amount of glyphosate (**chapter 5**). The increased expression of toxicity effects after a glyphosate pre-sowing application to a rye grass pre-culture compared with a direct soil application might indicate that 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. Glyphosate intoxication of non-target plants might be induced by root contact with these hot spots. The findings suggest an important and yet non-investigated role of glyphosate in plant residues in determining the risk for intoxication of non-target plant.

The globally increasing adoption of no-till or reduced tillage systems are becoming a driving force for increasing glyphosate use (Torresen *et al*., 1999). In such systems, glyphosate is applied pre-sowing for weed control and glyphosate may remain in root and shoot residues. Usually in these reduced tillage systems soil disturbance only occurs at sowing which might lead to incorporation of the glyphosate contaminated straw only to the upper soil layer where germination of following non-target crops will take place. Hence, roots of germinating seedlings may directly come in contact to the glyphosate contaminated weed residues with a subsequent phytotoxicity. To evaluate such risk, a pot experiment was conducted under controlled greenhouse conditions with the two contrasting soils. Glyphosate was supplied as glyphosate pre-treated rye grass plant material either as shoot or root residues (chopped to 1 cm) or homogenates (ground under liquid nitrogen) (**chapter 6**). Analysis of physiological parameters such as intracellular shikimate accumulation as metabolic indicator for glyphosate toxicity, biomass production and micronutrient status revealed, that detrimental effect linked to glyphosate toxicity originated from treated rye grass shoot straw or homogenate incorporated into the Arenosol but not into the Luvisol. This is most probably related to the difference in soil property between the two soils. At this level of glyphosate supply,

the detoxification capacity of the highly buffered calcareous subsoil with high Ca and Mg availability as potential ligands was high enough for an adequate immobilization and inactivation of glyphosate (Sprankle *et al*., 1975b). This might have played a primary role in preventing glyphosate toxicity in the Luvisol. On the weakly buffered acidic Arenosol with a low level of available Ca and Mg, the level of glyphosate supply was higher than the detoxification capacity. In addition to the difference in detoxification capacity between both soils, differences in nutrient bio-availability might have also aggravated the observed inhibition of nutrient acquisition. Therefore, only on the strongly weathered nutrient poor Arenosol such detrimental effects by glyphosate could be observed. The soil type dependent differences in toxicity by glyphosate enriched crop residues can also be related to differences in microbial community composition and soil texture between the two soils as these properties might play an important role in the decomposition rate of the shoot residues too.

In contrast to glyphosate treated shoot, application of glyphosate treated rye grass roots caused no plant toxicity as reflected by no plant growth depression or intracellular shikimate accumulation. This possibly was due to sublethal glyphosate supply via the root residue. The amount of root residues supplied (700mg dry matter kg^{-1} soil) was close to half the amount of shoot material supplied (1200mg dry matter kg^{-1} soil). But also higher phenol and lignin contents in roots might result in a slower decomposition rate of roots than shoot residues. As a consequence, a slower release rate of glyphosate stored in the root residues might explain the missing effect of root residues in this experiment. Thus, the findings suggest the importance of weed residues in transferring glyphosate from target to non-target plants, particularly in no-till or reduced tillage systems, with consequence of detrimental effects on intoxication of following crop plants.

All together, the achieved results of the model pot experiments are in correspondence with that of the reported field experiments. Further, the results revealed the important role of glyphosate desiccated weed plants in a soil as a glyphosate pool for intoxication of following crops. More information on transformation of these glyphosate enriched crop residues as “hot spots” and its glyphosate release during microbial decomposition are urgently needed for a better precaution and risk assessment of glyphosate for farmers practice.

Zusammenfassung

Die Organo-Phosphatverbindung Glyphosat ([N-Phosphonomethyl] Glycine) wird weltweit als nicht-selektives Herbizid mit breitem Wirkungsspektrum in einer Vor- und/oder Nachsaatbehandlung zur Kontrolle von Unkräutern im Gartenbau, Landwirtschaft, Forstwirtschaft und urbanen Flächen verwendet. Es bietet eine effektive Kontrolle der meisten annuellen und perennierenden Unkräuter und ist das weltweit meist verkaufte Herbizid. Einer der Gründe für die Beliebtheit von Glyphosat besteht in seinem Effekt auf die Wurzel- und Rhizomsysteme von Wurzelunkräutern nach einer Blattapplikation. Glyphosat wird als systemisches Herbizid innerhalb der Pflanze leicht vom Spross in die Wurzel verlagert und anschließend in die Rhizosphäre abgegeben. Im Boden wird Glyphosat rasch durch die Bildung von Wasserstoffbrückenbindungen und Ionen-Austausch an Tonminerale, Oxide und Hydroxide und organischer Substanzen festgelegt und damit inaktiviert. Dieses Sorption von Glyphosat im Boden hängt von Bodeneigenschaften wie u.a. dem pH-Wert, der Konzentration an di- und trivalenten Kationen in der Bodenlösung, Gehalten an Eisen-Humuskomplexen, den Gehalten an anorganischem Phosphat ab. Dieses Sorptionsverhalten stellt einen wesentlichen Unterschied zwischen Glyphosat und anderen Herbiziden dar und hat daher maßgeblich zu der Annahme beigetragen, Glyphosat habe durch eine schnelle Festlegung im Boden keine residuale phytotoxische Wirkung auf Nicht-Zielpflanzen bzw. Kulturpflanzen. Die Festlegung von Glyphosat an die Bodenmatrix kann jedoch unter bestimmten Bedingungen ein reversibler Prozess sein. Es gibt in der wissenschaftlichen Literatur für eine Reihe von Pflanzenarten Hinweise auf eine solche residuale phytotoxische Aktivität von Glyphosat im Boden und widersprüchliche Ergebnisse bezüglich der biologischen Verfügbarkeit von Glyphosat in Böden bzw. möglicher Risiken für Nicht-Zielorganismen, wie beispielsweise Folgekulturpflanzen und Bodenmikroorganismen. Bedenken bezüglich einer möglichen phytotoxischen Wirkung von Glyphosatrückständen auf Nicht-Zielpflanzen haben mit der durch die Einführung von Glyphosat-resistenten Kulturpflanzen und der pfluglosen Bodenbearbeitung verursachten ansteigenden Verwendung von Glyphosat zugenommen. Als Konsequenz aus diesen Bedenken gibt es inzwischen einen beachtlichen Umfang an wissenschaftlichen Untersuchungen in der

wissenschaftlichen Literatur (Fernandez et al., 2005; Huber et al. 2005; Neumann et al.; 2006; Bott et al., 2008).

Angesichts der zunehmenden Anzahl an bisher ungeklärten Beobachtungen von negativen Seiteneffekten nach einer Glyphosatapplikation (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) bestand das Ziel der hier vorliegend Arbeit in der Identifikation potenzieller Risikofaktoren für Nicht-Zielpflanzen bei der Applikation von Glyphosat in Agrarökosystemen.

Zu diesem Zweck wurden: (1) die Relevanz von Wartezeiten für die Aussaat von Kulturpflanzen nach einer Vorsaatbehandlung mit Glyphosat, (2) die potenzielle, durch veränderte pH-Werte in der Rhizosphäre induzierte Remobilisierung von an der Bodenmatrix festgelegtem Glyphosat, (3) die Rolle der Wurzeln von mit Glyphosat behandelten Unkrautpflanzen als Zwischenspeicher für Glyphosat und (4) die Relevanz einer Abgabe von Glyphosat aus sich zersetzenden Rückständen behandelter Unkrautpflanzen für phytotoxische Effekte auf die Folgekultur unter kontrollierten Gewächshausbedingungen auf zwei kontrastierenden Böden, einem schwach gepufferten, sauren Arenosol (Oberboden) und einem stark gepufferten, kalkhaltigen Luvisol (Unterboden), durchgeführt. Darüber hinaus wurden zwei Feldversuche durchgeführt, um Teile der in Modellversuchen gewonnenen Erkenntnisse auf ihre Relevanz unter Feldbedingungen zu prüfen.

In **Kapitel 3 und 5** sind die Resultate der Modellversuche unter kontrollierten Gewächshausbedingungen auf zwei kontrastierenden Böden und zwei Feldversuchen dargestellt und diskutiert. Alle durchgeführten Experimente zeigten, dass die Toxizität von Glyphosatrückständen für Kulturpflanzen mit abnehmenden Wartezeiten zwischen der Glyphosatapplikation auf Unkrautpflanzen und der Aussaat der Kulturpflanzen zunahmen.

In Modellversuchen unter Gewächshausbedingungen auf zwei kontrastierenden Böden und Sonnenblumen als Modell-Kulturpflanze, war das Wachstum und die Biomasseproduktion von Sonnenblumenkeimlingen bei einer Wartezeit von 0 Tagen nach einer Vorsaatapplikation vom Glyphosat stark eingeschränkt. Mit zunehmenden

Wartezeiten von 7-21 Tagen nahm die Einschränkung des Wachstums und der Biomasseproduktion ab. Diese negativen Effekte auf die Entwicklung und das Wachtum der Sommenblumen war in allen Experimenten mit erhöhten Konzentrationen an Shikimat im Wurzelgewebe als Indikator der Glyphosattoxizität verbunden.

Die Applikation von Glyphosat verursachte bei Sonnenblumensämlingen auch eine Einschränkung der Mangan (Mn)-Versorgung, die auch nach einer Wartezeit von 21 Tagen zwischen einer Vorsaatbehandlung mit Glyphosat und der Aussaat der Sonnenblumen vor allem auf dem Arenosol noch festzustellen war. Die Glyphosat-induzierte Beeinträchtigung der Mn-Versorgung der Sonnenblumen war im Vergleich zwischen den beiden gegensätzlichen Böden stärker auf dem sandigen, sauren, schwach gepufferten Arenosol mit niedriger Ca-Verfügbarkeit ausgeprägt als auf dem stark gepufferten, kalkhaltigen Unterboden. Dies deutet darauf hin, dass die Bodenart einer der bestimmenden Faktoren für die Stärke der Toxizität von Glyphosat für Nicht-Zielpflanzen sein kann. Diese Verminderung der Mn-Aneignung war nicht mit den korrespondierenden Unterschieden in den intrazellulären Shikimatkonzentrationen oder der Pflanzenbiomasseproduktion verbunden. Dies weist, zumindest bei den in diesen Versuchen verwendeten Glyphosataufwandsmengen, eher auf bodenartspezifische Unterschiede in der Mn-Verfügbarkeit als auf eine unterschiedliche Expression der Glyphosattoxizität auf den beiden untersuchten Böden hin. Auch die Glyphosat-induzierte Verminderung des Wurzelwachstums könnte die Mn-Aneignung und/oder Mn-Aufnahme vor allem auf dem sauren Sandboden mit niedrigen Gehalten an pflanzenverfügbarem Mn (Arenosol) beeinträchtigt haben.

Die Ergebnisse des Feldversuchs in Hirrlingen (Raum Tübingen) bestätigten die Relevanz von Wartezeiten nach einer Vorsaatbehandlung mit Glyphosat. Verzögerte Pflanzenentwicklung und Heterogenität im Auflaufen der Winter-Weizenpflanzen konnte verstärkt beobachtet werden, wenn Glyphosat bei einer kurzen Wartezeit von 2 Tagen vor der Aussaat im Vergleich zu einer Wartezeit von 14 Tagen vor der Aussaat des Winter-Weizens auf Unkrautpflanzen appliziert wurde.

Dieses heterogene Schadbild, das abgeschwächt, aber in durchaus vergleichbarer Art und Weise auch in Modellversuchen zu beobachten war, ist möglicherweise durch die

Konzentrierung von Glyphosat in jungen Wurzelzonen behandelter Unkrautpflanzen als sogenannte „hot spots“ und damit einer räumlich begrenzten Erhöhung der Glyphosatkonzentration in der Rhizosphäre der Unkrautwurzeln und/oder einer zeitlich verzögerten Abgabe von Glyphosat aus den sich zersetzenden Wurzelrückständen der behandelten Unkrautpflanzen erklärbar. Beide Mechanismen könnten zu einer Verzögerung der Festlegung und/ oder des Abbaus von Glyphosat in Böden und einer räumlich begrenzten Zone erhöhter Glyphosataktivität („hot spot“) beitragen. Entsprechend dieser Hypothese hängt die Expression von Glyphosat-induzierten Schädigungen von Nicht-Zielpflanzen nach einer Vorsaatbehandlung mit Glyphosat davon ab, ob die Wurzeln der Kulturpflanzen in einen Glyphosat „hot spot“ wachsen oder nicht (Römheld et al., 2008).

Bei einer kurzen Wartezeit von 2 Tagen zwischen der Glyphosatapplikation und der Aussaat zeigte eine visuellen Bonitur der Pflanzenschäden, dass bis 50% der Pflanzen Schäden aufwiesen, die wahrscheinlich durch Glyphosatrückstände im Wurzelraum ausgelöst wurden. Diese in der vegetativen Wachstumsphase entstandenen Schäden waren über die gesamte Wachstumsperiode visuell erkennbar und übereinstimmend mit Ergebnissen in der Literatur (Buehring et al., 2007) positiv mit Verlusten im Kornertrag korreliert.

Die Wachstumsdepressionen von Weizenpflanzen, die in Parzellen mit kurzen Wartezeiten (2 Tage) kultiviert wurden, war im Vergleich zu den Pflanzen, die in Parzellen mit langen Wartezeiten (14 Tage) kultiviert wurden, auch mit einem verminderten Nährstoffstatus (Ca, Mg, Zn und Cu) verbunden. Diese negativen Auswirkungen auf den Nährstoffstatus der Weizenpflanzen war auch von der Höhe der Glyphosataufwandmenge korrelliert und im Falle einer Applikationsrate von 6 L ha^{-1} im Vergleich zu einer einer Applikationsrate von 2 L ha^{-1} stärker ausgeprägt. Die Ergebnisse dieses Feldversuchs decken sich mit früheren Empfehlungen, die eine Wartezeit von 3 Wochen Wartezeit nach einer Vorsaatbehandlung mit Glyphosat zur Vermeidung bzw. Verminderung von Glyphosat-induzierten Schäden an Kulturpflanzen vorschlagen (Cornish, 1992).

Da Glyphosat in Böden ein vergleichbares Bindungsverhalten wie Phosphat zeigt, wurde die Hypothese aufgestellt, dass Prozesse, die zu einer Mobilisierung von Phosphat in der Rhizosphäre führen, wahrscheinlich auch geeignet sind, an die Bodenmatrix sorbiertes Glyphosat zu remobilisieren. Beispielsweise kann möglicherweise eine Ansäuerung der Rhizosphäre durch eine physiologisch sauer wirkende Ammonium-Stickstoffdüngung (NH_4^+) die biologische Verfügbarkeit von an die Bodenmatrix sorbierten Glyphosat erhöhen, vor allem wenn Glyphosat als Ca-Glyphosat vorliegt.

Um diese Hypothese zu überprüfen, wurde ein Experiment unter kontrollierten Bedingungen auf zwei Böden mit unterschiedlichen Eigenschaften, die mit verschiedenen Applikationsraten von Glyphosat vorinkubiert und mit stabilisierten Ammonium oder Nitrat als Stickstoffdüngungen gedüngt wurden, durchgeführt **(Kapitel 4)**.

Die Resultate dieses Experiments konnten jedoch die Hypothese einer Remobilisierung von im Boden festgelegten Glyphosat nicht bestätigen. Auf einem kalkhaltigen Luvisol konnte keine durch Düngung mit stabilisiertem Ammonium verursachte Remobilisierung von Glyphosat induziert werden. Eine phytotoxische Wirkung von Glyphosat bzw. eine intrazelluläre Akkumulation von Shikimat in Abhängigkeit einer Absenkung des Rhizosphären-pH-Werts oder eine Verminderung der Biomasseproduktion von Sonnenblumen wurde nicht beobachtet. Diese Ergebnisse können möglicherweise damit erklärt werden, dass die in diesem Modellversuch verwendeten Aufwandmengen an Glyphosat, da sie homogen mit dem gesamten Bodenvolumen gemischt wurde, zu niedrig gewählt waren. In der Realität wird Glyphosat nach einer Applikation auf die Blätter von Unkrautpflanzen von den Pflanzen rasch aufgenommen und innerhalb kurzer Zeit in die Wurzeln verlagert und verursacht so möglicherweise die Ausbildung von „hot spots“ mit lokal hohen Konzentrationen an Glyphosat.

Auf dem kalkhaltigen Luvisol, auf dem eine Bildung von Ca-Glyphosat-Verbindungen erwartet worden war, zeigte sich im Falle einer Stickstoffdüngung mit Ammonium lediglich eine minimale Wurzel-induzierte Veränderung des Rhizosphären pH-Werts (0,5 pH Einheiten). Darüber hinaus könnte auf dem Arenosol, auf dem eine geringe Bildung von Ca-Glyphosat-Verbindungen erwartet worden war, nach einer Vorinkubationszeit

von 21 Tagen Glyphosat weitgehend mikrobiell abgebaut und/oder an P-Bindungsstellen des sauren tropischen Boden stark festgelegt worden sein.

Die Abgabe von Wurzelexsudate (v.a. Carboxylaten) wird als pflanzliche Strategie zur Mobilisierung von Phosphat durch Chelatisierung und/ oder Desorption angesehen. Die Möglichkeit eines vergleichbaren Mechanismus wurde für eine Remobilisierung von Glyphosat angenommen. In der hier vorliegenden Studie zeigten sich in einem Modellversuch auf zwei unterschiedlichen Böden, die mit unterschiedlichen Aufwandmengen an Glyphosat vorinkubierten wurden, nach einer Applikation von Na-Citrat oder Zitronensäure keine eindeutigen Hinweise auf eine relevante Remobilisierung von Glyphosat mit phytotoxischen Auswirkungen.
Auf dem sauren Arenosol zeigten sich unabhängig von der Behandlung keine Unterschiede im Wachstum von Sonnenblumen. Auf einem Luvisol verursachte die Applikation von 10µmol g^{-1} Na-Citrat, aber nicht von Zitronensäure, in den Glyphosatbehandlungen eine Verminderung der Wurzelbiomasse. Diese Verminderung war jedoch nicht mit einer intrazellulären Akkumulation von Shikimat als Bio-Indikator der Phytotoxizität von Glyphosat verbunden. Möglicherweise konnte eine Remobilisierung von Glyphosat durch die Applikation von synthetischen Wurzelexsudaten nicht induziert werden, weil Glyphosat nach einer Vorinkubation von 21 Tagen bereits weitgehend mikrobiell abgebaut und/oder stark festgelegt war, oder keine ausreichende Perkolation der Wurzelexsudate in die Rhizosphäre der Sonnenblumen erreicht wurde **(Kapitel 4)**.

Nach dem bisherigen Stand des Wissens wird Glyphosat in Pflanzen, mit der beachtenswerten Ausnahme von Glyphosat-resistenten Sojabohnen, in keinem nennenswerten Umfang abgebaut, sondern bevorzugt in die meristematischen Wachstumszonen in Wurzeln und Sprosse verlagert. In diesen Wachstumszonen findet eine Akkumulation von Glyphosat statt und es können bis zu millimolare Konzentrationen im Gewebe erreicht werden. Diese inhomogene Verteilung von Glyphosat innerhalb der Pflanze (insbesondere der Wurzeln) kann in der Rhizosphäre

zu der Bildung von räumlich begrenzten Zonen mit sehr hohen Konzentrationen an Glyphosatrückständen („hot spots“) beitragen. In der Folgezeit kann dieses, in den Wurzeln von Zielpflanzen in hohen Konzentrationen gespeichertes Glyphosat, während des mikrobiellen Abbaus der Wurzelrückstände freigesetzt werden.

Um die Relevanz von Unkrautwurzeln für die Stabilisierung und anschließende verzögerte Abgabe von Glyphosat in die Rhizosphäre und einer Schädigung der Folgekultur zu evaluieren, wurden in Modellversuchen die Auswirkungen unterschiedlicher Wartezeiten (0-21 Tage) nach einer Glyphosatapplikation auf Weidelgras im Vergleich zu einer Applikation einer identischen Aufwandmenge an Glyphosat direkt in den Boden auf die Folgekultur (Sonnenblumen) auf zwei unterschiedlichen Böden (Arenosol/Luvisol) untersucht. Die Phytotoxizität von Glyphosat nach einer Vorsaatbehandlung für die Folgekultur (Sonnenblumen) war stark von der Art der Glyphosatapplikation abhängig. Wenn Glyphosat auf Weidelgras als Modellunkraut appliziert wurde, waren negative Effekte auf die Entwicklung und das Wachstum von Sonnenblumenkeimlingen, ein verminderter Nährstoffstatus von Mn und eine erhöhte intrazelluläre Akkumulation von Shikimat als Indikator von Glyphosattoxizität im Vergleich zu der Applikation einer identischen Aufwandmenge an Glyphosat direkt in den Boden signifikant stärker ausgeprägt **(Kapitel 5)**.

Die im Vergleich zu einer direkten Bodenapplikation von Glyphosat deutlich erhöhte Ausprägung von phytotoxischen Effekten einer Glyphosatapplikation auf Weidelgras deutet darauf hin, dass die Wurzeln von Glyphosat-behandelten Unkrautpflanzen auf den untersuchten Böden einen Speicherpool von Glyphosat in der Rhizosphäre darstellen können. In diesem Experiment war die biologische Verfügbarkeit von Glyphosat aus Wurzeln und Wurzelrückständen behandelter Unkrautpflanzen für Nicht-Zielpflanzen offensichtlich deutlich höher als die biologische Verfügbarkeit von Glyphosat aus dem Boden selbst.

Die weltweit zunehmende Verwendung von Anbausystemen mit reduzierter Bodenbearbeitung und Direktssaat ist einer der Faktoren für die zunehmende Verwendung von Totalherbiziden (v.a. Glyphosat), die in diesen Anbausystemen als unumgänglich angesehen werden (Torresen *et al*., 1999). In diesen Anbausystemen

wird Glyphosat möglichst kurzfristig vor der Aussaat auf Unkräuter appliziert und Glyphosat verbleibt möglicherweise bis zur mikrobiellen Zersetzung in Wurzel- und Sprossgewebe dieser Zielpflanzen.

Normalerweise erfolgt in Anbausystemen mit reduzierter Bodenbearbeitung nur während der Aussaat eine begrenzte Durchmischung des Bodens. Denkbare Konsequenzen einer reduzierten Bodenbearbeitung sind zum einen die Einarbeitung Glyphosat-belasteter Sprossrückstände in die obere Bodenschichten in denen die Keimung der Folgekultur stattfindet und/oder die Persistenz von räumlich begrenzten Zonen mit hohen Konzentrationen an Glyphosat aus Wurzeln und/oder Wurzelrückständen behandelter Unkrautpflanzen, die durch die sehr begrenzte Durchmischung des Boden intakt bleiben.

Aus diesen Gründen kann angenommen werden, dass Wurzeln keimender Kulturpflanzen in direkten Kontakt mit Glyphosat-belasteten Rückständen von Unkrautpflanzen kommen können und durch Glyphosat geschädigt werden. Um dieses Risiko abzuschätzen, wurde ein Gefäßversuch unter Gewächshausbedingungen auf zwei gegensätzlichen Böden (Arenosol, Luvisiol) durchgeführt. Die Applikation von Glyphosat erfolgte in Form von Glyphosat behandelten Blättern oder Wurzeln von Weidelgraspflanzen, die in separaten Töpfen mit Nährlösung vorkultiviert und anschließend in den Boden eingemischt wurden (Kapitel 6). Die Analyse physiologischer Parameter, wie der intrazellulären Akkumulation von Shikimate als Indikator der Glyphosattoxizität, der Biomasseproduktion und des Mikronährstoffstatus der Pflanzen, zeigte, dass die untergemischten Glyphosat-belasteten Blätter von Weidelgraspflanzen auf dem Arenosol, aber nicht auf dem kalkhaltigen Luvisol, negative phytotoxische Effekte auslösen können.

Diese Ergebnisse sind wahrscheinlich mit unterschiedlichen Eigenschaften zwischen den beiden Böden erklärbar. Möglicherweise war bei den in diesem Modellversuch verwendeten Aufwandmengen an Glyphosat auf dem stark gepufferten kalkhaltigen Unterboden (Luvisol) mit hoher Verfügbarkeit an Ca und Mg als potenzielle Liganden von Glyphosat, das Potenzial für eine Immobilisierung und Inaktivierung von Glyphosat ausreichend, um phytotoxische Effekte für die Folgekultur zu vermeiden (Sprankle et al.,

1975). Möglicherweise war im Gegensatz dazu auf dem schwach gepufferten, sauren Arenosol mit niedrigen Gehalten an Ca und Mg die applizierte Aufwandmenge an Glyphosat größer als das Detoxifizierungspotenzial des Bodens und verursachte deshalb Schäden an der Folgekultur. Neben Bodenart-spezifischen Unterschieden im Potenzial zur Detoxifizierung von Glyphosat spielten möglicherweise auch Unterschiede zwischen den beiden Böden bezüglich der biologischen Verfügbarkeit von Nährstoffen eine Rolle und verstärkten die durch die Applikation von Glyphosat induzierten negativen Effekte auf den Nährstoffstatus der Sonnenblumen, die auf dem stark verwitterten, nährstoffarmen Arenosol beobachtet werden konnten.

Die im Vergleich der beiden Modellböden beobachtete Unterschiede in der phytotoxischen Wirkung Glyphosat-belasteter Rückstände von Unkrautpflanzen auf die Folgekultur könnte auch von Unterschieden in der Zusammensetzung der Bodenmikroorganismen und/oder der Bodentextur und anderen Faktoren abhängen, die sich auf die Zersetzungsrate von pflanzlichen Rückständen im Boden auswirken.

Im Gegensatz zu Glyphosat-belasteten Weidelgrasblättern verursachte die Applikation von Glyphosat-belasteten Wurzeln auf beiden Böden für Sonnenblumen als Folgekultur keine Symptome von Phytotoxizität, Wachstumsdepressionen oder intrazelluläre Akkumulation von Shikimat (Toxizitätsindikator). Da die applizierte Menge an Glyphosat-belasteten Wurzelrückständen (700mg Trockenmasse kg^{-1} Boden) deutlich niedriger als die Menge an Glyphosat-belasteten Blättern (1200mg Trockenmasse kg^{-1} Boden) war, ist es möglich, dass mit den Wurzeln lediglich eine nicht toxische wirkende Aufwandmenge an Glyphosat appliziert wurde und deshalb keine negativen Auswirkungen beobachtet werden konnten. Darüber hinaus war das in der Vorkultur auf Weidelgras applizierte Glyphosat noch wesentlich stärker im Spross als in den Wurzeln akkumuliert.

Möglicherweise spielten auch die in Wurzelgewebe generell höheren Gehalte an phenolischen Verbindungen und Lignin und damit verbunden eine im Vergleich zu oberirdischen Pflanzenteilen langsamere mikrobielle Zersetzungsrate von Wurzeln für diese Ergebnisse eine Rolle. Möglicherweise verursachte die langsamere mikrobielle Zersetzungsrate von Wurzeln auch eine langsamere Abgabe von Glyphosat aus den

Wurzelrückständen und erklärt auf diese Weise den fehlenden negativen Effekt Glyphosat-belasteter Wurzelrückstände auf die Sonnenblumen als Folgekultur in diesem Experiment.

Insgesamt decken sich die Ergebnisse der Modellversuche mit den Beobachtungen und Ergebnissen der im Rahmen dieser Art durchgeführten Untersuchungen im Feld. Die Ergebnisse deuten die hohe Bedeutung von mit Glyphosat behandelten Unkrautpflanzen als Speicherpool von Glyphosat und damit als Risikofaktor für die Folgekultur an. Weitere Untersuchungen über den Umsatz und die Abgabe von Glyphosat aus belasteten Unkrautrückstände sind für eine verbesserte Risikoabschätzung und Vermeidung von unerwünschten Glyphosatschäden an Kulturpflanzen in der landwirtschaftlichen Praxis dringend notwendig.

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Research & Publications:

2008:

Tesfamariam, T, Römheld, V., Bott, S., Cakmak, I., and Neumann, G. (2008). 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* (corrected revision submited).

Bott, S.; Tesfamariam, T., Candan H., Neumann, G., Cakmak, I., and Roemheld, V.,(2008). Glyphosate-induced impairment of plant growth and micronutrient status in glyphosate-resistant soybean (*Glycine max* L.). *Plant and Soil*, 312: 185-194.

2007:

Tesfamariam, T, Römheld, V., Bott, S., Cakmak, I., Neumann, G. (2007). The Fate of Glyphosate in the Rhizosphere. *Rhizosphere2- International conference, Montpellier- France* (poster presentation).

Sebastian Bott, Tsehaye Tesfamariam, Günter Neumann, Ismail Cakmak,Volker Römheld (2007). Evidence for impaired micronutrient acquisition in glyphosate-resistant soybean

(*Glycine max* L.*). Rhizosphere2-International Conference, Montpellier-France* (poster presentation).

Tesfamariam T. ,Bott S ,Otzturk L. ,Eker S., Neumann G, Cakmak I., Römheld V. (2007). Possible Side Effects of the Herbicide Glyphosate on Trace Element Acquisition and Ecosystem Health. *Trace elements-International Conference, Helsinki-Finland* (oral presentation).

Tesfamariam T.; S. Bott, G. Neumann; V. Roemheld (2007). Dynamics of Glyphosate in the rhizosphere of target and non-target plants. *International Symposium on Glyphosate; Botucatu; SP; Brazil.* (oral presentation- presented by V. Roemheld).

2006:

Sebastian Bott, Tsehaye Tesfamariam, Günter Neumann, Volker Römheld (2006). Effects of different waiting times after pre-crop glyphosate treatment on sunflowers (*Helianthus annuus* l.). *plant nutrition meets plant breeding-international conference, hohenheim-germany* (poster presentation).

Zafrin Akter, Tsehaye Tesfamariam, Günter Neumann, Markus Weinmann, Volker Römheld. (2006). Development of microbiological methods to test soil Mn-oxidation and reduction capacity of rhizosphere microorganisms in relation to the severity of soil borne diseases. *Tropentage-International Conference, Bonn-Germany* (poster presentation).

Tesfamariam T., M. del bel Pereira, H. Sasmoto, A. Alexander, V. Roemheld (2006). Efficacy of an Organic Matter-Stabilized Fe Fertilizer for the Remedy of Fe Deficiency Chlorosis on Calcareous Soils. *13th International Symposium on Iron Nutrition and Interactions in Plants – Montpellier, France* (poster presentation).

2005:

Tesfamariam T., Römheld V., Neumann G. 2005. Phosphorus-deficiency induced root exudation of carboxylates contributes to molybdenum acquisition in the rhizosphere of leguminous plants. In: Hartmann A,, Schmid M., Wenzel W. Hinsinger P. (eds.). Rhizosphere 2004 – A tribute to Lorenz Hiltner. GSF-Report, Munich, Neuherberg, Germany, (extended abstract).

2003:

M. Sc. thesis entitled “Effect of P-Deficiency induced root exudates on Mo acquisition by leguminous plants”. University of Hohenheim, Germany.

Work Experience:

09/04 – 01/05: Research assistant of short term project at the Institute of Plant Nutrition; University of Hohenheim in collaboration with the University of Berlin.

1997-2001: Manager for Tesfa-Cooperative Farm, an Agricultural cooperative farm that focuses on Fruit and crop production on the Mereb river basin, Eritrea.

1994/95: Science teacher on Finote-Tibeb middle school.

1993-1997: General supervisor of Asmara branch office for Tesfa-Cooperative Farm as part-

time officer while studying under the University of Asmara, Eritrea.

Language Skills:

Tigrigna (Mother tongue), English (very good), Amharic (very good), German (basic).

Computer skills:

Microsoft office, SigmaStat, SPSS,

Extra Courses:

Summer 2006: ELLS Summer University Socrates Intensive Course Program under the topic "Soil-Plant-Microbe Interaction: Fundamental and Applications".

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Declaration

Hereby I declare that all the data and results presented in this dissertation are all original work of mine and are not copied or reproduced in any form from any other source. Only the resources and sources indicated herein are used for completion of the dissertation.

Hohenheim, 9th April 2009