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Re-Plant Problems in Long-Term No-Tillage Cropping Systems: Causal Analysis and Mitigation Strategies

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List of Abbreviations

Abbreviation	Full Text
%	Percent
®	Registered
°	Degree
°C	Degree Celsius
µL	Micro Litre
µM	Micromolar
µmol	Micromole
AG	Aktiengesellschaft
Al	Aluminium
AMPA	Aminomethyl-phosphonic acid
B	Boron
B.C.	Before Christ
BBF	www.sterixpert.de
bit	Binary digit
C	Carbon
$C_3H_8NO_5P$	*N*- phosphonomethyl-glycine, chemical formula of Glyphosate
Ca	Calcium
CA	Conservation Agriculture
$CaCl_2$	Calciumchlorid
CAL	Calcium-acetate-lactate
CAN	Calcium-ammonium nitrate
CaO	Calcium oxide
Cd	Cadmium
cDNA	Complementary DNA
CH_4	Methane
cm	Centimetre
CO_2	Carbon dioxide
Cr	Chromium

Cs/La	Cesium chloride- lanthanum chloride buffer solution
Cu	Copper
cv.	Cultivar
DNA	Deoxyribonucleic acid
DAS	Days After Sowing
DM	Dry Matter
DMSZ	Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany
dpi	Dots Per Inch
DPTA	Diethylenetriaminepentaacetic acid
DT50	Degradation time for 50% of a compound
e.g.	Exempli gratia
EDTA	Ethylenediaminetetraacetic acid
EPSPS	5-enolpyruvylshikimate-3-phosphate synthase
EUF	Electro-ultrafiltration
FAO	Food and Agriculture Organization
Fe	Iron
g	Gram
GA	Glyphosate and AMPA treatment
GAT	Dupont/Pioneer Hi-Bred, brand Optimum GAT Soybean
Gb	Giga bases
Gly	Glyphosate
GM	Genetically Modified
GMO	Genetically Modified Organism
GR	Glyphosate-Resistant
h	hour
H_2SO_4	Sulphuric acid
ha	hectare
HCl	Hydrogen chloride
Hg	Mercury
HirF	Hirrlingen Friedhof

HirG	Hirrlingen Gassäcker
HNO_3	Nitric acid
i.e.	id est
IBM	International Business Machines Corporation
Inc.	Incorporation
jpeg	Joint Photographic Experts Group
K	Potassium
K_2O	Potassium oxide
kg	Kilogram
kGy	Kilogray
km	Kilometre
kPa	Kilo Pascal
L	Litre
L.	(Carl von) Linné
LA Chemie	Landesanstalt für Landwirtschaftliche Chemie
log KOW	The octanol-water partition coefficient
LT	Long-term
LTZ	Landwirtschaftliches Technologiezentrum Augustenberg
M	Molar Mass
m	metre
Mg	Magnesium
mg	milligram
mL	millilitre
mm	millimetre
Mn	Manganese
Mol	Unit for molecular weight
nm	Nanometer (a unit of measurement of Ultra Violet Light)
N_2O	Dinitrogen monoxide
Ni	Nickel
nm	Nanometre
NR	Non-resistant

P	Phosphate
P_2O_5	Phosphorus pentoxide
Pa	Pascal
PAL	Phenylalanine ammonialyase
Pb	Lead
PEP	Phosphoenolpyruvate
pH	Potential hydrogen
Phe	Phenylalanine
pKa	Logarithmic acid dissociation constant
POE-tallowamine	Polyethoxylated tallowamine
Rem	Remmingsheimer Weg
RNA	Ribonucleic acid
RR	Roundup Ready® by Monsanto, San Francisco, USA
S	Sulphur
s	Seconds
SL	Schwarze Länder
SPAD	Soil plant analysis development
SPSS	Statistical Package for the Social Sciences (Statistic Software of IBM)
ST	Short-term
SW	Sülcher Wegle
t	tons
TB	TKS and sand mixture with biochar applied
TG	TKS and sand mixed with glyphosate
TGB1&5	TKS and sand mixture with glyphosate and biochar at rate of biochar 1% & 5% v/v
TKS®	www.floragard.de/fr/Produkte/Die-Bodenverbesserer
™	Trade Mark
Trp	Tryptophan
TTC	2,3,5- Triphenyl tetrazolium chloride
Tyr	Tyrosine

U.S.A.	The United States of America
UK	United Kingdom
US	The United States
US$	US Dollar
UV	Ultraviolet
v/v	Volume by volume
VDLUFA	Verband Deutscher Landwirtschaftlicher Untersuchungs- und Forschungsanstalten
w/v	Weight by volume
w/w	Weight by weight
WHO	The World Health Organisation
Zn	Zinc

Summary

No-tillage is considered as a promising alternative for tillage-based conventional farming, by saving energy-input and time, reducing groundwater pollution and counteracting soil erosion and losses of the soil-organic matter. However, in the recent past, no-tillage farmers in Southwest Germany repeatedly reported problems particularly in winter wheat production, characterized by stunted plant growth in early spring, chlorosis, impaired fine root development and increased disease susceptibility. These symptoms were particularly apparent on field sites with long-term (≥ 10 years) no-tillage history (LT) but not on adjacent short-term (≤ 2 years) no-tillage plots (ST). The effects could be reproduced in pot experiments under controlled conditions, with soils collected from the respective field sites in five different locations, providing a basis for causal analysis.

The expression of damage symptoms in pot experiments with sieved soils, excluded differences in soil compaction, induced by long-term no-tillage farming as a potential cause. Soil analysis revealed higher levels of soil organic matter in the topsoil, as expected for LT field sites and no apparent mineral nutrient deficiencies, both, on LT and ST soils. However, phosphate (P) deficiency was characteristic for plants grown on LT soils. Obviously, this was caused by the limited acquisition of sparingly soluble soil P, due to impaired root development but not by low P availability on LT soils.

In four out of five cases, gamma-ray soil sterilization did not affect the expression of plant damage symptoms on LT soils, excluding pathogen effects as a major cause. Soil application of biochar, at a rate of 5% (v/v), rapidly restored plant growth on LT soils, detectable already during the first week after sowing. This finding points to the presence of a phytotoxic compound since binding of soil xenobiotics by biochar is well documented. Accumulation of allelopathic compounds, originating from crop residues and root exudates remaining in the topsoil, is a problem related to no-tillage farming, particularly in cases of limited crop rotations or in monocultures, which also applied to the investigated field sites. However, a specific wheat auto-allelopathic effect is unlikely, since similar crop damage was also observed in soybean, sunflower, oilseed rape and various cover crops. Typical for allelopathic effects, in the pot experiments, plant damage

symptoms in winter wheat appeared rapidly during emergence and early seedling development. However, under field conditions, germination and early growth were usually not affected, and symptoms were first detectable during re-growth in early spring. Moreover, damage symptoms disappeared when soil sampling was performed in summer instead of early spring, suggesting degradation of the toxic compound, which is also not compatible with the hypothesis of long-term accumulation of allelopathic compounds. The observed temporal pattern of plant damage rather resembled residual effects, occasionally observed after application of certain herbicides with soil activity (e.g., sulfonylureas, propyzamide). Therefore, a systematic survey of herbicide residues was conducted for topsoils on six pairs of LT and ST-field sites.

Characteristic for no-tillage farming, glyphosate was the only herbicide, commonly and regularly used on all investigated field sites. The soil analysis revealed higher levels of glyphosate residues on all investigated LT, soils as compared with directly neighboured ST plots. Particularly on LT plots with strong expression of plant damage symptoms, high concentrations of glyphosate (2-4 mg kg^{-1} soil), and of its metabolite AMPA were detected in the 10 cm topsoil layer. This concentration range is characteristic for residual levels, usually observed several days after glyphosate applications but was still detectable in early spring, six months after the last glyphosate treatment, while only trace concentrations below the detection limit (0.05 mg kg^{-1} soil) were found in ST soils. Coinciding with the declining plant damage potential, residual glyphosate and AMPA concentrations on LT plots declined during the vegetation period until early summer. No comparable pattern was detectable for residues of other herbicides, such as pendimethalin and propyzamide. Degradation of glyphosate residues in soils correlates with microbial activity. Accordingly, reduced soil respiration as an indicator for microbial activity was detected in four out of five cases in soil samples collected from LT field sites, suggesting delayed glyphosate degradation as compared with ST plots.

Due to rapid adsorption, glyphosate usually exhibits extremely limited soil activity. However, at least trace concentrations of glyphosate and AMPA (1.5-3.5 µg L^{-1}) were detectable also in the potentially plant-available, water-soluble phase in spring samples, collected from LT field plots with high potential for plant damage. Nutrient solution

experiments, with 3-6 weeks exposure of winter wheat to the residual herbicide concentrations detected in the LT soil solution, revealed the development of chlorosis and similar to soil experiments, a 30%-50% reduction in fine root production, which surprisingly was mainly induced by AMPA and to a lesser extent by glyphosate itself. Accordingly, both, in hydroponics and LT soil experiments, the plant damage symptoms were not associated with shikimate accumulation in the root tissue as a physiological indicator for glyphosate but not for AMPA toxicity. The dominant role of AMPA toxicity also became apparent by the fact that, both, glyphosate resistant (GR) and non-resistant (NR) soybean plants were affected on LT no-tillage soils since transgenic GR plants are not resistant to AMPA.

A preliminary RNAseq gene expression analysis of the root tissue just prior to the appearance of visible plant damage symptoms, revealed down-regulation of genes involved in general stress responses, down-regulation of aquaporin genes (PIPs and TIPs) with functions in water uptake and root elongation, down-regulation of ethylene-related genes but up-regulation of cytokinin-related gene expression indicating interferences with hormonal balances. These changes in gene expression patterns relative to the untreated control were detected in plants treated with AMPA and glyphosate+AMPA but not with glyphosate alone. The findings suggest that long-term exposure to subtoxic levels of AMPA, as major glyphosate metabolite temporally accumulated in LT no-tillage soils, can finally interfere with metabolic processes essential for normal root development.

A series of pot and field experiments were initiated to test the potential of selected commercial formulations of plant growth-promoting microorganisms, based on strains of *Pseudomonas* sp., *Bacillus amyloliquefaciens*, and *Trichoderma harzianum*, for mitigation of plant stress symptoms, expressed on LT no-tillage field sites in spring. For members of the selected microbial genera, root growth-promoting effects, pathogen suppression, and glyphosate degradation potential have been reported. Unfortunately, plant growth promotion was detectable only on ST soils but was not successful on LT plots, both, in pot and field experiments, probably related to limited root development for microbial colonization and early summer drought under field conditions. As an alternative approach, incorporation of pyrolysis biochar from woody substrates at a rate of 5 % (v/v)

to the top 10 cm soil layer of LT soils, equivalent to approx. 35 t ha^{-1}, were able to restore plant growth completely in pot experiments and protected wheat plants from glyphosate overdose applications (up to 8 L Roundup Ultramax® ha^{-1}), even on artificial substrates with low potential for glyphosate adsorption. As a short-term mitigation strategy, field-testing with different biochar concentrations is recommended.

During the last two years, farmers also modified their no-tillage management strategies on the investigated field sites by introducing more variable crop rotations including, winter wheat, winter rape, maize and soybean and using mustard, pea, and *Crotalaria* as cover crops. Despite further annual applications of glyphosate (3 L ha^{-1} of a 360 g ai L^{-1} formulation), plant performance on the respective field sites was significantly improved. These observations suggest that limited crop rotation favored the development of a soil microflora with low degradation potential for glyphosate, leading to a decline in degradation rates of glyphosate soil residues and underline the importance of crop diversity management.

Zusammenfassung

Pfluglose Anbaumethoden werden oft als vielversprechende Alternative für Ackerbau mit wendender Bodenbearbeitung betrachtet, die zur Einsparung von Arbeitszeit und Energieverbrauch beitragen, der Grundwasserbelastung und Bodenerosion entgegenwirken und die Humusbilanz verbessern. In Direktsaatanbausystemen in Süddeutschland wurden jedoch in den letzten Jahren verstärkt Nachbauprobleme insbesondere beim Anbau von Winterweizen beobachtet, die sich in Wachstumsdepressionen, Chlorosen und Nekrosen, verminderter (Fein)-Wurzelbildung und lückiger Bestandesentwicklung der betroffenen Pflanzen äußern. Die Symptome wurden besonders deutlich auf langjährigen Direktsaatflächen (LT $\geq$ 10 Jahre) im Vergleich zu unmittelbar benachbarten Tauschflächen mit nur kurzzeitiger Direktsaatbewirtschaftung (ST $\leq$ 2 Jahre). Die Effekte konnten in Topfversuchen unter kontrollierten Bedingungen reproduziert werden und bieten so die Grundlage für eine Ursachenanalyse.

Die Ausprägung von Schadsymptomen in Topfexperimenten mit gesiebten Böden schließt den Einfluss einer verstärkten Bodenverdichtung durch langzeitig pfluglose Bewirtschaftung als mögliche Ursache aus. Bodenanalysen ergaben erwartungsgemäß die, für LT Direktsaat typischen, erhöhten Gehalte an organischer Substanz aber keinen offensichtlichen Mangel an Pflanzennährstoffen. Jedoch wiesen Pflanzen auf LT Böden regelmäßig Phosphat (P) Mangel auf, was offensichtlich durch verschlechterte Aneignung des schwerlöslichen Phosphats in Folge des gehemmten Wurzelwachstums bedingt war.

Bei vier von fünf LT Böden hatte eine Gamma-Sterilisierung des Bodens keinen Einfluss auf die Ausbildung von Schadsymptomen, was Krankheitserreger als Haupt-schadensursache ausschließt. Bodenapplikation von Biokohle (5% v/v) verbesserte dagegen schnell das Pflanzenwachstum auf LT Böden, was bereits in der ersten Woche nach der Aussaat nachweisbar war. Diese Beobachtung deutet auf Bodenkontamination mit einer phytotoxischen Substanz hin, da für Biokohle die Bindung organischer Schadstoffe in Böden nachgewiesen ist. Die Akkumulation allelopathischer Substanzen im Oberboden, die aus Pflanzenrückständen und Wurzelexsudaten stammen, ist ein gut dokumentiertes Problem in Direktsaatsystemen, besonders bei engen Fruchtfolgen oder

Monokulturen, was auch für die untersuchten Flächen zutraf. Allerdings ist ein Weizen-spezifischer Autoallelopathie-Effekt unwahrscheinlich, da Pflanzenschäden auch bei anderen Pflanzenarten wie Soja, Sonnenblume, Raps und Zwischenfruchtmischungen auftraten. Charakteristisch für allelopathische Effekte traten in Topfversuchen mit Winterweizen Schadsymptome schnell, schon während der frühen Keimlingsentwicklung auf. Unter Feldbedingungen war die Auflaufphase dagegen in der Regel nicht betroffen und Pflanzenschäden entwickelten sich erst zu Beginn der neuen Vegetationsperiode im zeitigen Frühjahr. Darüber hinaus verschwanden die Pflanzenschäden, wenn die Bodenproben für Topfversuche im Sommer und nicht im zeitigen Frühjahr genommen wurden, was auf einen Abbau der Schadsubstanz hinweist und nicht mit der Hypothese einer langfristigen Bodenakkumulation allelopathischer Substanzen erklärbar ist. Der beobachtete Zeitverlauf der Entwicklung von Schadsymptomen ähnelt eher den Nachbauproblemen, die unter bestimmten Bedingungen durch bodenaktive Herbizidrückstände, wie Sulfonylharnstoffe oder Propyzamid, ausgelöst werden können. Daher wurden in einer Übersichtsanalyse Herbizidrückstände im Oberboden auf sechs LT-, und ST-Standortpaaren untersucht.

Charakteristisch für pfluglose Anbausysteme war Glyphosat das einzige Herbizid, das regelmäßig auf allen Standorten eingesetzt wurde. Die Bodenanalyse ergab durchgängig höhere Glyphosatrückstandswerte auf den LT Standorten im Vergleich zu den benachbarten ST-Plots. Auf LT-Flächen mit besonders starker Ausprägung von Schadsymptomen, wurden besonders hohe Rückstandskonzentrationen (2-4 mg kg^{-1} Boden) gemessen, wie sie üblicherweise wenige Tage nach der Applikation auftreten, aber in diesen Fällen noch sechs Monate nach der letzten Glyphosatgabe nachweisbar waren, während die benachbarten ST Flächen nur Spurenkonzentrationen unterhalb der Nachweisgrenze aufwiesen. In Übereinstimmung mit dem abnehmenden Schadpotential der LT Böden, sank auch die Glyphosatrückstandsbelastung im Laufe der Vegetationsperiode zum Sommer hin ab. Für andere untersuchte Herrbizidwirkstoffe, wie Pendimethalin und Propyzamid, wurden keine vergleichbaren Verteilungsmuster gefunden. Glyphosatabbau in Böden korreliert mit der mikrobiellen Aktivität. Entsprechend war die Bodenatmung als Indikator für mikrobielle Aktivität bei vier von fünf untersuchten Standortpaaren auf den LT-Flächen im Vergleich zu den benachbarten

ST-Plots herabgesetzt, was auf einen verlangsamten Glyphosatabbau auf LT Standorten schließen lässt.

Aufgrund schneller Adsorption zeigt Glyphosat in der Regel keine oder nur sehr eingeschränkte Bodenaktivität. Allerdings waren auf LT Böden mit hohem Schadpotenzial, Glyphosat und AMPA zumindest in Spurenkonzentrationen (1.5 – 3.5 µg L^{-1}) auch in der wasserlöslichen und damit potenziell pflanzenverfügbaren Phase nachweisbar. Nährlösungsversuche mit Winterweizen, der über 3-6 Wochen den Herbizidspurenkonzentrationen, die in der LT Bodenlösung nachgewiesen wurden, ausgesetzt war, entwickelten Chlorosen und zeigten ähnlich wie bei den Bodenversuchen 30 – 50 % vermindertes Feinwurzelwachstum, was überraschenderweise in erster Linie durch AMPA und nicht durch die Glyphosatexposition verursacht wurde. Übereinstimmend wurde weder in Hydroponik-, noch in Bodenversuchen Shikimatakkumulation im Wurzelgewebe als spezifischer Indikator für Glyphosat-Toxizität nachgewiesen. Die bestimmende Rolle der Toxizität von AMPA wurde auch bei Topfversuchen mit Glyphosat-resistenten und nicht-resistenten Sojalinien deutlich, die in beiden Fällen Schadsymptome auf LT Böden ausbildeten, da transgene, Glyphosat-resistente Sojasorten nicht gleichzeitig resistent gegenüber AMPA-Toxizität sind.

Eine erste RNAseq Genexpressionsanalyse im Wurzelgewebe, unmittelbar vor Ausbildung visueller Schadsymptome, ergab verminderte Expression von Genen der generellen Stressantwort, von Aquaporinen (PIPs und TIPs) mit Funktionen bei der Wasseraufnahme und beim Wurzelstreckungswachstum, von Genen des Ethylenstoffwechsels aber eine erhöhte Expression von Cytokinin-Genen, was auf Wechselwirkungen mit hormonellen Gleichgewichten hinweist. Diese Veränderungen der Genexpression relativ zur unbehandelten Kontrolle, wurden in Pflanzen mit AMPA-, und AMPA+Glyphosat-Exposition aber nicht bei ausschließlicher Glyphosatexposition nachgewiesen. Die Ergebnisse deuten darauf hin, dass Langzeitexposition gegenüber subtoxischen AMPA Konzentrationen, die besonders im Frühjahr in den Böden der LT Flächen als Folge des verzögerten Abbaus akkumulieren, zu Störungen von Stoffwechselfunktionen führt, die für die normale Wurzelentwicklung essentiell sind.

In einer Reihe von Topfversuchen wurde das Potenzial ausgewählter, kommerzieller Formulierungen mikrobieller Pflanzenstärkungsmittel, basierend auf Stämmen von *Pseudomonas sp., Bacillus amyloliquefaciens,* und *Trichoderma harzianum* getestet, um die Stress-Symptome vom Pflanzen, die im Frühjahr auf LT Böden auftraten, zu vermindern. Für Vertreter der ausgewählten Mikroorganismengattungen sind Wurzelwachstumsstimulierung, Pathogen-Antagonismen und die Fähigkeit zum Glyphosatabbau dokumentiert. Unglücklicherweise trat eine Stimulierung des Pflanzenwachstums nur auf den ST Böden auf, während auf LT Böden, weder in Topfversuchen, noch im Feld, fördernde Effekte beobachtet wurden, was möglicherweise auf mangelnde Wurzelbesiedlung in Folge der Hemmung des Wurzelwachstuns und auf Frühsommertrockenheit im Feld zurückzuführen war.

Als alternativer Ansatz, wurde die Einarbeitung einer Pyrolyse-Biokohle aus Holzabfällen getestet, die in Topfversuchen bei einer Applikationsdosis von 5% (v/v) im Oberboden, entsprechend ca 35 t ha^{-1}, die Ausbildung von Schadsymptomen bei Winterweizen auf LT Böden komplett verhinderte und eine Schútzwirkung gegen Glyphosatüberdosierung (bis 8 L Roundup Ultramax ha^{-1}) sogar auf Substraten mit minimalem Adsorptionpotenzial vermittelte. Als mögliche kurzfristige Schutzmaßnahme ist daher die Untersuchung unter Feldbedingungen mit unterschiedlichen Biokohle-Applikationsdosen angeraten.

Während der vergangenen beiden Jahre wurden Änderungen des Fruchtfolgemanagements auf den betreffenden Flächen eingeführt, mit vielfältigeren Fruchtfolgen, die Winterweizen, Winterraps, Mais und Crotolaria einschließen und auch Zwischenfruchtgemenge aus Erbsen und Gelbsenf getestet. Trotz weiterem, jährlichem Glyphsoateinsatz mit Aufwandmengen von 3 L ha^{-1} einer 360 g ai L^{-1} Formulierung, hat sich die Pflanzenentwicklung auf den betroffenen Flächen unzwischen signifikant verbessert. Diese Beobachtungen weisen darauf hin, dass die bislang praktizierten, engen Weizen/Raps Fruchtfolgen die Entwicklung einer Bodenmikroflora mit vermindertem Glyphosatabbaupotenzial begünstigt haben, was die Bedeutung eines Biodiversitäts-managements auch für Kulturpflanzen unterstreicht.

1 Introduction

1.1 History of tillage in agricultural practice

Agriculture is the science and practice of cultivating the soil to grow crops for food production. With the aid of modern scientific techniques, we have achieved high crop productivity. State of the art machinery made farming efficient, latest plant varieties increased production, and agrochemicals provided an effective solution against pests and weeds. In developed countries, feed and food shortage are no more problems. This industrial agricultural production is efficient, but there are still a lot of unsolved problems concerning ecological sustainability. In Agronomic practices, tillage has played a vital role throughout the history of agriculture. During tillage, the soil is manipulated mechanically to prepare the ground for plantation. In 3000 B.C., a wooden plow was used in the Euphrates and Nile rivers (McKyes, 1985). First, it was pulled by man and then later by animals.

There are also several Biblical references about the use of a plow. One of them is: "They shall beat their swords into plowshares" (Isaiah 2:4). This plow was just a branch of a tree, which was used to scratch the soil surface without mixing the soil layers (Derpsch, 1998). Plows with the ability to invert soil surface and help in weed control were first developed in the 17^{th} century and became more and more sophisticated in the 18^{th} and 19^{th} centuries. Of note, at the end of the 18^{th} century, the British, Dutch and Germans developed a tool in the shape of a moldboard. It is the perfect tool to turn the soil by 135^{o} and, thereby, be efficient in weed control. This plow was used to control widespread weed all over Europe, and it helped to mitigate the famine at the end of 18^{th} century. It became a symbol of "modern" agriculture and used by Agriculture Institutes. In the museum of the University of Hohenheim, located in Stuttgart Germany, one of these plows from 1884 is displayed. It was spread in America, Asia, and Africa through colonial powers and gained vital importance (Derpsch, 1998). Nowadays, deep and intensive tillage is used commonly and is referred to as conventional tillage, or cultivation. Therefore, some of the equipment utilized for this purpose are called cultivators.

1.1.1 Advantages of tillage

Due to multiple significant benefits, tillage has been continued till now. It provides weed control through breaking the soil and reduces the soil compaction (Hobbs *et al.,* 2008). Tillage enhances root growth and development (Varsa *et al.,* 1997). It can quickly incorporate crop residues into the soil and provide a faster increase in organic matter for short-term (Hobbs *et al.,* 2008). Tillage destroys shelters of pests and disrupts their life cycle, exposes pests to unfavorable conditions including predators and improves soil aeration (Oisat, 2015).

1.1.2 Disadvantages of tillage

Along with benefits, tillage also brings problems. Tillage destroys the soil structure, increases soil erosion, reduces the population of beneficial organisms such as mycorrhizal fungi in soil by disrupting their life cycle, induces moisture loss, delays when planting can begin, incurs high costs for both machinery and energy, and increases pollution (Oisat, 2015). Crop production based on intensive tillage negatively affects and damages the quality of natural resources, including water, soil, terrain, the associated ecosystem, and biodiversity. Of total CO_2, N_2O and CH_4 emissions (the greenhouse gasses which lead climate change), 30% stem from Agriculture (IPCC, 2007). In addition, regular cultivation of soil leads to the depletion of soil quality, which in the medium to long-term is not sustainable for economics and environment (Basch *et al.,* 2008). In conclusion, intensive tillage cannot continue to keep sustainable agriculture, and a shift is required towards conservation agriculture.

1.2 Conservation agriculture (CA)

Conservation agriculture (CA) is a combination of ideas, practices, and technology to manage the ecosystem, develop sustainable production, increase agriculture profit, and ensure the availability of food without damaging environmental resources. CA conserves and improves natural resources like fauna, flora, and wildlife without a reduction in yield (FAO, 2015). It also improves biodiversity and the various processes of nature both above and below ground.

There are three principals of CA defined by FAO.

1. Continuous minimum mechanical soil disturbance. (No-tillage, direct seeding or seeding through broadcasting, direct placement of planting material in the soil. Minimal soil disturbance by both cultivation and harvest operations)

2. Permanent cover of organic matter. (Crop residues and cover crops)

3. Diverse crop rotation.

To correspond the principals of CA alternative of conventional tillage is conservation tillage, which includes no-tillage or zero-tillage, non-inversion, and minimal-tillage (strip-tillage, and mulch-tillage). In the case of no-tillage cultivation (Figure 1), the soil is not disturbed except for seeding. In the event of non-inversion or minimal-tillage, reduced cultivation is done by using cultivator like chisel plow, etc. (Jones *et al.*, 2006).

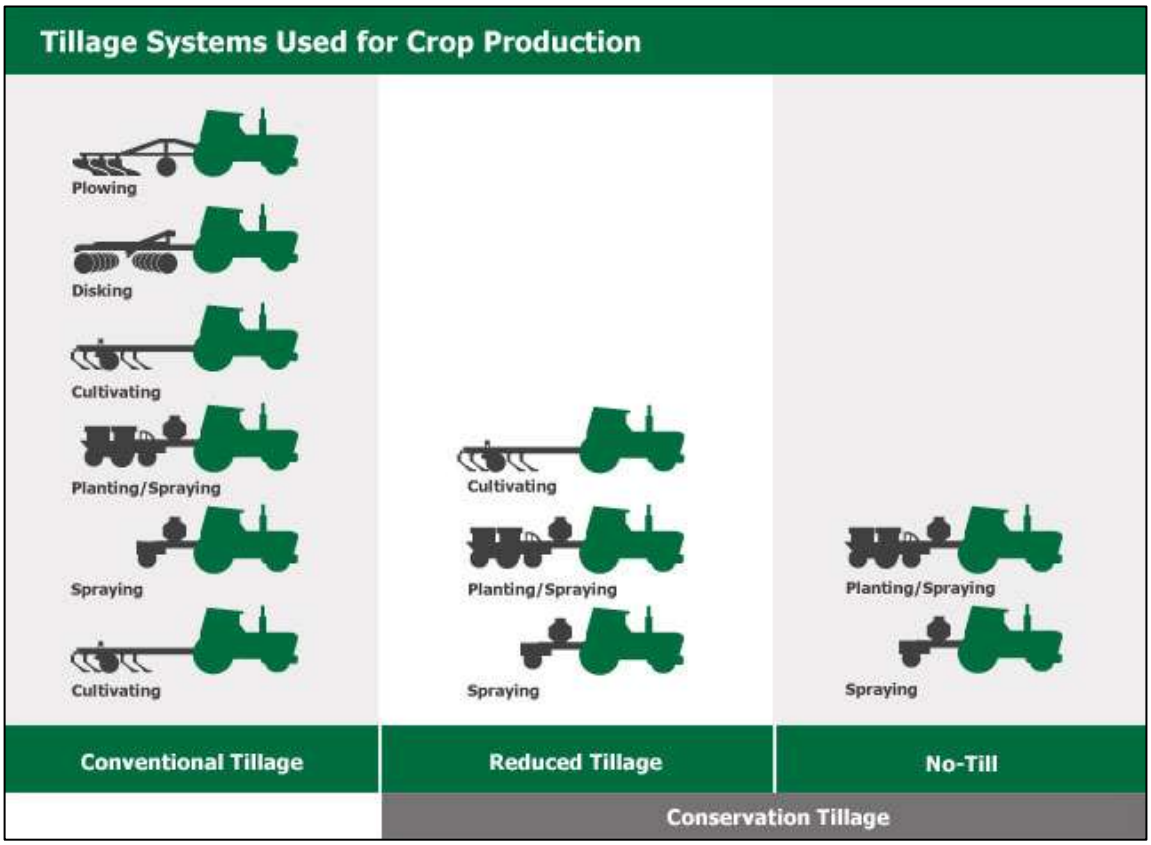

Figure 1: Agricultural practice in conventional and conversation tillage. Courtesy; Dow AgroSciences.

1.3 No-tillage

No-tillage is often understood as a system where only seeding is done without tillage, but, actually, it is an entirely different system where not just one factor, tillage, but a complete set of factors must be changed. Different machinery for seeding is necessary, machinery, which is capable of cutting through residues of previous crops. In addition, adaptations are required in crop variety selection, fertilization, and weed and pest management. In essence, it can be defined as a system of planting (seeding) crops into the untilled soil by opening a narrow slot, trench or band of only sufficient width and depth to obtain proper seeding coverage. No other soil tillage is done. Further, the aim should be permanent no-tillage, not occasional tillage or tillage in alternative seasons. To achieve this, crop rotation and cover crops are essential; the soil must have the undisturbed cover of crops or green manure (Derpsch and Friedrich, 2009).

1.3.1 No-tillage History

No-tillage is an ancient practice in agricultural management. It was used by primitive cultures because man does not have enough muscle power to till large areas manually. One of the most indigenous cultures, the Incas in the Andes of South America, used sticks to make seeding holes in the soil and covered the seeds by foot. A large number of farmers in Central and South America (Derpsch, 1998) are still using a similar method today. Slush mulch or "tapado" is another system practiced in Central America and Mexico today and for centuries before. It is a no-tillage system developed by small landholders (Thurston *et al.*, 1994). After the rain, seeds are thrown on the topsoil underneath a dense stand of vegetation, e.g., Mexican Sunflower (*Tithonia diversifolia*), and, then, the plants are cut and left on top of the seeds. After a few days, the plants on top are dried and the seeds germinate without any tillage being performed (Derpsch, 1998).

In Europe and worldwide, modern no-tillage started in 1955 with the development of herbicide Paraquat® in the United Kingdom (UK). This concept developed because of increasing research activities on no-tillage. By 1973/74, in the UK 200,000 hectare (ha) was under no-tillage. After ten years, it increased to 275,000 ha (Derpsch, 1998). In 1962,

research on no-tillage started in Netherlands with the aim to find ways to make field work simple and improve farm economy by saving time and energy. In Germany, research on no-tillage began in 1966 (Derpsch and Friedrich, 2009). After 18 years of research, no-tillage was found more profitable due to the lower cost for machinery, lower operating expenses, lower initial cost, which is required for machinery, labor, and other variable and fixed cost (Derpsch, 1998). In a well-managed no-tillage system, yield comparable with conventional tillage systems can be obtained. Even in cases of a lower yield, higher profit is still expected due to lower input costs. Based on total process cost, no-tillage is economical and can be further improved (Tebruegge and Böhrnsen, 1997). In Germany till 2010, 44% of the agricultural areas were under conservation tillage (Table 1), and about 50% of winter rape (*Brassica napus*) and 50% of winter wheat (*Triticum aestivum*)/barley (*Hordeum vulgare*) was grown under no-tillage management (Schmitz and Graevert, 2012).

Table 1: Soil tillage methods on arable land in Germany 2010. (Destatis, Statistisches Bundesamt, Wiesbaden 2015, www.destatis.de).

Soil Tillage Method	Arable Land (1000 ha)
Conventional soil tillage (ploughing)	6,6082
Conserving soil tillage (e.g., by grubbing, harrowing)	4,4693
Direct seeding method (zero tillage)	1463

In France, INRA and ITCF started experiments on minimum and no-tillage techniques in 1970 (Derpsch and Friedrich, 2009). They made advancements in no-tillage in Europe, and, until 2008 the area under no-tillage in France was 200,000 ha (Derpsch *et al.*, 2010). In Spain, no-tillage research started in 1982, and the no-tillage system proved more productive than tillage and minimal tillage in southern Spain's clay soil because of the low energy consumption and moisture conservation. In 1967 to 1982 long-term experiments with plowing and direct drilling showed higher yield in winter bean, winter wheat, and spring oats (*Avena sativa*) managed with no-tillage. In maize (*Zea mays*) and spring barley 15% and sugar beet (*Beta vulgaris*) 20% yield was reduced due to no-

tillage. No-tillage was adopted in Spain more than the in rest of the Europe. It was practiced on 650,000 ha for annual crops growing in Spain, mainly wheat and barley (Derpsch *et al.,* 2010).

In the USA, research on conservation tillage started in the 1930s with early chisel plow in Great Plains for the purpose to alleviate soil damage caused by wind erosion after the famous "dust bowl." In 1950, successful application of no-tillage was reported (Philips and Philips, 1984). Therefore, intensive research started on no-tillage in combination with chemical weed control. No-tillage was promoted and facilitated by the publication of "No-tillage farming" by Philips and Young in 1973. It reached to 19.4 million ha by 1996/97 (Hebblethwaite, 1997) and it kept advancing as shown in Table 2.

In 1971, with the cooperation of GTZ (German aid), no-tillage experiments started in Brazil. Maize, wheat, soybeans (*Glycine max*), barley, sorghum (*Sorghum bicolor*), sunflower (*Helianthus annuus*), beans (*Phaseolus vulgaris*) are the main crops being grown with no-tillage technology. In 1974, Argentina started no-tillage farming with the goal to find a better way of double cropping of soybean and wheat (Derpsch, 1998). In both countries, no-tillage adaptation was rapid and dominating the cropland. According to Derpsch and Friedrich (2009), the main advantage of no-tillage is a possibility of production without dragging the soil, and it improves soil biological, chemical and physical properties.

On a global level, only 2.8 million ha were under no-tillage in 1973/74. Within 10 years, this area grew to 6.2 million ha in 1996/97 and increased to 38 million ha (Derpsch, 1998). In 2010, the estimated area under no-tillage worldwide was 111 million ha (Derpsch *et al.,* 2010). According to FAO (AQUASTAT, 2016) estimates of different years from 1960 until 2015, the total area under CA worldwide is 156.991 million ha. It is managed with different conservational tillage practices including no-tillage. The area under no-tillage in all over the world is steadily increasing. The highest adaptation is in MERCOSUR countries, a larger percentage of total area is under no-tillage, i.e., Argentina 80%, Brazil 50%, Paraguay 90%, and Uruguay 82% (Gianessi, 2014). In US 38.8 million ha (almost 25% of cropland area) are reported under no-tillage (Dobberstein, 2014).

Table 2: Area under no-tillage in various in countries with > 100,000 ha (Derpsch et al., 2010).

Country	Area (ha) 2008/2009
USA	26,500,000
Brazil	25,502,000
Argentina	19,719,000
Canada	13,481,000
Australia	17,000,000
Paraguay	2,400,000
China	1,330,000
Kazakhstan	1,200,000
Bolivia	706,000
Uruguay	655,100
Spain	650,000
South Africa	368,000
Venezuela	300,000
France	200,000
Finland	200,000
Chile	180,000
New Zealand	162,000
Colombia	102,000
Ukraine	100,000
Total	110,755,100

1.3.2 Significance of No-tillage

As compared to conventional tillage, the no-tillage system is beneficial in the conservation and improvement of natural resources. With the use of no-tillage reduction in production costs and increasing yield (Tebrügge, 2001) leads to higher net profit. No-tillage is cheaper than conventional tillage due to fewer field operations (80% fuel and

60% lesser labor requirements) and a 50% less cost for machinery, which also lasts longer due to less operational hours per year. Further, once a no-tillage system is established, less technical skill, chemical and fertilizer inputs are required (Baker *et al.*, 2007).

Soil structure is improved with the least possible physical disturbance and with the addition and preservation of organic matter and soil fauna and flora, e.g., earthworms. Water loss, runoff of soil and applied chemicals, soil compaction, and the wind and water erosions are controlled (Triplett and Dick, 2008). Due to organic matter cover, the soil has better internal drainage and infiltration, higher earthworm populations and higher water holding capacity. Under no-tillage, growth conditions for plants are improved with better nutrient availability and soil temperature moderation. Proper mixing of potassium and phosphorus by earthworms increases nutrient availability in the root zone. Soil temperature remains lower in summer and higher in winter (Baker *et al.*, 2007). No-tillage is a sink for greenhouse gasses, reduced runoff of agrochemical and contamination of water, less use of fossil fuels, agrochemicals, and fertilizer (Baker *et al.*, 2007), leads to environment-friendly and sustainable agriculture.

1.3.3 Challenges in no-tillage adaptation

Along with several advantages, there are also some challenges to deal with when establishing a no-tillage system. No-tillage is a shift of various factors and agricultural practices from conventional tillage. Farm machinery needs to change or upgrade and land leveling, which makes it expensive to start. More skills are required to start this system and perform agricultural operations, especially to deal with pests and diseases.

As a rule, higher the soil disturbance leads to a reduction in weed infestation (Boström, 1999) and less soil disturbance contributes in developing a weed seed bank (Cardina *et al.*, 2002: Moonen and Barberi, 2004). The major reason for tillage is weed control to reduce competition for early crop growth (Triplett and Dick, 2008). In the case of no-tillage, weed infestation and seed banks do increase (Légère *et al.*, 2011). Grasses are an additional problem in no-tillage systems (Locke *et al.*, 2002: El Titi, 2003). In the absence of tillage, major weed control is possible by the application of chemicals (herbicides), which tend to be selective towards weeds, which are resistant against formulations (Baker

et al., 2007). Therefore, the ultimate limiting factor in no-tillage is weed management (Soane *et al.,* 2012).

In a no-tillage system, applying non-selective herbicides, mainly glyphosate mostly controls weeds. Glyphosate is the most widely used herbicide worldwide (Powles, 2008) in both conventional and no-tillage system. It is sold under different trade names, based on the manufacturer and formulation. The most widely used product is Roundup® (Monsanto, St. Louis, Missouri, USA).

1.4 Glyphosate

1.4.1 Significance of glyphosate

Glyphosate is an active ingredient of more than 750 commercial products (Saltmiras *et al*., 2015) for agricultural, forestry, residential and urban applications. It is a broad-spectrum herbicide, frequently used for the pre-emergence application. In the case of orchards, vineyards forestry, and glyphosate-resistant (GR) crops glyphosate is also applied in the standing culture or as a post-emergence herbicide, respectively. It is used for weed control in perennial and annual plants, broad- leaf weeds, grasses, grains, orchards and forestry, aquatic weed control and infrastructure (railway track). Furthermore, glyphosate is approved for use in vegetables, orchards, vine, ornamentals, forest, and lawn. It is also used to synchronize and accelerate the ripening of forage cereals. Glyphosate is reported to control 76 of world's most damaging weeds. It can provide control for 300 weeds in more than 100 crops (Franz *et al*., 1985).

Glyphosate is the worldwide most frequently used herbicide (Saltmiras *et al.,* 2015). Its use was boosted with the introduction of glyphosate-resistant (GR) crops in 1996 and with changes of management practice to no-tillage and reduced tillage (Cerdeira and Duke, 2006). It was estimated that the use of glyphosate in the European Union raises the annual welfare of 1.4 billion euros (Schmitz and Garvert, 2012). Annual global production figures for glyphosate have recently been estimated at 825,800 tons (Benbrook, 2016). Others estimated even higher production volumes, surpassing 1 million ton annually

(Székács and Darva, 2012; Bøhn *et al*., 2015). In 2017, the global market of glyphosate is expected to reach 1.35 million tons (Global Industry Analysts, 2011).

In 2007, the production capacity of Chinese companies was 323,400 tons, which increased to 835,000 tons in 2010 with 37% annual growth rate (Székács and Darvas, 2012). The global sale value of glyphosate was US$ 4.7 billion (Borggaard, 2011). The amount of glyphosate used is increasing globally and there is a tendency to use it as a sole herbicide (Woodburn, 2000) particularly in cropping systems based on GR crops. In Denmark, the use of glyphosate was 35% of total pesticide applied in 2008 (Borggaard, 2011). In Italy only in 2011, more than 120 tons of glyphosate were sold mainly for use in vineyards (Napoli *et al.,* 2015). In the United Kingdom, 40 to 80% of cereals and oilseed rape were treated with glyphosate as a pre harvest herbicide (Cook *et al.,* 2010). In Germany (Table 3), use of glyphosate increased from 1999 to 2010. The annual growth rate was 20% between 1999 and 2008. It dominated the herbicide market being covering 40% of national use (Steinmann *et al.,* 2012). According to winter survey of 2010/11, Steinmann *et al.* (2012) reported glyphosate application on 27.5% agricultural area (arable land and grassland) and 35% of the total arable area. It was mainly applied to grow oilseed rape, winter barley, and pluses. Grassland, forage crops and potatoes (*Solanum tuberosum*) were less exposed to it. The survey reported the diverse application of glyphosate, not just for weed control but also as a multifunctional agronomic tool.

According to Franz *et al.* (1997), glyphosate is regarded as an environment-friendly herbicide. It is less likely to leach and contaminate ground water than many other products because of its strong sorption and rapid inactivation in the soil quickly after application. It is effective against all weeds with relatively limited recognized resistance evolution, except areas with the intensive use of GR cropping systems. It was known to be noncarcinogenic and has low acute human and animal toxicity. It was also reported to have limited effects on soil macro-, and microorganisms (Borggaard and Gimsing, 2008; Forlani *et al.,* 2008; Powles, 2008).

Table 3: Glyphosate use in Germany applied on the area to different crops in 2008 (Steinmann et al., 2012).

Crop	Calculated application area (ha*1000)	Calculated share of applied area (%)	Calculated amount of Glyphosate (t)	Share of total glyphosate applied (%)
Grassland	133.5	3.3	165.5	4.0
Winter wheat	702.5	23.2	658.8	15.8
Silage maize	389.7	25.2	347.9	8.3
Oilseed rape	1200.6	87.2	1149.2	27.5
Winter barley	898.7	65.9	837.9	20.1
Rye/ triticale	382.1	35.0	335.6	8.0
Forage crops	77.0	12.5	76.1	1.8
Spring cereals	230.8	41.7	264.4	6.3
Maize	146.6	33.6	144.9	3.5
Sugar beet	111.9	31.0	124.3	3.0
Potatoes	26.1	10.5	25.6	0.6
Pulses	40.8	72.1	39.9	0.9

1.4.2 Glyphosate-Resistant Crops (GR)

In 1996, Monsanto developed glyphosate-resistant (GR) soybean called Roundup Ready® Soybean which was genetically modified (GM) to resist against glyphosate application. After development of GR soybean, Monsanto continued to develop GR cotton (*Gossypium* spp.), GR maize, GR canola, GR alfalfa (*Medicago sativa*) and GR sugar beet (Dill *et al.,* 2008) and following this, other companies made similar varieties and named them Gly-Tol™ (Bayer CropScience), Optimum GAT (Pioneer HiBred) and Agrisure GT (Syngenta AG). Thus, the postemergence application of the glyphosate-based herbicides became possible in a broad range of important crops. It adopted quickly, particularly in no-tillage farming associated with a corresponding increase in glyphosate use (Figure 2B). The area under GMOs is rapidly growing (Table 4, Figure 2A) and it reached to 120

million ha where 80% of them were herbicide resistant crops virtually all being glyphosate resistant crops (Duke and Powles, 2010). In 2013, the area under herbicide resistant crops increased to 99.4 million ha with high net profit (ISAAA GM, 2014).

Table 4: Total crop production share of GR cultivars in different years and countries (Duke and Powles, 2010).

GR Crop	**GR Share**	**Year**	**Country**
Soybean (*Glycine max* L.)	90%	2009	USA
Soybean (*Glycine max* L.)	90%	2003	Argentina
Cotton (*Gossypium hirsutum* L.)	70%	2009	USA
Canola (*Brassica napus* L. & *B. rapa* L.)	70%	2008	Canada
Sugar beet (*Beta vulgaris* L.)	60%	2008	USA

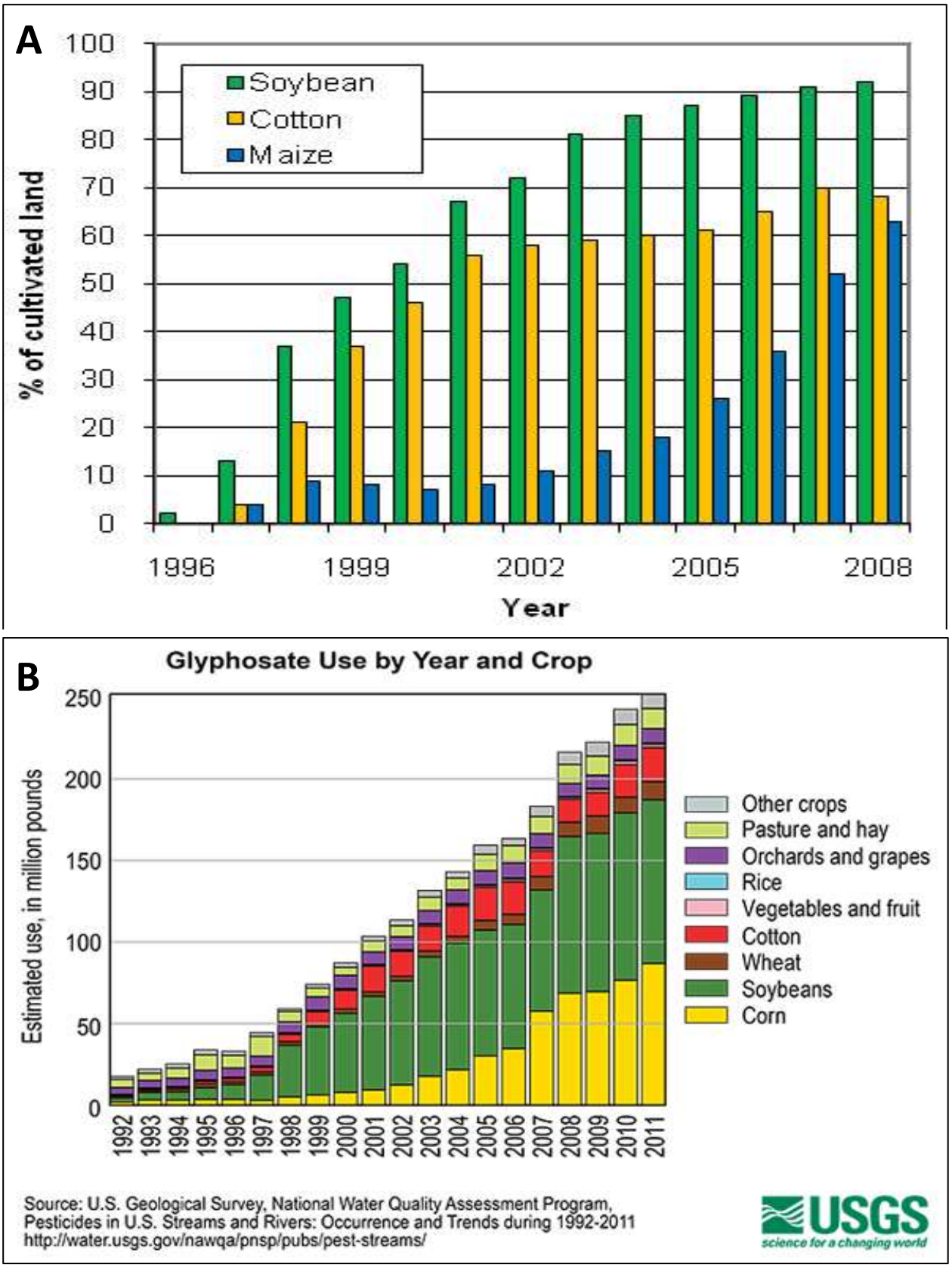

*Figure 2: (**A**) GR crops from 1996 to 2008 in the United States based on USDA ERS, 2009 (Duke and Powles, 2010). (**B**) Glyphosate use in different crops during 1992 to 2011 in the United States based on U.S Geological Survey.*

1.4.3 Chemical and Physical Properties

Glyphosate (*N*- phosphonomethyl-glycine) is a phosphonomethyl derivative of the amino acid glycine. It is an odorless, white crystalline solid, a weak organic acid (Table 5) belongs to the group organophosphates. The molecular mass is 169.07 g mol^{-1} with the formula $C_3H_8NO_5P$. Its molecule forms a zwitterionic structure. It is amphoteric and has a central basic secondary amino function, with acidic functions on both ends formed by a mono-carboxylic acid and dibasic phosphonic acid (Figure 3).

Due to high polarity, glyphosate is insoluble in organic solvents, such as ethanol, acetone, and benzene (Franz, 1985) but also its solubility in water is relatively low. To increase water solubility, glyphosate is usually formulated as ammonium, isopropyl ammonium, potassium, sodium or trimethylsulfonium salts (Székács and Darva, 2012). Surfactants are used to increase glyphosate's penetration in plant cells.

The formulated herbicide is stable under ambient temperature conditions (-20 °C to 40 °C), non-volatile, photo-stable with limited soil persistence. In many agricultural soils, the half-life of glyphosate is typically less than 60 days but ranges from 1 to 197 days (Giesy *et al.*, 2000) depending on environmental conditions.

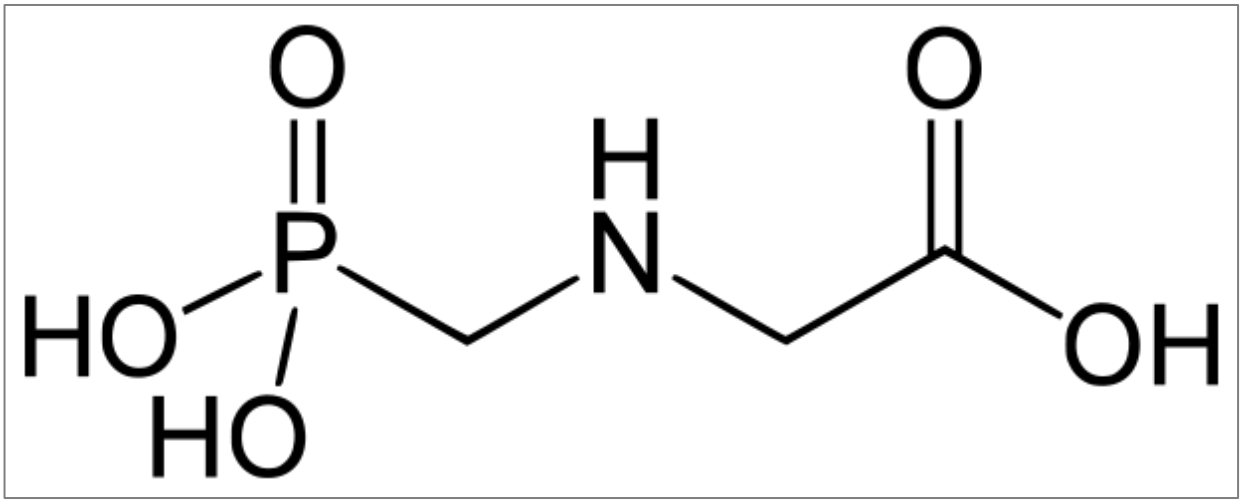

Figure 3: Glyphosate (N- (phosphonmethyl) glycine) - Chemical structure.

Table 5: Chemical and physical properties of Glyphosate (WHO, 1994).

Boiling point	Decomposing
Color	White
Explosiveness	Not explosive
Flammability	Not flammable
Henry's law constant	< 7 X10-11
Melting point	184.5 ^{o}C and Decomposing at 187 ^{o}C
Molar absorptivity	0.086 liter mol^{-1} per cm at 295 nm
Molecular Mass	169
Octanol-water partition coefficient (log KOW)	-2.8
Odor	None
pH	2.5 (1% solution) Water
Physical state	Crystalline powder
pKa values	< 2, 2.6, 5.6, 10.6
Solubility in water	10- 100 mg L^{-1} at 20 ^{o}C
Specific gravity (density)	1.704 at 20 ^{o}C
Surface tension	0.072 N/m 0.5% (w/v) at approx. 25 °C
Vapor pressure	< 1 x 10-5 Pa at 25 ^{o}C

The Roundup Ultra® formulation is active against most annual and perennial weeds with a dosage 2 to 4 L ha^{-1} containing 960 to 1920 g of active ingredient. However, for some perennial weeds and woody species, higher dosages and single plant application instead of broadcast spraying are required (Bott, 2010).

1.4.4 Mode of Action

1.4.4.1 Primary Effects

In general, glyphosate is classified as an inhibitor of aromatic amino acid biosynthesis via inhibition of the shikimic acid pathway as a primary mechanism (Figure 4) (Duke and Hoagland, 1985; Panettieri *et al.,* 2013). However, it is not completely clear if this is the only mode of action. Glyphosate is readily absorbed and translocated within the plant, preferentially to the young growing tissues (Cranmer, 1988). In higher plants and many microorganisms, the shikimate pathway has vital importance to link primary and secondary metabolism, initiated by condensation of phosphoenolpyruvate (PEP) with erythrose-4-phosphate (Herrmann and Weaver, 1999). The end products of the pathway are the aromatic amino acids tryptophan (Trp), tyrosine (Tyr) and phenylalanine (Phe), essential for the synthesis of proteins (Comai and Stalker, 1986). Tryptophan is the precursor for the synthesis of indole-acetic acid (IAA) as one of the most important growth-promoting phytohormones (Yamada *et al.,* 2009). Phenylalanine is a major precursor for the synthesis of secondary phenolic compounds via phenylalanine ammonialyase (PAL) for the production of phenolic acids, coumarins, flavonoids, lignins, tannins and quinones (Duke and Hoagland, 1985). Within the shikimate pathway, glyphosate competitively inhibits the key enzyme 5-enolpyruvylshikimate-3-phosphate synthase, which catalyzes the reaction of shikimate-3-phosphate and phosphoenolpyruvate to form 5-enolpyruvyl-shikimate-3-phosphate (Panettieri *et al.*, 2013). This pathway blockage causes shikimic acid accumulation, which is also widely used as an indicator for the detection of glyphosate toxicity (Neumann *et al.,* 2006; Reddy *et al.*, 2010). The lack of the essential amino acids Trp, Tyr, and Phe inhibits biosynthesis of proteins, which rapidly affects photosynthesis with the most abundant chloroplast proteins, leading to leaf necrosis and finally death of the plant (Duke *et al.,* 2003; Duke and Powles, 2008). Due to the ubiquitous occurrence of the shikimate pathway in plant metabolism, the herbicidal effect is observed in all plant species (Székács and Darva, 2012).

1.4.4.2 Secondary Effects

Due to the general inhibition of protein synthesis, a wide range of metabolic pathways and processes are affected by glyphosate in a pleiotropic way, including reduction of chlorophyll and porphyrin synthesis, inhibition of photosynthesis, respiration and nitrate assimilation, reduction in synthesis of nucleic acids and inhibition of anthocyanin formation (Cole, 1985). Reduced uptake of amino acids, nucleotides, and glucose, caused by glyphosate has been demonstrated in cell cultures (Brecke & Duke, 1980). Glyphosate also affects hormonal balances including IAA, ethylene, and cytokinins, (Cañal *et al.*, 1987; Cole, 1985; Duke *et al.*, 1979; Lee, 1980).

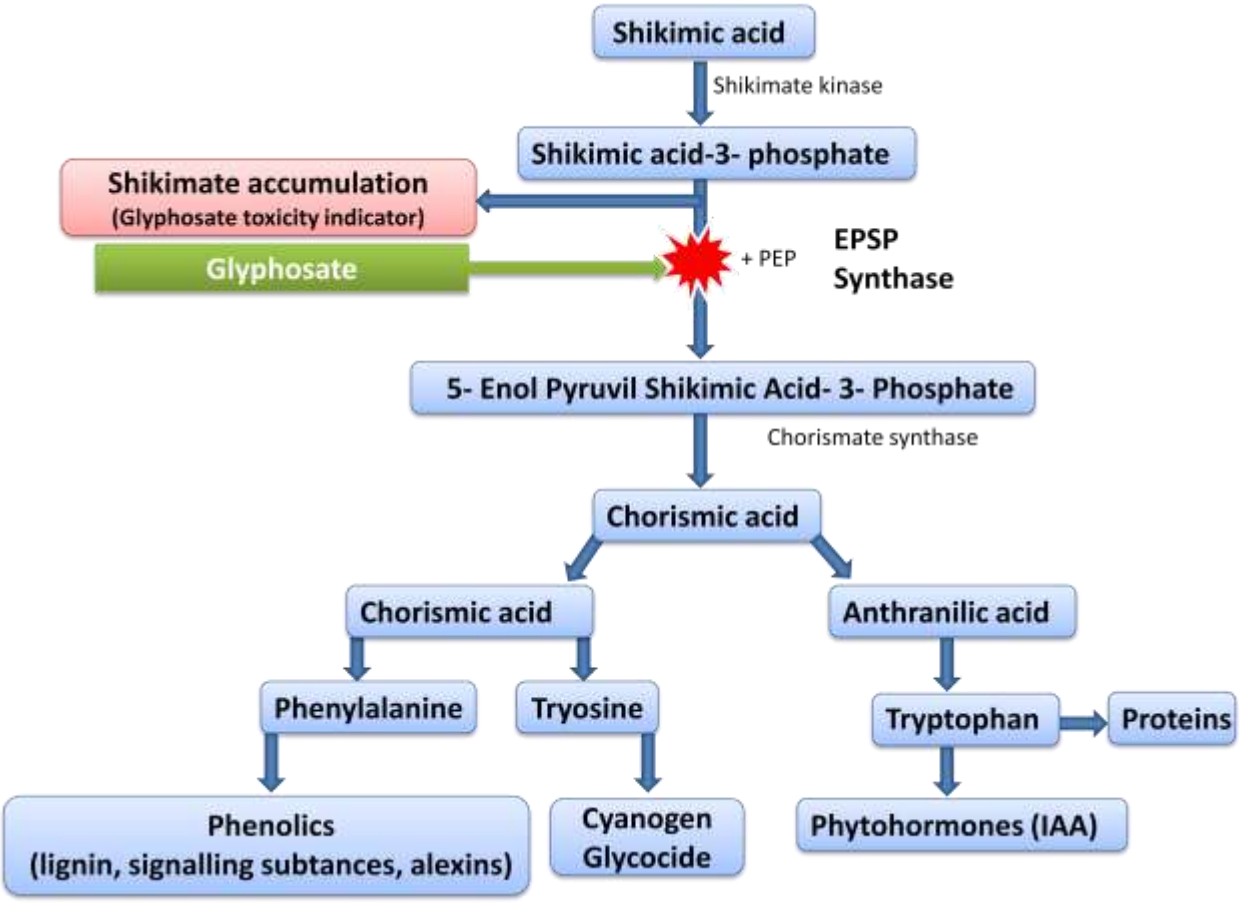

Figure 4: Shikimic acid pathway and its inhibition by glyphosate (adapted from Dill, 2005).

1.4.5 Glyphosate uptake translocation in plants

After foliar application, glyphosate is quickly absorbed by the foliage of treated seeds and translocated to root and shoot meristems and young growing tissues (Grangeot *et al.*, 2006). However, in many plants self-limited translocations of glyphosate has been

observed after foliar application particularly at higher doses of glyphosate. It may be due to toxic effects of the internal translocation processes (Geiger *et al.,* 1999; Hess, 1999; Majek, 1980). Usually, the uptake is initially rapid and then slower. The herbicide absorption through the plasma membrane into the symplast involves passive and active transport mechanisms using phosphate carriers (Franz *et al.*, 1997, Caseley and Coupland, 1985). Gougler and Geriger (1981) reported the involvement also of amino acid transport systems in glyphosate transport across the plasma membrane.

1.4.6 Glyphosate in Soil

Glyphosate enters into the soil by direct contact during spraying, being washed off the leaf surface after the foliar application or as plant root exudates and lysates (Kremer *et al.*, 2005). It is rapidly adsorbed to the soil matrix by the formation of complexes with metal cations, i.e., Fe^{2+}, Cu^{2+}, Mn^{2+}, and Ni^{2+} (Andréa *et al*. 2003), representing the main mechanism of glyphosate detoxification in soils. In a study on glyphosate adsorption in three soils with illitic, kaolinite and smectic clay minerals, glyphosate adsorption and presence of clay minerals could be related (Dion *et al.,* 2001). Some studies showed that glyphosate adsorption in soil is not linked or negatively related to soil organic matter (Gerritse *et al.,* 1996; Vereecken, 2005). Western Australian studies on glyphosate adsorption on sandy soils indicate that it is possible for soil organic matter (e.g., humic acids) to compete for adsorption sites and counteract adsorption of glyphosate (Gerritse *et al.,* 1996). According to a review of Borgaard and Gimsing (2008), soil organic matter has a controversial and dual role in soil sorption of glyphosate. Soil organic matter can reduce glyphosate sorption by blocking sorption sites. It can also increase absorption because high organic matter enhances the poorly ordered aluminum and iron oxides, which have high glyphosate sorption capacity. However, the main sorption sites for glyphosate are on the surface of aluminum and iron oxides, edges of layer silicates and poorly ordered aluminum silicates. Glyphosate adsorption was tested on three topsoils having different cation exchange capacity, textural fraction and amorphous Fe and Al oxides. It revealed amorphous Fe and Al oxides and organic matter controls glyphosate interaction with soils (Morillo *et al.,* 1999). Soil with permanently charged clay minerals

like illite, smectite, and vermiculite adsorb less glyphosate as compared to soil with high Fe and Al contents (Gimsing and Borgaard, 2007).

1.4.7 Glyphosate Degradation

Glyphosate is biologically degraded in soils. In the laboratory, the half-lives (DT50) ranges from 1 to 40 days and forms the intermediate metabolite aminomethyl-phosphonic acid (AMPA). AMPA is more persistent with DT_{50} ranging from 24 to 75 days (Mamy *et al.,* 2005) in most cases. The primary route of glyphosate degradation is microbial, although photodegradation and chemical degradation can take place to a smaller extent (Tu *et al.,* 2001). Barrett and McBride (2005) demonstrated abiotic degradation of glyphosate and AMPA by Mn oxide birnessite. Microbial degradation preferentially proceeds under aerobic but also under anaerobic conditions, favored by high temperature (Heinonen- Tanski, 1989; Rueppel *et al.,* 1977). During this degradation, microorganisms are using the herbicide for acquiring phosphorus rather than as a carbon source (Franz *et al.,* 1997). The degradation of the herbicide is related to microbial activity, and its degradation rate is correlated with the rate of soil respiration (Franz *et al.,* 1997). Higher adsorption of glyphosate in soil leads to slower degradation (Sørensen *et al.,* 2006) due to the limitation of bioavailability.

There are two pathways of glyphosate degradation:

a) decarboxylation, (catalyzed by oxidoreductases) forming the intermediate metabolite AMPA;

b) dephosphorylation, (catalyzed by C-P lyases cleaving the carbon-phosphorous bond) forming intermediate metabolite sarcosine and glycine (Székács and Darva, 2012).

The AMPA pathway is commonly seen in mixed soil bacterial cultures (Rueppel *et al.,* 1977) and the glycine pathway is characteristic for *Pseudomonas* and *Arthrobacter* sp. (Jacob *et al.,* 1988). It is not clear which of these pathways is more common. However, in soils treated with glyphosate, AMPA is commonly detected (Rueppel *et al.*, 1997; Borggaard and Gimsing, 2008) and it is more mobile in soil (Duke and Powles, 2008)

while the presence of sarcosine is rare possibly due to its quicker degradation (Moshier and Penner, 1978). AMPA formation is rapidly mediated by microbial activity but not by chemical action in the water and in various loams soils. It finally degrades to carbon dioxide (Rueppel *et al*., 1977; Sprankle *et al*., 1975). Chemical processes of degradation are not effective due to the presence of a highly stable carbon-phosphorus bond (Gimsing *et al*., 2004). Nevertheless, the mechanisms of AMPA degradation are still not completely understood (Kononova and Nesmeyanova, 2002).

Bacterial glyphosate degradation has been also reported for strains of *Flavobacterium*, *Agrobacterium*, *Bacillus*, *Rhizobium* and *Achromobacter*, while degradation by fungal strains (e.g., *Trichoderma*, *Penicillium*, and *Fusarium*) is less well documented (Arfarita *et al*., 2013), although an important role of fungal glyphosate degradation has been postulated already by Krzyśko-Lupicka *et al.*, (1997). This is in line with reports on increased fungal populations after soil application of glyphosate (Araujo *et al.*, 2003).

There are reports of glyphosate leaching (Napoli *et al.*, 2015) and delayed degradation (Helander *et al.*, 2012) after field application. This delayed decomposition can be due to partly binding to soil matrix and formation of complexes with metallic ions (Al, Fe, Mn, and Zn) (Vereecken, 2005). Panettieri *et al.* (2013) investigated the influence of glyphosate on microbial activities and found differences in results obtained from incubated soil and agricultural plots. Those differences were explained as an effect of meteorological factors like temperature variations, light intensity, wind and rain strength on the degradation of agrochemicals. These changes could be related to stimulation or inhibition of microbial communities and/or related to activation of other patterns of chemical oxidation. Temperature also plays a vital role in degradation as reported by Stenrød *et al.* (2005). During the period of thawing, microbial activity increases subsequently higher degradation rate but decreases during freezing period. Degradation of glyphosate reduced between 6 to 10% with 10 ^{o}C decrease in temperature.

In aquatic systems, the primary mode of degradation of glyphosate is microbial, and half-life is 12 days to 10 weeks (Ruppel *et al.*, 1977), but degradation in water is slower than soil due to fewer microbes (Ghassemi, 1981).

1.5 Ecological risk assessment of glyphosate

Glyphosate was stated to be a "unique ideal herbicide" and a "once in a century herbicide" (Duke and Powles, 2008). Early findings, mostly before 2010, justified that glyphosate was widely recognized as having a low impact on the environment, on workers who deal with the chemical, and on consumers through food (Cuhra *et al*., 2016). In recent years, the established assumptions on the glyphosate safety have come under revision. It is found to have more profound and complex toxicological effects on the environment, workers, and consumers due to high residues in food. These differences in conclusions have initiated a global scientific debate on glyphosate. Now glyphosate is a controversial product, and more studies are urgently required (Soil Association, 2016; Nguyen *et al.*, 2016).

Glyphosate residues in feed and food are a major concern since GR crops accumulate glyphosate (Bøhn *et al*., 2014) and preharvest application of glyphosate on crops leave higher concentrations of its residues. These glyphosate contaminated crops are being used for cattle feed. Therefore, glyphosate residues can be found in cattle as a potential health risk for them and end consumers human beings (Cuhra *et al*., 2015). Glyphosate has been found to have antibiotic qualities (Abraham, 2010). The effect of glyphosate on microorganisms heterogeneous and dependent on the presence of shikimate pathway.

There are increasing numbers of reports suggesting glyphosate as ecological risk, e.g., it can be hazardous for vertebrates (Paganelli *et al*., 2010), it has adverse effects on the availability of plant nutrient uptake, can exert non-target effects on susceptible crops (Bott *et al*., 2011) and impact on rhizosphere microorganisms and plant pathogens (Kremer and Means, 2009). Additional risks are the development of resistant weeds (Owen, 2008), accumulation or delay in degradation, contamination of ground and surface water through leaching and runoff (Helander *et al*., 2012). Glyphosate-based herbicides can adversely affect aquatic invertebrate ecology (Cuhra *et al*., 2013). It has also shown a negative impact on amphibian larvae (tadpoles) (Relyea, 2006) and earthworms (Gaupp-Berghausen *et al.* 2015). These effects can last longer with persistence of the herbicide, and recent research suggests that glyphosate persists longer with the return of crop residues on the field containing glyphosate to the soil (Mamy *et al*., 2016).

Some other studies indicate that commercial glyphosate-based formulations are more toxic than glyphosate itself because of surfactants (Coalova *et al*., 2014) and a potential carcinogenicity, which is still a matter of controversial discussions.

1.5.1 Glyphosate damage to non-target plants

Through spray, drift glyphosate may reach to non-target plants in minor but effective dose. In wheat, 10% of the labeled usage rate of glyphosate caused >90% yield losses (Deeds *et al.*, 2006). Drift dose of glyphosate produced lower biomass of young wheat plants (Kutman *et al.*, 2013).

In soil, glyphosate is generally less bioavailable due to soil adsorption. However, after pre-sowing application, weeds absorb it, and it remains stable in weed residues being temporarily protected from microbial degradation (Bott *et al*., 2011). Subsequently, it can be released from the damaged roots of dying target plants (Neumann *et al.*, 2006) with the potential to damage adjacent plants and seedlings by contact contamination (Tesfamariam, 2009). Also, Coupland and Casely, (1979) reported the release of glyphosate through root exudates. They detected the significant amount of ^{14}C-glyphosate in the surrounding solutions of the intact root of quackgrass (*Elytrigia repens*).

1.5.2 Glyphosate Resistant weeds (GR)

Some scientists considered the evolution of weed resistance to glyphosate is unlikely because of its unique mechanism of action (Bradshaw *et al.*, 1997). They were proven wrong in 1996 when in Australia the first population of GR *Lolium rigidum* was reported (Pratley *et al.*, 1996). Next year in 1997 in Malaysia, GT goosegrass (*Elevsine indica*) (Lee and Ngim, 2000), followed by GR horseweed (*Conyza Canadensis*) in the United States (VanGessel, 2001), GR Italian ryegrass (*Lolium multiflorum*) in Chile (Perze and Kogan, 2003). The glyphosate resistance in weeds continued, and by 2014, the number of GR weeds species reached 32 weeds worldwide, including 15 species in the United States (Heap, 2015). There are two most common identified mechanisms of glyphosate resistance in GR weeds. The first mechanism is alternations of target site through a genetic mutation in EPSPS in the way that either EPSPS was no longer inhibited by

glyphosate or over-expressed. The second mechanism is a reduced translocation of glyphosate to meristems (Powles and Yu, 2010). The development of GR weeds is a direct consequence of the massive use of GR cropping systems exclusively based on the herbicidal action of glyphosate and limited crop rotation (Green and Owen, 2010)

1.5.3 Plant Nutrients

Cropping systems, where glyphosate is being used for weed management, have been shown to induce a deficiency of Fe, Mn, Zn and B (Neumann *et al.,* 2006). This can be a result of an effect of glyphosate on the composition of soil microbial communities, associated with changes in soil nutrient dynamics (Johal and Huber, 2009; Kremer and Means, 2009). In the upper Midwest of the United States even growing incidence of potassium deficiency was noticed in maize grown in rotation with GR soybeans (Lane *et al.,* 2012) with frequently applied glyphosate. Fungi can take up and sequester potassium in the fungal biomass (Weed *et al.,* 1969), which was found to be stimulated by glyphosate application. Also, glyphosate-induced impairment of micronutrient uptake and transport in plants has been described. Internal micro nutrient immobilization has been discussed as the putative cause, induced by the ability of glyphosate to form stable complexes with Fe and Mn and other metals (Cakmak *et al.*, 2009).

1.5.4 Toxicity on Other Organisms

There are different opinions in the literature about the safety of this herbicide as some reports have not found any significant risk to human and animal health by the use of glyphosate since its target enzyme EPSPS (5-enolpyruvyl-shikimate synthase) is absent in animals (De Roos *et al*., 2005; Solomon *et al*., 2007). Some other studies revealed toxicity of sub-lethal exposures of glyphosate in fish (Szarek *et al*., 2000, Guilherme *et al*., 2012), earthworms (Gaupp-Berghausen *et al*. 2015; Yasmin and D'Souza, 2007; Verrell and Buskirk, 2004), mice (Prasad *et al*., 2009) human cell lines (Koller *et al*., 2012) and workers exposed to glyphosate formulations (Bolognesi *et al*., 2009).

The entry of glyphosate into the food chain has been facilitated with the invention of GR crops and pre-harvest applications of the herbicide. In GR soybean high concentrations of

glyphosate were detected (Bøhn *et al*., 2014) reaching up to 100 mg kg^{-1} (Biotech, 2013). Reports are confirming the presence of glyphosate in groundwater, human and animal urine, human breast milk, and farmed animal meat (Krüger *et al*., 2014, 2013; Honeycutt and Rowlands, 2014; Borggaard and Gimsing, 2008; Niemann *et al*., 2015). The ingestion of lowest concentrations (0.1 mg mL^{-1}) (Krüger *et al*., 2013) of glyphosate can disturb the normal gut bacterial several studies raised concerns about the effects of glyphosate on gut microbiota (Ackermann *et al*., 2015; Shehata *et al*., 2013). In 2014, Krüger *et al*. detected Glyphosate residues in malformed euthanized one-day-old Danish piglets and suspected correlation to glyphosate.

There are different views about a potential carcinogenicity of glyphosate and/or its formulations. In 1985, the United States Environmental Protection Agency (U.S EPA, 1985) studied the effect of glyphosate on the tumor in mice. They concluded glyphosate as possibly carcinogenic to humans and assigned Group C. In 1991, they re-evaluated the study and shifted it to Group E, which indicates none- carcinogenic in humans (IARC, 2015). Similarly, the Joint Meeting on Pesticide Residues, sponsored by the Food and Agriculture Organization of the United Nations and the World Health Organization (JMPR, Geneva, 2006) and then the United States Environmental Protection Agency (U.S. EPA, 2013) also declared that glyphosate was unlikely to be carcinogenic to humans. In 2014 on behalf of the European Union, the German Federal Institute for Risk Assessment reviewed all toxicological studies of glyphosate in animals and humans. They concluded the absence of carcinogenic or mutagenic properties of glyphosate (BRf, 2015). Contradictory, in 2015, on behalf of World Health Organization (WHO), International Agency for Research on Cancer investigated and found evidence of carcinogenic properties of glyphosate in animals. They declared glyphosate as "probably carcinogenic to humans." It was categorized in Group 2A. This category is used for pesticides showing sufficient evidence of carcinogenicity in animals but limited evidence in humans (non-Hodgkin lymphoma) (Guyton *et al*., 2015; IARC, 2015). In a recent study, Chang and Delzell (2016) reported the absence of a relationship between glyphosate exposure and risk of cancer. The exact mechanism of the genotoxic effects of glyphosate formulations is unknown (Yadav *et al*., 2013).

The European Commission announced in 2016 an extension of the current approval of glyphosate for a limited period. The final decision about the future of glyphosate is subjected to further findings of the European Chemical Agency. However, this current extension contains three recommendations, which include a ban on toxic co-formulations of glyphosate-based products called POE-tallowamine, a minimization in the use of glyphosate in public parks, public playgrounds, and gardens and a minimization the use of glyphosate to speed up the harvest.

1.5.5 Aquatic Ecosystem

Glyphosate may reach to aquatic systems wind-driven or through the accidental drift of the herbicide spray, as suspended particulate matter or through surface runoff (Feng *et al*., 1990). Glyphosate and AMPA were amongst the first major pollutants of surface waters (IFEN, 2006). In the mid-1990s, glyphosate was listed among pesticides, which were of potential concern in surface water contamination in the Mediterranean region of Europe (Barcelo, 1997). In two tributaries of river Ruhr in North-Rhine-Westphalia, Germany, glyphosate, and AMPA were found in concentration up to 590 ng L^{-1} (Skark *et al*., 1998). In Norway, glyphosate and AMPA were detected in 54% of tested water samples (Ludvigsen and Lode, 2001a). Similarly, in 2002 in the United States and Midwest, glyphosate was detected in 35 to 40% surface water samples with a maximum concentration of 8.7 µg L^{-1} and AMPA in 53 to 83% of the samples with a maximum concentration of 3.6 µg L^{-1} (Battaglin *et al*., 2005). In Italy, the concentrations of glyphosate and AMPA in surface water exceeded the 0.1 mg L^{-1}, which is the European maximum threshold (Europaeu, 2013). Worldwide glyphosate represents the most detected herbicide residues in freshwater ecosystems, of which AMPA is represented most in France (Villeneuve *et al*., 2011). There are reports of significant off-target displacements of glyphosate, functional and structural changes in the freshwater biota, consistent with the decrease in water quality (Pérez *et al*., 2007; Vera *et al*., 2010). Glyphosate is toxic to microalgae and other aquatic microorganisms (Ma *et al*., 2003).

1.5.6 Microorganisms

Several studies have noted variable effects of glyphosate on soil microbial community function (Bünemann *et al*., 2006; Duke *et al*., 2012; Soil Association, 2016; Nguyen *et al.*, 2016). Some reported no significant (Rosenbaum *et al*., 2014) or even positive impact of glyphosate on microbial communities (Araújo *et al*., 2003). In contrast, reports confirmed mid-range concentrations of glyphosate (10-100 mg kg^{-1}) (Nguyen *et al*., 2016) could suppress soil microbial biomass (Andréa *et al*., 2003; Lancaster *et al*., 2010). These effects can be temporary (Zabaloy *et al*., 2008; Nye *et al*., 2014) due to changes in chemical bioavailability with aging, fluctuations in environmental (Brock *et al*., 2008). Busse *et al*. (2001) found contradictions in results of in vitro and field study due to rapid binding of glyphosate with soil colloids. In a recent meta-analysis, Nguyen *et al.*, (2016) could not confirm that glyphosate has a consistently positive or adverse impact on soil microbial communities. They reported that the impact of glyphosate on microbial communities depends on the time of incubation, a dose of glyphosate applied and soil characteristics. Therefore, they concluded that the toxicity or safety of glyphosate to the whole soil microbial biomass and activity should not be generalized. It should be qualified with details of the conditions under which glyphosate is applied.

1.6 Aim of the study

In no-tillage farming in Southwest Germany, intensified problems had been observed in the recent years. The problems were apparent mainly in winter wheat/oilseed rape cropping systems. These plant damages featured growth depressions, chlorosis, necrosis, and reduced formation of fine roots. The damage symptoms could be partly assigned to short waiting times after weed control with a total herbicide like glyphosate before sowing the subsequent crop. Under unfavorable conditions, this can entail contamination with glyphosate from root and plant residues of the declining weed population and can promote the development of pathogens within the dying weeds (green bridge) (Smiley *et al.*, 1992, Neumann *et al.*, 2006; Tesfamariam *et al.*, 2009; Bott, 2010).

However, observations on adjacent field plots (Figure 5), which had been managed for different periods with no-tillage, suggested that, besides the described short-term waiting

time effects, obviously additionally long-term effects were responsible for the occurrence of damage symptoms. The cause for these long-term effects was unknown. By contrast, to the short-term waiting time effects, which were usually appearing in autumn shortly after emergence, the long-term effects with similar damage symptoms were characteristic for the beginning of the growth period in early spring. The effects have repeatedly been-observed with increasing frequency in different years on different field sites, both, in winter wheat and winter rape associated with yield losses of 30% and more. An examination of the underlying factors was therefore of great practical interest for the further development and improvement of no-tillage farming. In preliminary trials, it was possible to reproduce the damage symptoms under laboratory conditions (Figure 6). This was of substantial significance for a causal analysis of the underlying mechanisms under controlled conditions.

Figure 5: Spring damage in closely neighbored winter wheat plots with long-term no-tillage history, 2008 (Hirrlingen, Tübingen).

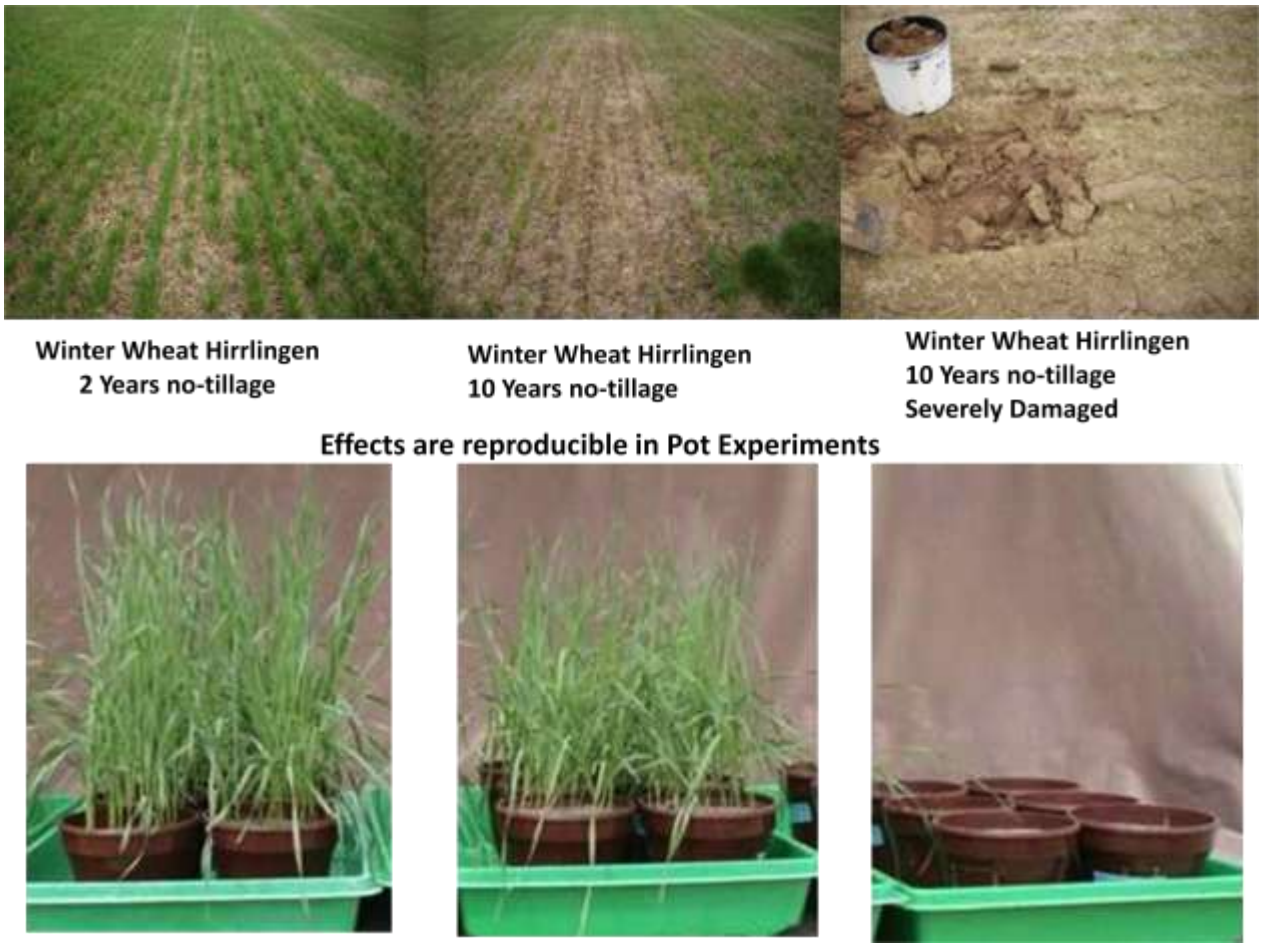

Figure 6: Reproduction of damage effects in winter wheat in soils with long-term no-tillage cropping history in pot experiments under controlled environmental conditions.

Therefore, the objectives of this study focused on (i) the identification of causes for the observed damage symptoms and (ii) initiating the exploration of potential counteractive measures using experiments under laboratory and field conditions. Based on these objectives, three working hypotheses were formulated.

Hypothesis I

The damage effects have a biotic provenience by the promotion of pathogens with low host specificity.

Hypothesis II

The damage effects have an abiotic provenience caused by toxic impacts of allelopathic compounds or herbicide residues in soil as a consequence of long-time herbicide application.

Hypothesis III

Soil supplementations with plant growth-promoting microorganisms with root growth stimulating properties or adsorbents for toxic organic compounds are capable of alleviating plant damages.

2 Material and Methods

2.1 Experiments in soil culture

Soil samples were collected from selected no-tillage field sites with neighbored plots characterized by a different history of no or reduced tillage farming: (i) long-term 8-15 years (LT); (ii) short-term 1-5 years (ST).

2.2 Sampling of field soils

Samples were collected yearly during spring (April) and autumn (November/December) from the field sites located in Southwest Germany in the administrative district Tübingen.

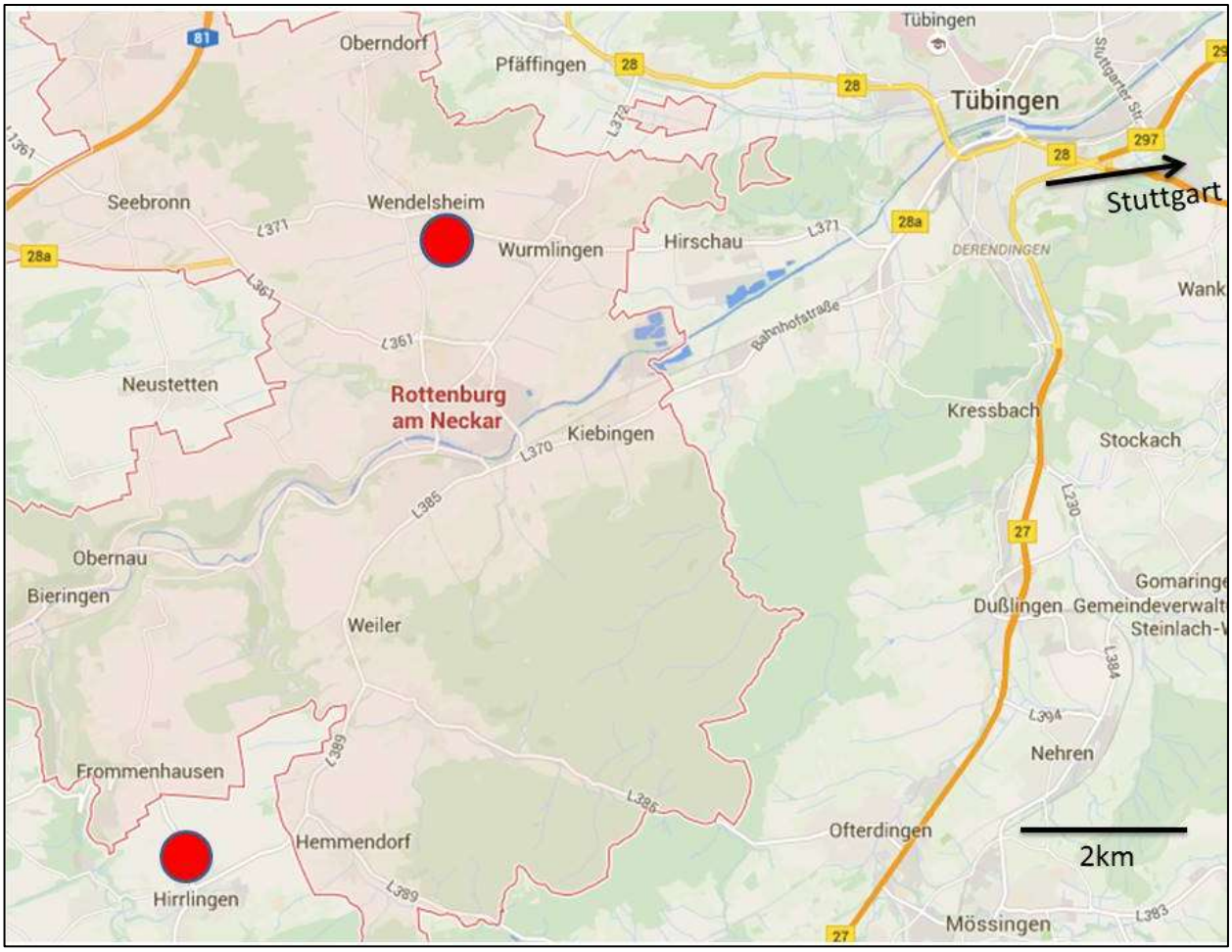

Figure 7: Location of no-tillage field sites used for soil sampling in Southwest Germany, administrative district Tübingen (Google Maps).

Sampling for pot experiments was conducted in: (i) Windelsheim, with paired plots at the local subdistricts Remmingsheimer Weg (Rem) and Sülcher Wegle (SW); and (ii) in Hirrlingen, with paired plots at Schwarze Länder (SL), Gassäcker (HirG) and Hirrlingen Friedhof (HirF) (Figure 7, Table 6). Pooled samples of topsoil (0-15 cm) were collected from various parts of long-term (LT) and short-term (ST) no-tillage plots with each 8-12 sampling points per plot.

*Table 6: Location and management of no-tillage field sites in the local sub districts used for soil sampling. *Locations further investigated in the study. GL= Conversion from grassland*

Local subdistrict	No-tillage management	Cadastral unit	No-tillage cultivation period
Hirrlingen			
*Gassäcker	Long-term	1431	1992
*Gassäcker	Short-term	1430	2010 GL
*Schwarze Länder	Long-term	1358	1992
*Schwarze Länder	Short-term	1359/60	2008 GL
*Friedhof	Long-term	304, 300	1999
*Friedhof	Short-term	303	2006
Beim Steinbruch	Long-term	493, 495	1992
Beim Steinbruch	Short-term	494	2008
Eichenberg	Long-term	1058, 1054-56	1992
Eichenberg	Short-term	1057	2008
Wendelsheim			
*Remmingsheimer Weg	Long-term	1533-1536	1998
*Remmingsheimer Weg	Short-term	1537	2010
*Sülcher Wegle	Long-term	3237/1-3241	1998
*Sülcher Wegle	Short-term	3247-3259	2005

2.3 Soil storage and preparation

Fresh soil samples were stored at 2 ^{o}C - 8 °C in darkness. For setup of the pot experiments, they were further homogenized and sieved 2 mm mesh size to break soil aggregates and Remove crop residues.

2.4 Soil analysis

Determination of nutrient status, microbial activity, and herbicide residues.

2.4.1 Soil chemistry

Analyzed by the certified laboratory of the LA Chemie, University of Hohenheim, Stuttgart, Germany. Macronutrients, such as phosphorus (P) and potassium (K) were measured by spectrophotometry (Gericke and Kurmies, 1952) and electro-ultrafiltration (EUF) after calcium-acetate-lactate (CAL) extraction (Schüller, 1969). Magnesium (Mg) was determined in 0.0125 M $CaCl_2$ extracts. The micronutrients Iron (Fe), manganese (Mn) and zinc (Zn) were measured by atomic absorption spectrometry after calcium chloride/ DPTA extraction (VDLUFA, 2004). Soil pH was measured in 0.01 M $CaCl_2$, and the carbon content was determined by elemental analysis.

2.4.2 Soil respiration

Microbial respiration in soil was measured during seven days, using an automated "Respicond" system (Type Company, Town, State) according to (Nordgren, 1988). Samples of 20 g fresh soil from autumn sampling stored at 2 °C (see 2.1, 2.2) were adjusted to 20 % (w/w) soil moisture before analysis. The evolution of CO_2 originating from soil respiration in sample jars was measured by CO_2 capturing in KOH (0.3 M) traps. This results in a decrease of the conductance in the hydroxide solution, which is measured with platinum electrodes in each incubation vessel. The conductometer signals were digitalized via a converter attached to a microcomputer, which then calculated CO_2 evolution and evolution rate.

2.4.3 Herbicide residues

Residues of more frequently applied herbicides such as glyphosate, the glyphosate metabolite AMPA, pendimethalin and propyzamide were measured from freshly collected pooled soil samples (500 g, see 2.1, 2.2) by the certified lab of the Agricultural Technology Center (LTZ) Augustenberg, Karlsruhe, Germany. For determination of water-soluble residues of glyphosate and AMPA, water extracts (100 g L^{-1} demineralized water) were prepared from fresh soil samples adjusted to a moisture level 20% (w/w). After sedimentation of the solid soil fraction, the supernatant was cleared by filtration (Whatman GF/D glass-fiber filters and Blue ribbon filters, Macchery and Nagel, Düren, Germany) and subsequently evaporated to dryness using a rotary evaporator and a speed-vac concentrator. The soluble herbicide fraction was analyzed in the dried residue of the supernatants, and the concentration in the soil solution was calculated back to a soil moisture level of 20 % (w/w).

2.5 Soil sterilization

Freshly collected soil samples (see 2.1, 2.2) were exposed to gamma ray sterilization (26.2 kGy) in in 2.5 kg plastic bags (BBF irradiation service, Kernen, Germany).

2.6 Plant culture

Plant culture was performed in pot experiments with two crops under controlled conditions in a growth chamber, adjusted to a 16 hours light period, a 25° C / 20° C day/night temperature regime with 55% - 60% air humidity and light intensity of 300 µmol $m^{-2}s^{-1}$.

2.6.1 Test plants

Investigated crops comprised winter wheat (*Triticum aestivum* cv. Isengrain), glyphosate-resistant (GR) soybean (*Glycine max* cv. BSR Valiosa RR) and it's near isogenic, parental, non-resistant line *Glycine max* L. cv. BR16 Conquista (Bott *et al.*, 2008).

2.6.2 Pot experiments on LT and ST no-tillage soils

Experiments were conducted in plastic pots (10 x 11 cm top radius, 7 cm bottom radius) filled with, 500 g of freshly sieved soil (see 2.2). Soil moisture was adjusted to 70% of the maximum water-holding capacity. The soil was not fertilized to keep it similar to field conditions. In wheat and soybean experiments, 20 and 10 seeds, respectively, were sown at a depth of 0.5-1.0 cm. The soil surface was leveled after sowing and subsequently covered with a layer of fine quartz sand to reduce mechanical disturbance during watering and evaporation. The final weight of each pot was recorded, and daily replacement of water losses was performed gravimetrically with distilled water.

2.6.3 Soil amendments with biochar

A pyrolysis biochar produced from a mixed woody substrate obtained from landscape conservation work (Pyreg GmbH, Doerth, Germany; Holweg, 2011) was used for the experiments. Biochar was mixed into the soil in pot experiments (see 2.6.2) at a rate of 5% (v/v).

Table 7: Chemical composition (A) and appearance (B) of the biochar employed for the experiments.

A

Total-N	[% DM]	0.58
P		0.32
K		0.89
Mg		0.45
CaO		2.77
S		0.03
Organic matter	mg kg^{-1} DM	52.32
Pb		8.02
Cd		0.09
Cu		13.56
Zn		83.91
Hg		0.00
Cr		22.57
Ni		34.54
Dry Matter	%	69.36
pH	8.32	
Density	4.34 mL g^{-1}	

B

2.6.4 Detoxification of glyphosate by biochar amendments

Four pot experiments were conducted to test the effect, concentration-dependency and application methods of biochar in the growth substrate on glyphosate toxicity to winter wheat. To induce glyphosate toxicity, the herbicide was applied as Roundup Ultramax® at a rate 6 L ha^{-1} as calculated by Bott *et al.* (2011) and mixed into the substrates followed by biochar applications in different concentrations (v/v). Soil samples were collected from Hirrlingen, Gassäcker (2.1, Table 6). Sowing was performed one day after glyphosate application with 400 g substrate and 10 seeds per pot.

A. Glyphosate was applied to soil and after one day, different doses of biochar (0%, 5%, 10%, 20% v/v) were added and mixed homogeneously.
B. Since glyphosate soil contamination under field conditions is usually restricted to the uppermost soil layers (Aletto *et al*., 2010), glyphosate and biochar were applied only to upper 5 cm of the topsoil representing approximately 50% of the total soil volume (200 g). The Remaining 200 g untreated soil was filled into the pots as a 5 cm bottom layer. Treatments comprised: i) Control (untreated soil), ii) Gly, iii) Gly+Biochar 5%, iv) Gly+Biochar 10%, v) Gly+Biochar 20% (v/v).
C. As a worst-case scenario, a 1:1 peat culture-substrate / quartz sand mixture TKS®2 (Floragard Vertriebs GmbH, Oldenburg, Germany) representing a growth medium with minimal glyphosate inactivation by adsorption was used for an experiment using cylindrical pots with a size of 18 x 9.5 cm. Glyphosate (Roundup Ultramax® 8 L ha^{-1}) and biochar (0%, 1% and 5% w/v) were applied to a 200 g top-layer of the substrate (see 2.6.4 B), while the bottom of the culture vessels was filled with untreated substrate followed by sowing of 10 seeds of winter weed per pot.

Table 8: Nutrients in TKS® Floragard Vertriebs GmbH, Oldenbur, Germany.

pH	Salinity (g L^{-1})	N (mg L^{-1})	P_2O_5 (mg L^{-1})	K_2O (mg L^{-1})	Structure
5.6	1.6	290	160	340	Medium coarse

2.6.5 Application of microbial bio-effectors

To test mitigation potential of plant damage on soils with long-term no-tillage management by inoculation with plant growth-promoting microorganisms, one pot experiment and three field experiments were conducted. Commercial bioeffectors containing microbial species with a proven potential for root growth promotion and glyphosate degradation according to literature reports were selected.

i) Proradix ® (PRO, Sourcon Padena, Tübingen, Germany) contains a formulation of the bacterial strain *Pseudomonas* DMSZ 13134.

ii) Trichostar® (TRI, Gerlach Natürliche Düngemittel, Hannover, Germany) contains a spore formulation of the fungus *Trichoderma harzianum*.

iii) FZB 42®, RhizoVital® (RHI, ABITEP, Berlin Germany) contains an endospore formulation of the bacterium *Bacillus amyloliquefaciens*. In the pot experiment (see 2.6.2), bio-fertilizers were applied by fertigation according to the recommendations of the manufacturer's soil samples collected from the LT and ST no-tillage field sites at Hirrlingen, Friedhof (see 2.1, Table 6). For the field experiments, LT no-tillage sites in Hirrlingen, (Schwarze Länder), and Wendelsheim (Sülcher Wegle, Remmingsheimer Weg, see 2.1, Table 6) were selected. The microbial bio-effectors and distilled water controls were applied by fertigation according to manufacturer's instructions to 1 m^2 plots selected for intense plant damage with three replicates per treatment followed by visual scorings of plant damage after four weeks.

2.7 Plant analysis

Data on plant growth, such as seedling emergence plant height, and chlorosis scoring were recorded at different growth stages. Destructive determinations of root and shoot biomass, root length, mineral nutrient status, and metabolites were conducted at final harvest.

2.7.1 Seedling emergence

Numbers of emerged seedlings were counted at equal time intervals during the germination period, and emergence percentage was calculated relative to the number of initially sown seeds.

2.7.2 Plant height

Plant height was measured from the shoot base to the top of the longest leaf (wheat) and to the shoot vegetation point (soybean) with the help of a measuring tape.

2.7.3 Root and shoot biomass:

Depending on plant growth and expression of symptoms, plants were harvested after a growth period of two to four weeks. Shoots were cut at the stem base, fresh weight was recorded and the plant material was oven-dried at 60 °C for dry weight determination and analysis of mineral nutrients. Root systems were washed out of the soil, gently dried between layers of paper towels to remove excess moisture and fresh weight was recorded. From the total root material of each pot, a representative sample of 1 g fresh weight was stored in 20% ethanol for analysis of root morphology. The Remaining parts of the root system were oven-dried at 60 °C for dry weight determination.

2.7.4 Root morphology

Root samples were spread and separated carefully in the water on a transparent plastic tray to exclude root overlapping. After that, the root samples were digitalized by scanning (Epson Perfection V700 Photo, Epson, USA). Scanner settings: 400 dpi, 8 bit gray scale, image format: jpeg. The digitalized images were analyzed for, total root length, average root diameter and root length in different diameter classes using the root analysis software WinRHIZO^{TM} (Regent Instruments Inc. Quebec, Canada).

2.7.5 Chlorosis scoring

Green values were recorded from the youngest fully developed leaves with a SPAD-50 plus meter Konica Minolta, Tokyo, Japan). For each pot, 30 measurements were taken and the average was recorded.

2.7.6 Mineral analysis of shoot tissue

Mineral analysis of shoot tissues was performed according to Tesfamariam *et al.* (2009). Freshly harvested shoots were oven-dried at 60 ^{o}C and then homogenized in a mechanical grinder (MM301, Retsch, 2005, Haan, Germany). The fine powder was transferred to a muffle furnace at 500 ^{o}C for 4 hours for ashing. After cooling, the samples were extracted with 2 mL of 3.4 M HNO_3 (v/v) and then heated again until dryness. They were further dissolved in 2 mL of 4 M HCl, afterward diluted 10 fold with hot distilled water and

boiled for 2 minutes. For measurement of Fe and Mn, 0.1 mL Cs/La buffer was added to 4.9 mL of ash solution. For P measurement, according to Gericke and Kurmies, (1952), 3 mL color reagent, molybdate-vandate-solution, was added to a sample of the ash solution. Mn, Fe, Zn and Cu were determined by atomic absorption spectrometry (UNICAM 939, Offenbach / Main, Germany). The concentration of P was measured by spectrophotometry (Spektralphotometer, U-3300, Hitachi, 1994, Tokyo, Japan), Ca and K with flame photometry (ELEX 6361, Eppendorf, 2001, Hamburg, Germany).

2.7.7 Shikimate analysis

Shikimate analysis was performed according to Tesfamariam *et al.* (2009). Root samples were frozen in liquid nitrogen right after harvest. Root material was homogenized using mortar and pestle with the addition of orthophosphoric acid (1 mL 100 mg^{-1} fresh weight). Subsequently, the samples were centrifuged for 5 min at 14,000 × g. The supernatant was diluted with 2.5 mM H_2SO_4 and subjected to HPLC analysis (HPLC System SIL-20AC, Shimadzu, Portland, Oregon, USA). Separation was performed by ion exclusion chromatography using an Aminex 87H column (Bio-Rad, Richmond, CA, USA), designed for organic acid analysis. A sample volume of 20 µL was injected into the isocratic flow (0.5 mL min^{-1}) of the eluent (2.5 mM H_2SO_4) at 40 °C. Organic acids were measured by direct UV detection at 210 nm. Shikimic acid was identified and quantified by comparison of retention time and absorption characteristics with a known standard.

2.8 Experiments in hydroponics

Two hydroponic experiments were performed to test the plant effects of long-term root-exposure to trace concentrations of glyphosate and AMPA detected in the water-soluble phase of soil extracts.

2.8.1 Plant culture

Seeds of winter wheat were pre-germinated in rolls made from sheets of filter paper (58×58 cm, MN710, Macherey and Nagel, Düren, Germany), folded lengthwise two times to obtain a 4-layer paper strip. The strips were soaked with 60 mL of distilled water, and

ten seeds were placed at a distance of 2 cm along the upper edge of the strip, which was subsequently folded, forming a paper roll with the seeds inside. The paper rolls were placed in upright position into a plastic germination box (30×20×10 cm) and incubated at 25 °C in darkness for 4 days, followed by 24 h incubation in a climate chamber with a 16h light period (300 µmol m^2 s^{-1}), 60% relative humidity and 24°C/18°C day/night temperature regime. After that, the seedlings were transferred to hydroponic culture, performed in black 2.5 L plastic pots. Each ten seedlings were fixed at the shoot base with foam strips into perforated PVC lids, covering the plastic pots filled with nutrient solution. The nutrient solution modified after Hoagland and Arnon (1950): 2000 µM $Ca(NO_3)_2$, 700 µM K_2SO_4, 500 µM $MgSO_4$, 250 µM KH_2PO_4, 100 µM KCl, 20 µM Fe-EDTA, 1 µM H_3BO_3, 0.5 µM $MnSO_4$ x H_2O , 0.5 µM $ZnSO_4$ x 7 H_2O, 0.2 µM $CuSO_4$ x 5 H_2O, 0.01 µM $(NH_4)Mo_7O_{24}$ x $4H_2O$. The nutrient solution was replaced every interval of 1-2 day and continuously aerated with pipes connected to an aquarium pump.

2.8.2 Herbicide treatments

Both, glyphosate its major soil metabolite AMPA were applied in the concentration range (1.5-5 µg L^{-1}) detected in the soil solution of long-term no-tillage soils (see 2.4.3).

The cumulative effects of applications daily applications (untreated control, glyphosate, AMPA, glyphosate+AMPA) on winter wheat were assessed after a culture period of four weeks. An additional time course experiment was conducted with sequential harvests at 21, 28, 35 and 41 days after sowing (DAS). To counteract effects of glyphosate/AMPA inactivation by complexation with the high concentrations of cationic nutrients in the growth medium, nutrient solution was supplied at intervals of three days, while water was the culture medium for the remaining time (Table 9).

Table 9: Weekly plan for application of herbicides, nutrient solution and distilled water in the hydroponic culture of winter wheat.

Day 1	Day2	Day3	Day4	Day5	Day6	Day7
Herbicide	Herbicide	Herbicide	Herbicide	Herbicide	Herbicide	Herbicide
Nutrient Solution	Distilled Water	Distilled Water	Nutrient Solution	Distilled Water	Distilled Water	Nutrient Solution
						

2.8.3 Monitoring of plant growth

After harvest, plants were analyzed as described in section 2.7.

2.8.4 Root vitality staining (TTC)

Staining was performed according to Chen *et al.* (2006) using 2,3,5-triphenyl tetrazolium chloride (TTC), which is reduced by dehydrogenase activities forming a red-coloured agent and formazan dye as an indicator for metabolic activity. For staining, roots washed for 10 minutes in distilled water, were incubated in darkness for 24 h in TTC solution (0.08% TTC in 0.05 M sodium phosphate buffer, pH 7.4). After staining, fresh weight was recorded and formazan was extracted from root segments located (0-1 cm behind the root tip and 1-3 cm behind the root tip. Extraction was performed with 10 mL of 95% (v/v) ethanol followed by incubation in a water bath at 80 °C for 20 min. After cooling, the formazan concentration was recorded spectrophotometrically at 485 nm.

2.9 Transcriptome analysis of wheat roots exposed to trace concentrations of glyphosate and AMPA

Winter wheat (*Triticum aestivum* cv. Isengrain) was grown in hydroponics (see 2.8.1) with the addition of 5 µg L^{-1} glyphosate (G), 2.5 µg L^{-1} AMPA (A), a combination of both and a control treatment, free of the herbicides (see 2.2).

2.9.1 Harvest of root tissue

At 19 DAS, the complete root system of each plant was harvested with a razor blade, shortly dried between paper towels to remove excess moisture, weighed, and quickly frozen in liquid nitrogen. From each pot, root samples were pooled and stored at -80 °C until further processing.

2.9.2 RNA isolation

A representative sample of frozen root tissue of three replicates was ground in liquid nitrogen. Total root RNA was extracted using the RNeasy® Mini Kit-Qiagen (Qiagen, Hilden, Germany). The extracted total RNA was tested spectrophotometrically for quality and quantity with the Nanodrop 2000/200c system (Thermo Fisher Scientific Inc. v 1.4.2). The RNA integrity was determined by 2100 Bioanalyzer instruments (Agilent Technologies, USA).

2.9.3 RNA-Seq analysis

Total RNA was processed for conversion to cDNA and library creation using the TruSeq RNA Sample Preparation Kit v2 (illumina® Inc. USA). The obtained cDNA was further processed for libraries preparation, and the prepared RNA libraries were paired-end sequenced (100x) using the illumina® HiScanSQ system.

2.9.4 Data processing

The initial data output was 14.13 Gb (giga bases) in AMPA (A), 13.09 Gb in Glyphosate (G), 6.42 Gb in Glyphosate+AMPA (GA) and 8.02 Gb in control. Further, these sequence

data were processed with RobiNa (Lohse *et al*., 2012). After filtering and alignment of the data, the data volume reduced in different treatments as 2.94 Gb in A, 1.31 Gb in G, 1.3 Gb in GA and 1.64 Gb in control. The processed data were used for determination of fold change values and relative read numbers using DESeq (Version 1.8.3) (Anders and Huber, 2010). The public available wheat transcriptome database from IWGSP1.23 was employed for mapping. The reference transcriptome was annotated via Mercator (http://mapman.gabipd.org/web/guest/mercator) and distributed into plant functional categories (bins) according to Mapman (Usadel *et al*., 2009). Metabolic pathways were visualized with Mapman 2.5.IR2 and its integrated module Pageman. In Pageman, significant differences were determined using the Wilcoxon Rank Sum test and the Benjamini-Hochberg procedure (PageMan Z-score below -1.96 or above 1.96, MapMan probability below 0.05). Each metabolic pathway was presented as a bin. The total changes in bins were compared in all treatments using color codes.

2.10 Statistics

All experiments were performed with four replicates using completely randomized designs. Statistical analysis was conducted using the Sigma Plot® 12 statistics software package (Sigma plot, Systat Software Inc. U.S.A) and SPSS® by IBM.

3 Results

3.1 Limited performance of winter wheat as affected by long-term no-tillage farming

3.1.1 History

The investigated area is located in the valley of the "Neckar" river and the mountain region "Schwäbische Alb" approx. 10-15 km South of Tübingen, 340-380 m above sea level (Figure 8, Figure 9). Typical soil types in the region are heavy clay-loam soils partially covered with loess topsoil layers. The average annual temperature is 9 °C with average precipitation of 600 mm, frequently associated with drought periods during spring and early summer.

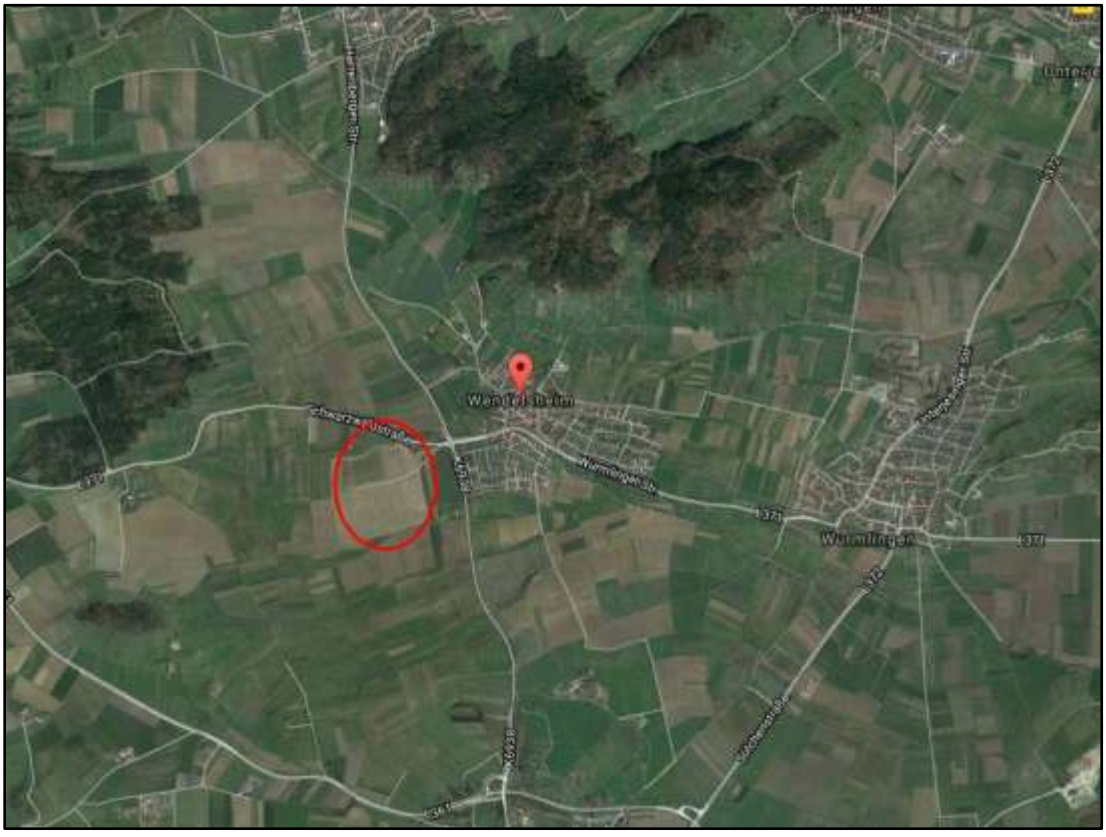

Figure 8: Satellite map of the investigated field site Wendelsheim (Google Maps).

Figure 9: Satellite map of the investigated field sites Hirrlingen (Google Maps).

Some of the investigated field sites have a long-term no-tillage history (LT) of meanwhile 10-20 years. During the investigated period, (2010-2012) winter wheat (75 %) and winter rape (25%) were the predominant crops, even with several years of winter wheat mono cropping for economic reasons in some cases. For historical reasons, very small plot sizes of 0.15 ha and less were quite abundant in the investigated area, forcing the farmers to increase their cropping areas by exchanging field sites. In many cases, this created heterogeneous fields consisting of plots with 10-12 years no-tillage history (LT) directly neighbored by plots with only 2-3 years short-term (ST) no-tillage management. In 2008, limited plant development in early spring on LT plots as compared to adjacent ST plots was observed for the first time in winter wheat (Figure 10) and later in winter rape also.

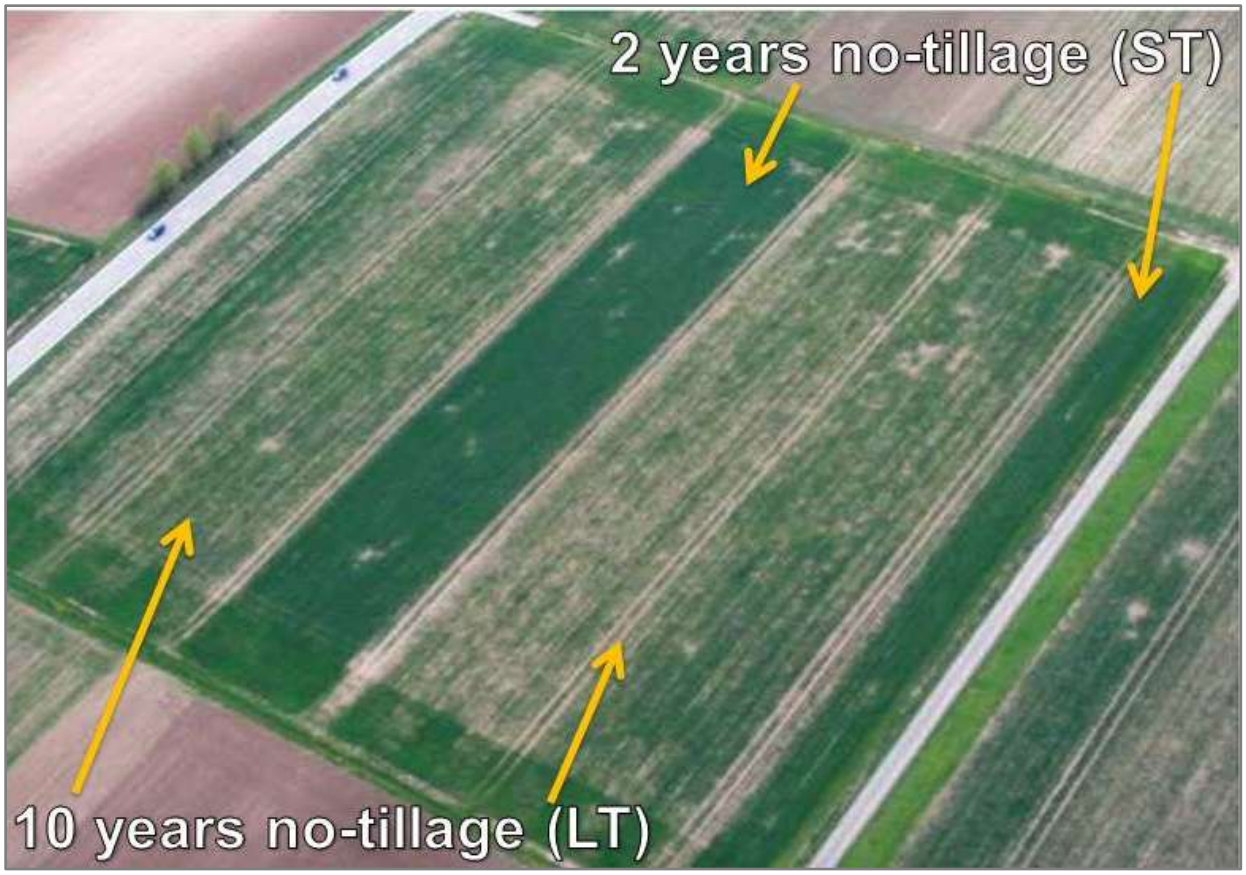

Figure 10: Arial view of plant growth on the long-term (LT) no-tillage field site "Schwarze Länder" after 11 years and neighboring plots with short-term no-tillage (2 years. ST) (Courtesy; Dr. K. Weiss, Tübingen).

3.1.2 Symptoms of plant damage observed on LT no-tillage field sites

In the LT no-tillage plots, problems were repeatedly observed on different field sites between 2008 and 2012 (Figure 11). Frequently, plants germinated and developed well after sowing in autumn, even without strong symptoms of frost damage during the winter period. However, symptoms of stunted growth, chlorosis and dying back selectively appeared in the LT no-tillage plots during re-growth in early spring, while directly neighbored ST plots remained unaffected (Figure 11).

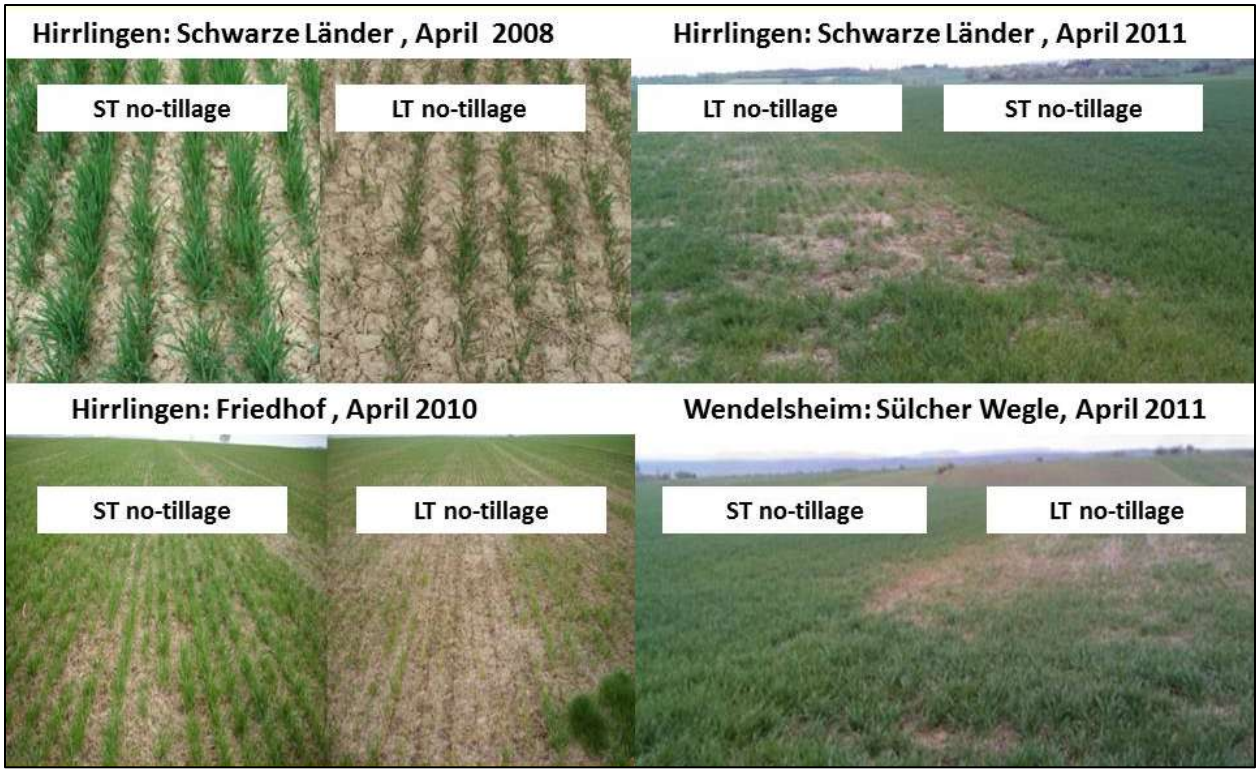

Figure 11: Repeated expression of winter wheat damage in spring on different no-tillage field sites in South West Germany with long-term (11-15 years) no-tillage cropping history.

The closer examination of damage symptoms in LT plots revealed weak seedling development, stunted shoot growth with chlorosis and necrosis on older leaves, and strongly impaired root growth with extremely limited development particularly of fine roots (Figure 12). During further plant development, the weakest seedlings frequently died, and virus infections were particularly abundant in LT plots.

Figure 12: Habitus of winter wheat on the long-term (LT) no-tillage field site REM and root growth in ST and LT plots.

3.2 Causal analysis of plant damage on long-term no-tillage field sites

A major focus of the present study was the characterization of factors determining the observed symptoms of plant damage on the LT no-tillage field sites, by comparison with neighbored ST no-tillage plots without expression of plant damage.

3.2.1 Soil fertility

Soil fertility is a major factor determining plant growth. In no-tillage farming, the duration of no-tillage can change nutrient availability and soil bulk density with impact on plant growth and development (Wilhelm *et al*., 1982).

During the examination period, fertilization management on the respective field sites was mainly focused on the application of nitrogen fertilizers as calcium-ammonium-nitrate (CAN) in Hirrlingen and ammonium-urea solution in Wendelsheim. The available soil P (phosphorus) status (CAL extraction) was characterized as sufficient, and therefore, no P

application was performed during the examination period. In Hirrlingen, additionally, micronutrient fertilization was performed (2 x 8 kg ha^{-1} $year^{-1}$) “Epso Combi Top” (K&S Kali GmbH, Kassel, Germany) for winter wheat and “Epso Micro Top” (K&S Kali GmbH, Kassel, Germany) for oilseed rape.

The commonly observed damage symptoms of impaired root growth stunted plant growth, chlorosis and necrosis may indicate potential deficiencies of other nutrients not applied as fertilizers: i.e., impaired root growth is typical for magnesium (Mg) or potassium (K) deficiency. Limitations of phosphorus (P) and zinc (Zn) can cause inhibition of shoot growth. Deficiencies of Mg and Manganese (Mn) can induce chlorosis of older leaves, and severe deficiency of K causes chlorosis and necrosis (Marschner’s, 2012).

To address the question whether damage symptoms on the LT field plots could be related to soil nutrient limitation, comparative soil analyses were performed on selected LT and ST no-tillage plots in the years when damage symptoms were observed (Table 10). However, no apparent nutrient deficiencies or toxicities were detectable in all investigated soils. While available P was usually higher in LT soils as compared with short-term soils, similar or lower levels were detected for the remaining nutrients in the LT soils but without a regular pattern for the investigated soil pairs.

In addition, soil pH is a major factor determining the solubility of mineral nutrients and toxic elements. However, in all soils, the pH remained in a narrow range between 6.8 and 7.1. As expected for LT no-tillage soils, the humus content in the topsoil layer was increased by 1-1.5 %.

Taken together, the reported findings suggest that the observed damage symptoms could not be attributed to limited soil fertility of the LT no-tillage soils.

Table 10: Analysis of mineral nutrients, pH and humus in long-term (LT) and short-term (ST) no-tillage soils (see 2.4.1 for methodology) collected from the field sites Schwarze Länder (SL) 2008 & 2011 and Remmingsheimer Weg (REM) 2011. Phosphorus (P) and potassium (K) were extracted using the calcium-acetate-lactate (CAL) method. Magnesium (Mg) was measured in $CaCl_2$ extracts. The micronutrients iron (Fe), manganese (Mn) and zinc (Zn) were measured after calciumchloride/DPTA extraction (CAT). Soil pH was measured in $CaCl_2$ extracts, and humus percentage was determined by elemental analysis (EA).

Soil Nutrients, pH& Humus	Year 2008		Year 2011				**Standard values**
	Field Site SL		**Field Site SL**		**Field Site REM**		
	LT no-tillage	ST no-tillage	LT no-tillage	ST no-tillage	LT no-tillage	ST no-tillage	
Phosphorus (P_2O_5 mg 100 g^{-1})	9.1	8.8	25.0	17.0	34.0	20.0	10-20 (in heavy soils) [1]
Elemental P (P_2O_5x0.4364)	4.0	3.8	10.9	7.4	14.8	8.7	4.36 - 8.73 (in heavy soils) [1]
Potassium (K_2O mg 100 g^{-1})	46.0	33.0	44.0	48.0	49.0	52.0	21 - 30 (in heavy soils) [1]
Elemental Potassium (K_2Ox0.8301)	38.2	27.4	36.5	39.8	40.7	43.2	17.43 - 24.90 (in heavy soils) [1]
Magnesium (mg 100 g^{-1})	43.0	41.0	28.0	30.0	17.0	41.0	11 - 15 (in heavy soils) [1]
Iron (mg kg^{-1})	66.2	94.6	59.9	66.6	30.4	67.9	Approx. 70 (in all soils) [2]
Manganese (mg kg^{-1})	224.0	332.0	124	207	61.9	242	40 - 60 (with pH > 6,5) [3]
Zinc (mg kg^{-1})	3.5	6.6	5.10	6.60	2.70	4.40	1 - 3 (in all soils) [3]
Soil organic carbon %	2.4	2.0	5.16	4.11	5.45	4.04	1.3 - 2.7 (in heavy soils) [4]
pH ($CaCl_2$)	7.0	6.7	7.1	7.0	7.1	7.1	5.7 - 6.7 (Humus > 4%)[4] 6.6 - 7.2 (Humus < 4%)[4]

[1]LTZ Augustenberg, 2011, [2]Steiermark, 2016, [3]Landwirtschaftskammer, 2008, [4]LfL, 2012

3.2.2 Soil structure

One of the basic soil properties affected by tillage is the bulk density. Higher bulk density reduces soil porosity by changing the ratio of water-to-air capacity proportionally in favor of water capacity (Badalíková and Kňákal, 2000). Badalíková (2010) reported increased soil bulk density along with decreased total porosity as result of reduction in soil tillage. In winter wheat, root length and density as well as plant height and aboveground biomass was reduced with increase in soil density (Wilhelm *et al*., 1982), symptoms similarly observed in the present study. However, soil organic matter influences the extent of possible compaction and determines the moisture content at which maximum compaction occurs: with higher organic matter, there is less maximum compaction and a higher moisture requirement to cause maximum soil compaction (Lull, 1959). In most cases, according to the principle of conservation tillage, no-tillage systems soils are high in topsoil organic matter. Accordingly, also on the investigated LT field sites higher topsoil humus contents have been determined as compared to ST plots (Table 10). Moreover, activity of earthworms with a positive impact on soil structure is frequently enhanced in no-tillage systems (Kemper *et al.*, 2011).

However, as a striking observation, the symptoms of plant damage observed on LT field plots could be reproduced in pot experiments with homogenized and sieved soil samples (2 mm mesh size) taken from the respective field sites (Figure 13). Since no soil structure effects can be expected in homogenously sieved soils, these findings demonstrate that soil structure was obviously not a critical factor for the expression of damage symptoms.

Figure 13: Inhibition of wheat (cv. Isengrain) growth in pot experiments taken from different field sites with LT no-tillage history as compared with soils from neighboured ST plots.

3.2.3 Symptoms of plant damage in pot culture

3.2.3.1 Habitus

Interestingly, plant damage on LT field plots in pot experiments was not only reproducible for the location of different field sites and the years when plant damage occurred (Figure 13), but also the variation in damage intensity on a specific LT plot was reflected in corresponding results in pot culture (Figure 14). In later stages of plant development, plants grown on LT soils in pot experiments also showed similar symptoms of necrosis in older leaves as observed under field conditions (Figure 15).

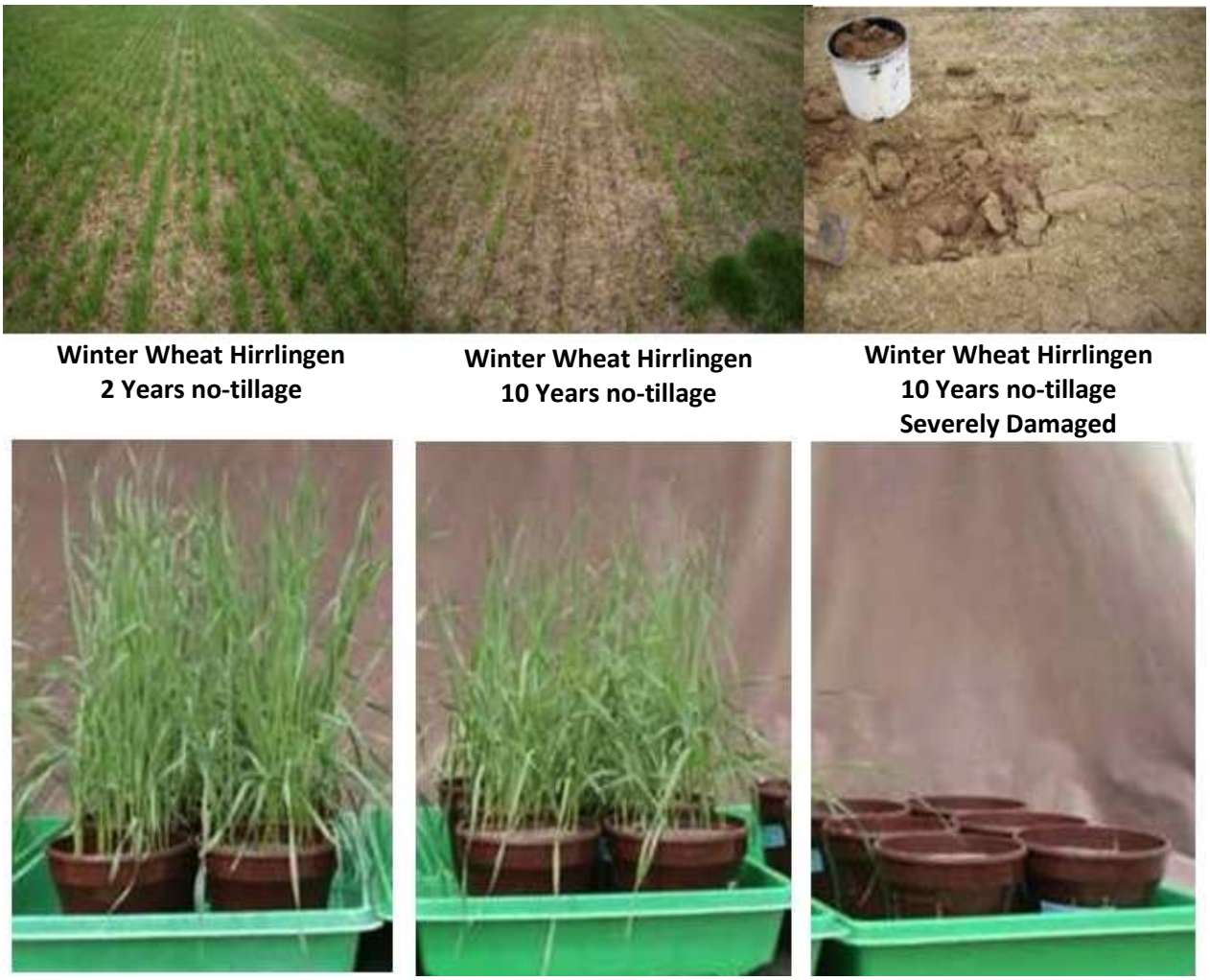

Figure 14: Variation in intensity of plant damage on long-term no-tillage field plots (Hirrlingen, Friedhof) under field conditions and in pot experiments conducted with the respective soils.

Figure 15: Necrosis and chlorosis of older leaves in winter wheat (cv. Isengrain) grown on the soil of a long-term no-tillage (LT) plot in Wendelsheim, Remmingsheimer Weg (REM) under field conditions (left) and in pot culture (right).

3.2.3.2 Germination and seedling growth

Germination (emergence) was not regularly affected in pot experiments with LT field soils (Figure 16). However, plant height (Figure 13) and shoot biomass (Figure 17) on LT soils was generally lower than on soils collected from ST plots.

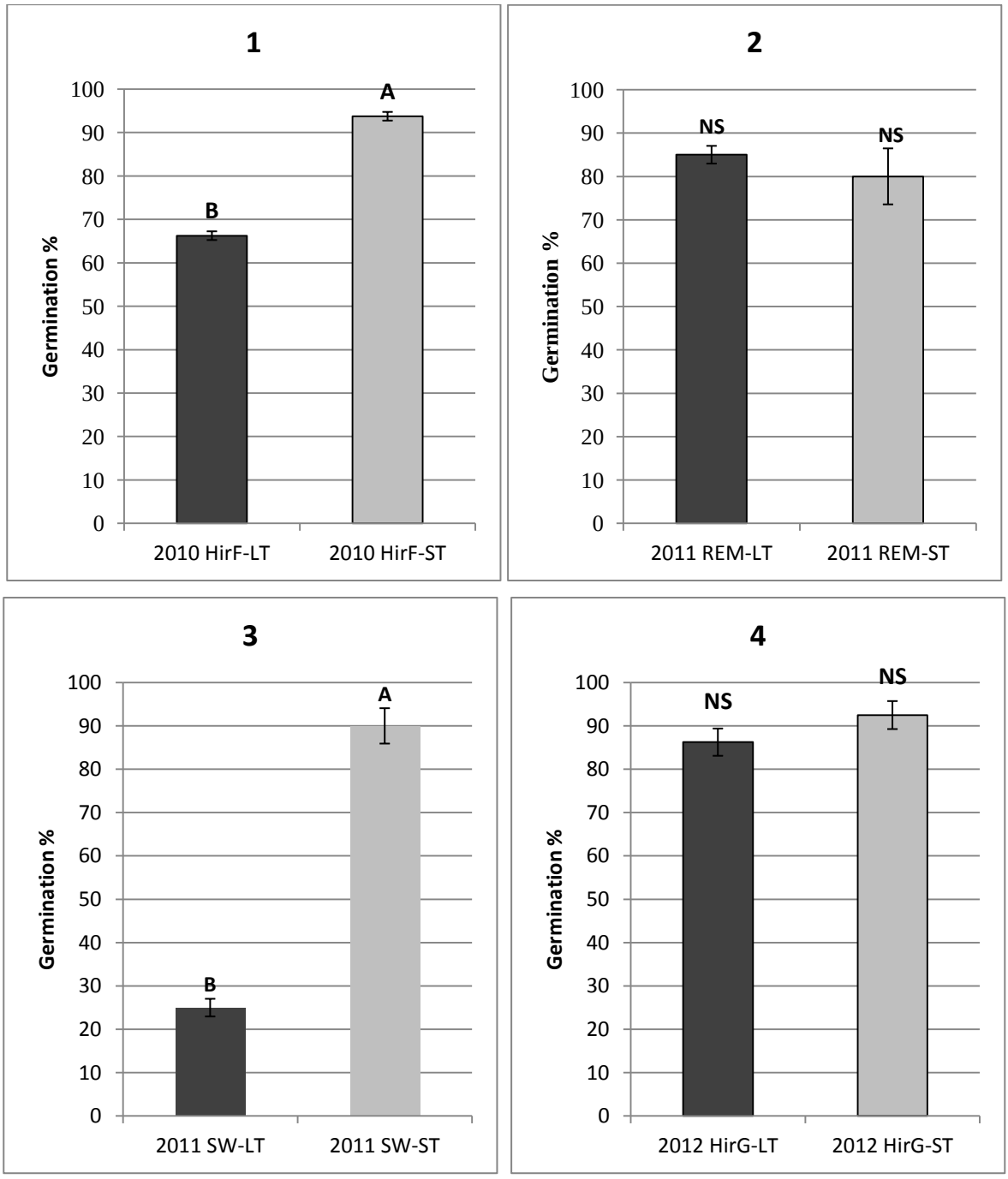

Figure 16: Germination percentages of winter wheat (cv. Isengrain) after 16 days growth in soil samples collected in spring from long-term and short-term no-tillage fields in different years. ***(1)*** *Hirrlingen Friedhof (HirF) 2010* ***(2)*** *Remmingsheimer Weg (REM) 2011* ***(3)*** *Sülcher Wegle (SW) 2011* ***(4)*** *Hirrlingen Gassäcker (HirG) 2012. Values are means of 4 replicates ± SE. Means with different letters are significantly different. NS = not significant. (t- test, α = 0.05).*

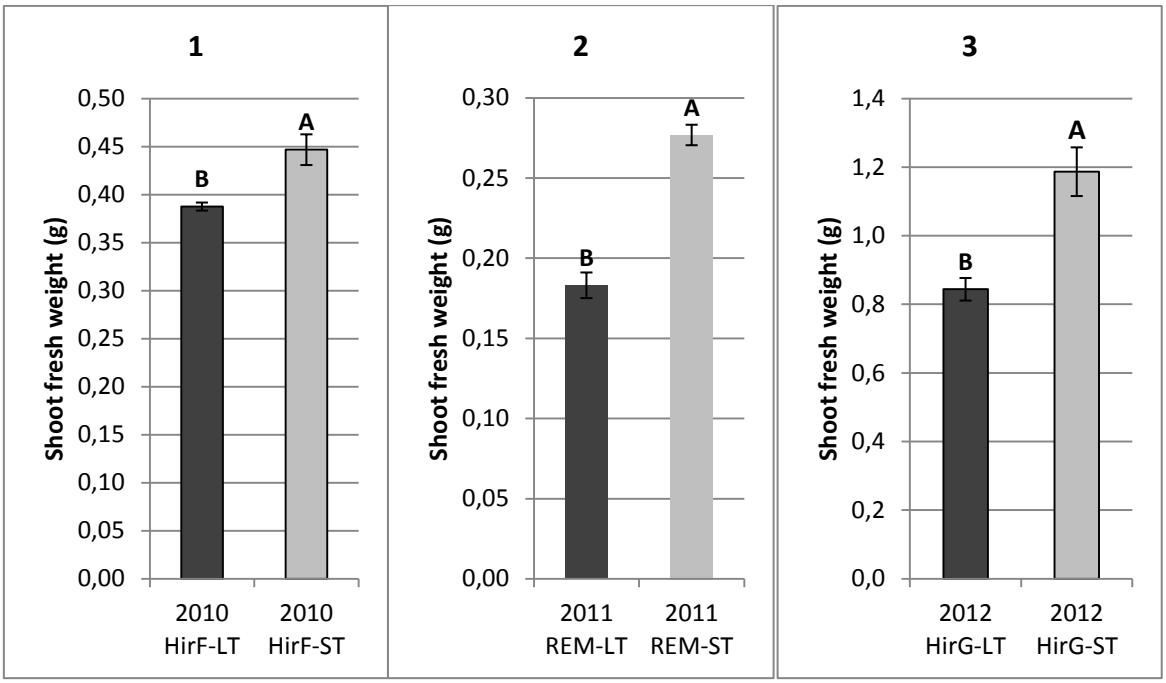

*Figure 17: Shoot biomass production per plant in winter wheat (cv. Isengrain) (**1**) after 21 days of growth on soil collected from Hirrlingen Friedhof (HirF) 2010 **(2)** after 18 days of growth on soil collected from Remmingsheimer Weg (REM) 2011 and **(3)** after 21 days of growth on soil collected from Hirrlingen Gassäcker (HirG) 2012. Values are means of 4 replicates ± SE. Means with different letters are significantly different (t- test, α = 0.05).*

3.2.3.3 Root growth and morphology

Similar to the field observations (Figure 12) root growth was particularly affected on soils from LT field plots. While average root diameter (Figure 18) and frequently also root biomass was increased in soils from LT field sites, but total root length was drastically reduced by 40-60 % (Figure 19). This finding indicates a higher proportion of fine roots in plants grown on the soil from LT plots.

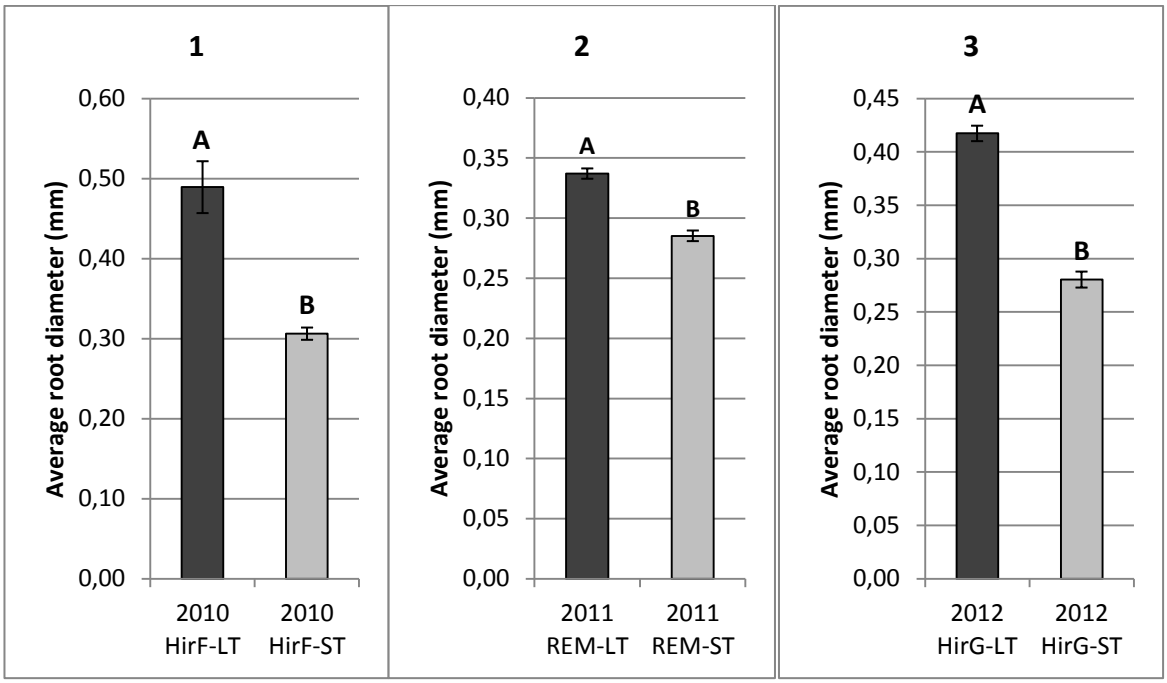

*Figure 18: Average root diameter of winter wheat (cv. Isengrain) grown in pot culture on soils collected from two field sites **(1)** after 21 days of growth on soil collected from Hirrlingen Friedhof (HirF) 2010 **(2)** after 18 days of growth on soil collected from Remmingsheimer Weg (REM) 2011 and **(3)** after 21 days of growth on soil collected from Hirrlingen Gassäcker (HirG) 2012 with long-term (LT) and short-term (ST) no-tillage history. Values are means of 4 replicates ± SE. Means with different letters are significantly different (t- test, α = 0.05).*

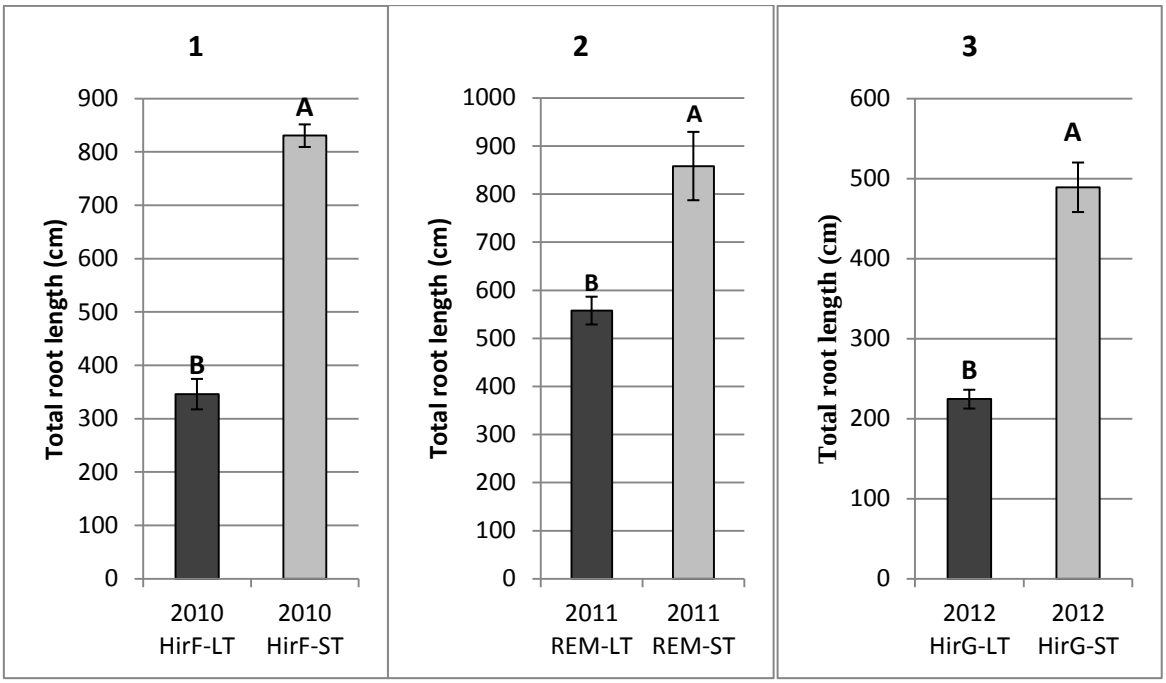

*Figure 19: Root length of winter wheat (cv. Isengrain) grown in pot culture on soils collected from two field sites **(1)** after 21 days of growth on soil collected from Hirrlingen Friedhof (HirF) 2010 **(2)** after 18 days of growth on soil collected from Remmingsheimer Weg (REM) 2011 and **(3)** after 21 days of growth on soil collected from Hirrlingen Gassäcker (HirG) 2012 with long-term (LT) and short-term (ST) no-tillage history. Values are means of 4 replicates ± SE. Means with different letters are significantly different (t- test, α = 0.05).*

The negative impact of long-term no-tillage on fine root production was confirmed by analysis of different root diameter classes (Figure 20) showing a 30-50 % reduction of the finest root fraction but an increased contribution of thicker roots to the total root length on LT soils (Figure 20).

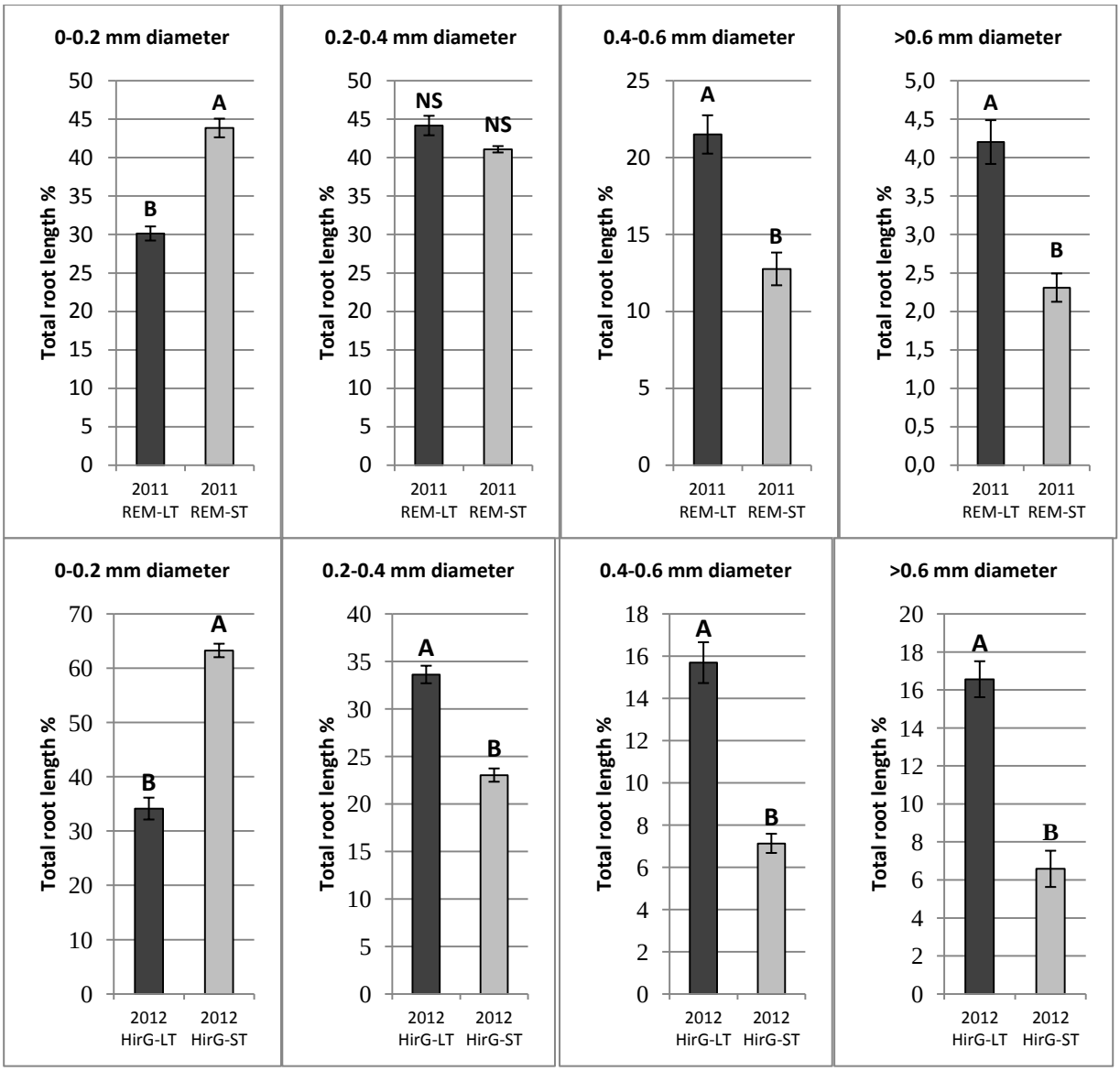

*Figure 20: Root length distribution in different root diameter classes of winter wheat (cv. Isengrain) grown in pot culture on two field sites **(Upper row)** after 18 days of growth on soil collected from Remmingsheimer Weg (REM) 2011 and **(Lower row)** after 21 days of growth on soil collected from Hirrlingen Gassäcker (HirG) 2012 with long-term (LT) and short-term (ST) no-tillage history. Values are means of 4 replicates ± SE. Values are means of 4 replicates ± SE. Means with different letters are significantly different. NS = not significant. (t- test, α = 0.05).*

3.2.4 Plant nutritional status

In winter wheat, 70% of total root length is found in the topsoil (0-30 cm) with the highest nutrient levels in most agricultural soils (Manske and Vlek, 2002). Particularly acquisition of sparingly soluble nutrients, such as phosphate (P) strongly depends on root growth and expression of fine root structures to exploit larger soil volumes and for efficient

expression of adaptations for chemical nutrient mobilization (Neumann and Römheld, 2002). Accordingly, improved P acquisition efficiency was found in wheat genotypes with high root length densities (Manske *et al*., 2000). However, even with the highest rooting densities, usually less than 20 % of the topsoil volume is exploitable by plant roots (Neumann and Römheld, 2002). Therefore, the acquisition of nutrients with low solubility is particularly affected by stress factors limiting root growth. Accordingly, in the present study, particularly P acquisition was limited in winter wheat plants grown on LT no-tillage soils (Figure 21) due to the massive reduction of fine root production (Figure 20). Although, as compared with ST soils, the available P levels in LT soils were even higher (Table 10). The P status of the plants grown on LT soils was in the deficiency range but sufficient for plants on ST soils (Figure 21). By contrast, no regular patterns were detectable for other nutrients (Table 11).

Table 11: Nutrient status (based on shoot dry weight) of winter wheat (cv. Isengrain) grown in pot culture on two field sites: Hirrlingen, Friedhof (HirF) 2010 and Wendelsheim, Remmingsheimer Weg (REM) 2011 with long-term (LT) and short-term (ST) no-tillage history and the nutrient deficiency limit (Bergmann, 1988). Values are means of 4 replicates ± SE. Means with different letters are indicating significant differences at α = 0.05% (t- test), NS = not significant.

Nutrients	**Deficiency limit**	**Hirrlingen, Friedhof 2010**		**Wendelsheim,Remmingsheimer Weg 2011**	
		LT no-tillage	ST no-tillage	LT no-tillage	ST no-tillage
P (mg g^{-1})	04	3.4±0.06 (B)	4.2±0.06 (A)	3.8±0.13 (B)	5.0±0.11 (A)
K (mg g^{-1})	32	46.0±1.15 (B)	56.5±1.23 (A)	59.4±2.06 (B)	65.6±1.14 (A)
Ca (mg g^{-1})	02	6.6±0.08 (B)	8.5±0.11 (A)	7.6±0.23 (A)	5.1±0.31 (B)
Mg (mg g^{-1})	1.5	2.7±0.05 (A)	2.4±0.04 (B)	1.6±0.06 (NS)	1.7±0.05 (NS)
Fe (mg kg^{-1})	25	240.3±77 (NS)	250.3±19 (NS)	125.9±27.6 (NS)	138.7±36.8 (NS)
Mn (mg kg^{-1})	35	166.1±3.86 (A)	71.5±0.70 (B)	18.7±0.65 (A)	14.0±0.75 (B)
Zn (mg g^{-1})	15	37.7±0.69 (NS)	37.2±0.55 (NS)	25.9±1.05 (NS)	26.3±0.61 (NS)
Cu (mg kg^{-1})	05	10.2±0.24 (B)	13.2±0.41 (A)	18.7±0.65 (A)	14.0±0.75 (B)

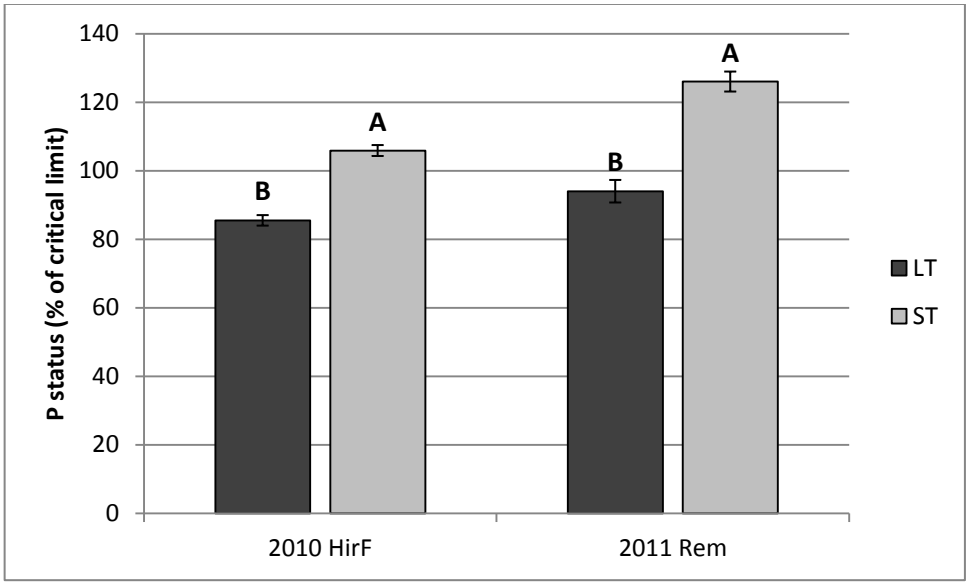

Figure 21: Phosphorus status of winter wheat (cv. Isengrain) grown in pot culture on two field sites (Wendelsheim, ***(1)*** *after 21 days of growth on soil collected from Hirrlingen Friedhof (HirF) 2010 and* ***(2)*** *after 18 days of growth on soil collected from Remmingsheimer Weg (REM) 2011, with long-term (LT) and short-term (ST) no-tillage history relative to the critical shoot P concentration for P deficiency (Bergmann, 1988). Values are means of 4 replicates ± SE. Means with different letters are significantly different (t- test, α = 0.05).*

3.3 Plant pathogens as potential causes for plant damage on long-term no-tillage soils

A shift from conventional tillage to no-tillage influences various soil factors, such as moisture content, temperature, bulk density, organic matter distribution or physical structure of crop residues. These changes in soil properties can also affect the composition and activity of soil-microbial populations (Rothrock, 1992). Particularly in long-term no-tillage systems, substantial alterations in root pathogen populations have been reported, including increased severity of *Pythium* and *Rhizoctonia* root rot or take-all disease (Bailey *et al.*, 2000; Smiley *et al*., 2009). Under no-tillage, soil-borne pathogens surviving in previous year-crop residues can make diseases more problematic, and monoculture or late sowing with germination in wet and cool soils bears the risk of highest disease severity (Smiley *et al*., 2009).

In the present study, damage symptoms on long-term no-tillage plots were usually observed in early spring, providing ideal cold and wet weather conditions for the development of many root pathogens. High soil organic matter (Table 10) and in some cases even wheat monoculture are additional factors promoting the growth of pathogens. However, on aerial plant parts, classical symptoms of fungal (rust, mold, mildew, leaf spot,) or bacterial diseases (leaf spot, canker, blight) were not detectable, although, in some years, barley yellow dwarf virus infections have been identified in later spring. On the other hand, the observations of impaired fine root production and root thickening (Figure 12, Figure 18) are comparable to symptoms of *Rhizoctonia* or *Pythium* root rot (Smiley *et al*., 2009). Impaired root growth with a high visible fraction of course roots was comparable to pathogen attack. In winter wheat, *Rhizoctonia* root rot symptoms are stunted growth, root rot and delayed maturity. It is caused by *Rhizoctonia solani* AG-8. Disease severity increases with no-tillage and annually planting wheat or planting winter wheat too late. Similarly, *Pythium* root rot is caused by *Pythium* spp., which expresses its symptoms as seed rot, damping-off, stunting and delayed maturity and cool, wet soil, plant winter wheat annually and/or too late, can lead to highest disease severity (Smiley *et al*., 2009). Under no-tillage, surviving soil borne pathogens of the pervious year in crop residues make the disease more problematic. This result many plant pathogens increased to damaging levels. *Fusarium* spp. is major soil borne pathogen of wheat and Bailey *et al*. (2000) reported its higher incidence under no-tillage, though root diseases severity was low in no-tillage as compared to conventional tillage. Stunt nematodes are also parasitic nematodes, infecting and feeding on roots of cereals, grasses and other plant species. They feed on epidermal cells and root hairs, mostly in the region of cell elongation. This can cause similar symptoms of leaf chlorosis (Figure 15), root thickening and inhibition of root elongation (Figure 12, Figure 19) (Smiley, 2006).

3.3.1 Effect of soil sterilization

Due to the reproducibility of plant damage symptoms, observed on long-term no-tillage field plots, in pot experiments (see 3.2), it was possible to assess a potential involvement of pathogens by experiments on sterilized soils. For many soil sterilization methods, a major drawback is a possible alteration of soil chemical and physical properties and

chemical changes in soil organic matter. Autoclaving is the most commonly used method by use of high temperature and pressure, i.e., 120°C at 103 kPa. However, the efficiency is low and can be improved by repeated autoclaving two or three times, but this can cause significant modifications of soil structure and chemistry, making comparisons with unsterilized controls difficult. On the other hand, poisons and fumigants are highly effective sterilizers, but they frequently change the soil chemistry by leaving toxic residues (McNamara *et al*., 2003). Gamma (γ) irradiation affects soil organisms by direct ionization of cells and by the creation of harmful radicals within the extracellular water and intercellular fluids (Jackson *et al*., 1967). For plant growth, degradation or sorption experiments, soil sterilization with γ- irradiation is a reliable method due to the absence of contaminations and comparatively small effects on soil physical and chemical properties. The optimal dose of γ- irradiation is essential for the desired sterilization success, since at lower doses, many microorganisms may survive, while higher doses increase the risk of changes in soil properties. At a dose of 15 kGy fungi and actinomycetes and at higher doses between 20-70 kGy also soil bacteria can be eliminated (McNamara *et al*., 2003). Therefore, gamma ray doses of 26 kGy were employed for all soil sterilization experiments conducted in the present study, with three pairs of long-term and short-term no-tillage soils and winter wheat as a test crop.

3.3.1.1 Germination

On two out of three investigated soil pairs, long-term no-tillage negatively affected germination percentage (Figure 22, Figure 23). On the LT no-tillage soil collected from HirF in 2010, germination percentage of winter wheat at two weeks after sowing was not improved by soil sterilization, thereby excluding pathogens as a cause for reduced germination.

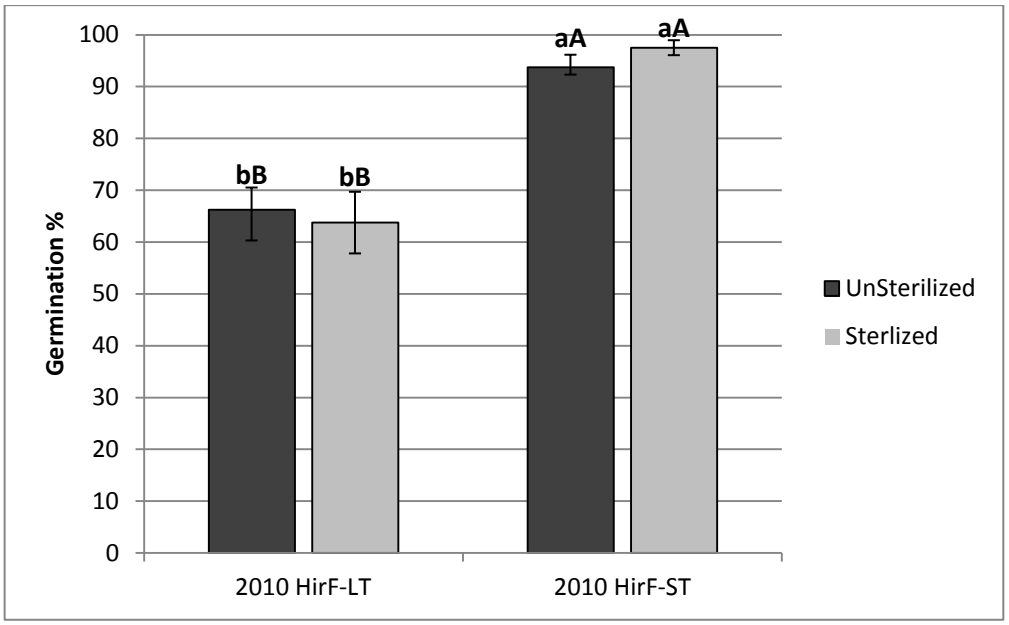

Figure 22: Effect of soil sterilization on germination % of winter wheat (cv. Isengrain) at 2 weeks after sowing in LT and ST no-tillage soils (field site: HirF2010). Values are means of 4 replicates ± SE. Mean values with different letters are indicating significant differences where small letters compare sterilization and big letters compare tillage duration. Two-way ANOVA ($p<0.05$) followed by Tukey's test ($\alpha = 0.05$).

By contrast, germination on LT no-tillage soil collected from the SW field site was significantly improved by soil sterilization (Figure 23), as a definite indication for the presence of soil pathogens affecting germination on this soil.

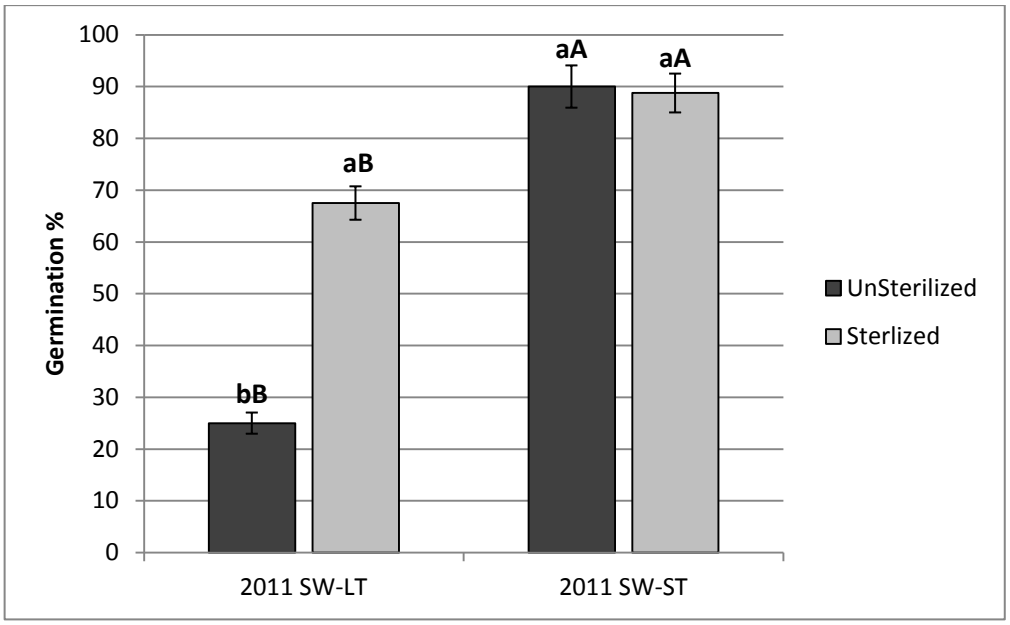

Figure 23: Effect of soil sterilization on germination % of winter wheat (cv. Isengrain) at 16 days after sowing in LT and ST no-tillage soils (field site: SW2011). Values are means of 4 replicates ± SE. Mean values with different letters are indicating significant differences where small letters compare sterilization and big letters compare tillage duration. Two-way ANOVA ($p<0.05$) followed by Tukey's test ($\alpha = 0.05$).

3.3.2 Root growth

During further seedling development, long-term no-tillage management affected root growth particularly. On long-term no-tillage soils collected from the field sites REM and HirF both, root length (Figure 24) and fine root development (Figure 25) were significantly reduced. No significant differences were recorded between sterilized and unsterilized soils.

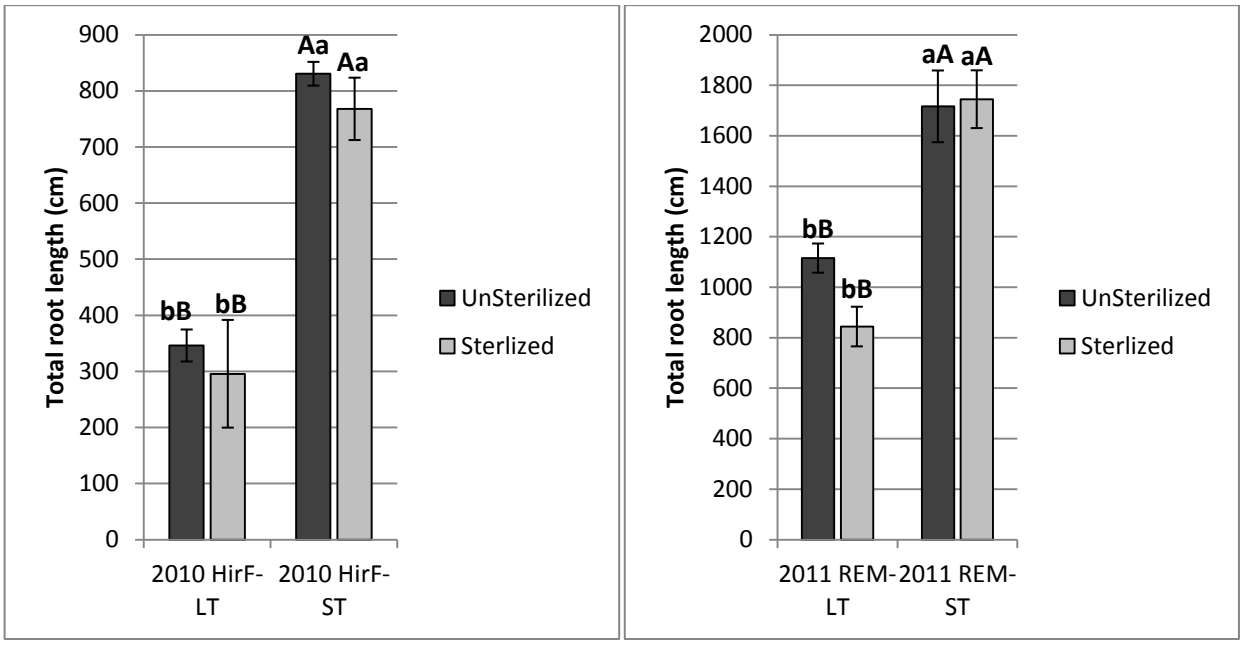

Figure 24: Effect of soil sterilization on total root length of winter wheat (cv. Isengrain) at 3 weeks after sowing in LT and ST no-tillage soils (field sites: REM2011 and Hir2010). Values are means of 4 replicates ± SE. Mean values with different letters are indicating significant differences where small letters compare sterilization and big letters compare tillage duration. Two-way ANOVA (p<0.05) followed by Tukey's test (α = 5%).

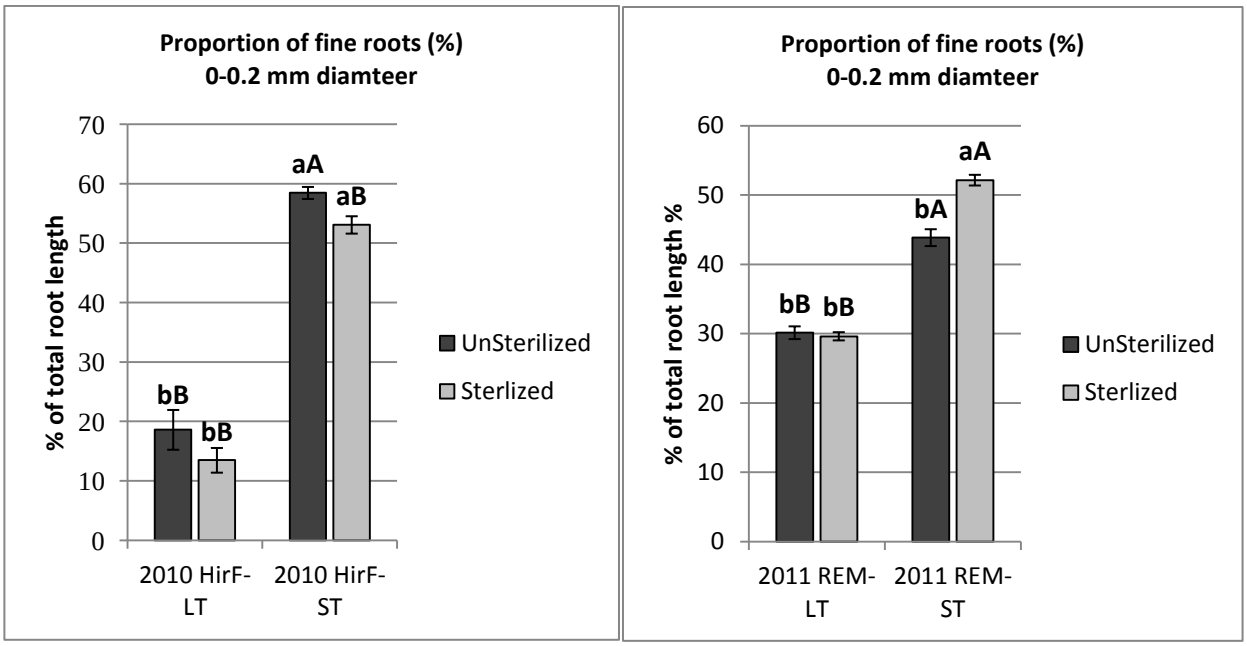

Figure 25: Effect of soil sterilization effect on fine root growth of winter wheat (cv. Isengrain) after 3 weeks of sowing in LT and ST soils (Field sites: HirF2010 and REM2011). Values are means of 4 replicates ± SE. Mean values with different letters are indicating significant differences where small letters compare sterilization and big letters compare tillage duration. Two-way ANOVA (p<0.05) followed by Tukey's test (α = 5%).

In summary, the soil sterilization experiments with three soil pairs characterized by long-term or short-term no-tillage history revealed a positive effect of soil sterilization on plant performance only in one case on soil collected from the field site SW2011, where germination was improved by soil sterilization on the long-term no-tillage soil. In all other cases, soil sterilization did not affect. These findings suggest that increased pathogen pressure on LT no-tillage soils was not the primary cause for the observed symptoms of plant damage.

3.4 Allelopathic interactions as potential causes for plant damage on long-term no-tillage soils

Allelopathy refers to both detrimental and beneficial biochemical interactions among all classes of plants including those mediated by microorganisms (Molisch, 1937). Negative allelopathic effects are caused by the release of inhibitory substances into the environment by living plants via root exudates, leaching, volatilization, and decomposition of plant residues (Rice, 1984), termed as allelochemicals (Wittaker and Feeny, 1971). These substances can be helpful in pest and disease control and reduction of competition. Wheat (*Triticum aestivum*) is a well-characterized allelopathic plant species. Allopathic effects of root exudates, straw, affect various agricultural weeds. Aqueous extracts of residues and range of simple phenolics and hydroxamic acids have been discussed as active compounds (Wu *et al*., 2001). Autotoxicity is an intraspecific type of allelopathy; it occurs when the same species inhibit germination and growth of a plant species through the release of inhibitory substances (Putnam, 1985). For example, the root exudates and leachates from the straw of wheat and oats exhibit an autotoxic potential on seedling growth (Schreiner and Reed, 1907; Wu *et al*. 2001). These auto-allopathic substances from living winter wheat and straw can accumulate in soils with continuous wheat cropping and in long-term no-tillage farming due to the accumulation of straw, finally causing auto-inhibitory effects on germination, seedling development, and root and shoot growth (Wu *et al.*, 2001). This situation may also apply to the long-term no-tillage field sites investigated in the present study.

By sequestering plant-available organic constituents in the soil solution, added carbon can remove allelochemicals from the soil *in situ*. This approach can be employed to identify the presence of phytotoxic organic compounds in soils by growing test plants with and without soil amendments of carbon (Inderjit and Nilsen, 2003). Accordingly, in the present study, a pyrolysis biochar (Table 7) commonly used for soil improvement was employed as a carbon material with the potential to adsorb organic phytotoxins (Sun *et al*., 2012), and added to a long-term no-tillage soil collected from the field site HirG2012 (Table 6). The addition of biochar at a concentration of 5% (v/v) completely removed the inhibitory effects on plant growth observed during emergence and early growth of winter wheat (Figure 26).

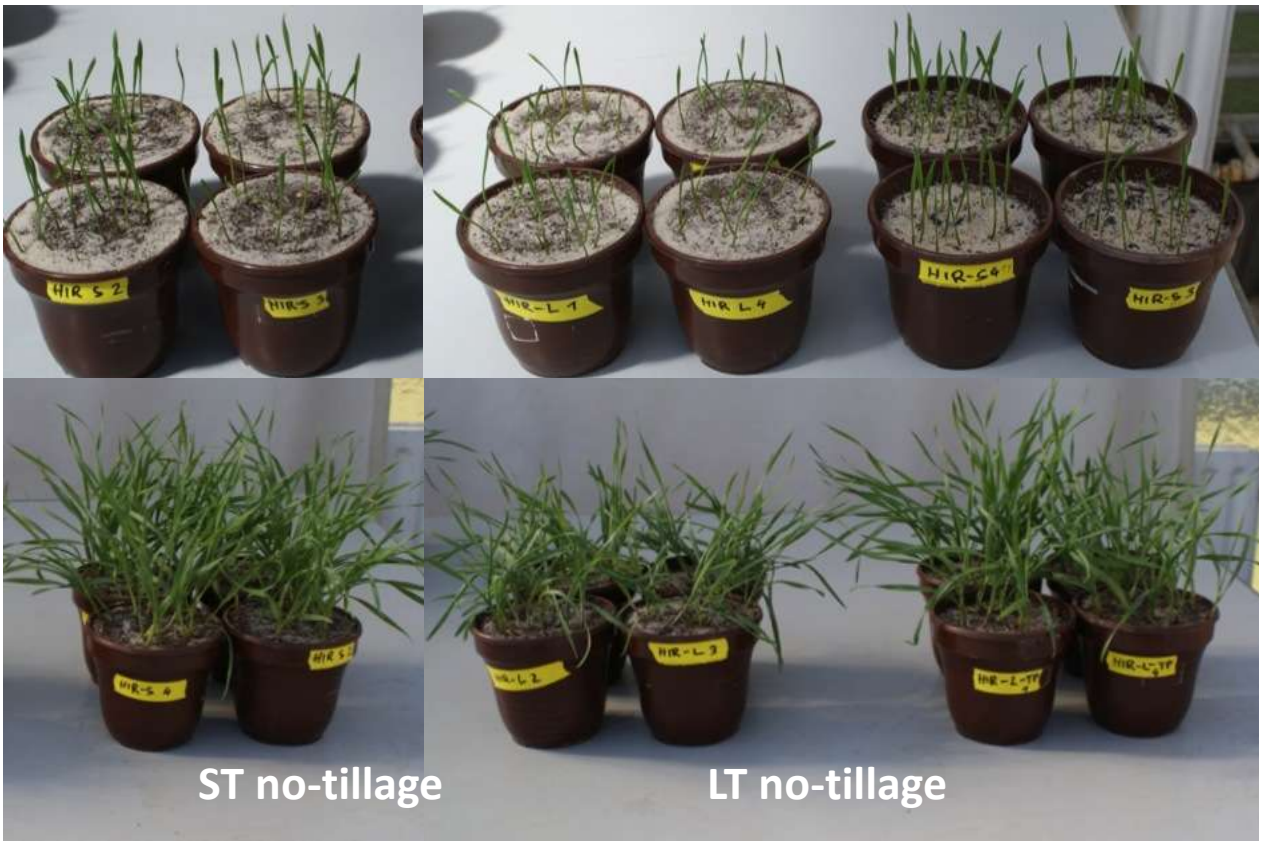

Figure 26: Mitigation effect of biochar amendment [5% v/v] on plant damage of winter wheat (cv. Isengrain) during emergence and early growth on long-term no-tillage soil collected from the field site HirG2012.

The rapid mitigation effect of the biochar treatment was detectable already during the first week after sowing (Figure 26, upper row) suggests efficient immobilization of a toxic compound in the LT no-tillage soil rather than a nutritional effect e.g. by release of

minerals sequestered in the biochar (Table 7), expected to require much longer incubation periods. Additionally, biotests with different plant species (wheat, soybean, sunflower) were performed to test a putative allelopathic potential of long-term no-tillage soils. In a pot experiment with soybean (*Glycine max*) as test plant similar damage symptoms appeared as previously observed in winter wheat, comprising stunted shoot growth, chloroses, reduced leaf number, inhibited root elongation and less fine root development (Table 12) induced by a culture period of four weeks on long-term no-tillage soil collected from the REM2011 field site.

Table 12: Growth of soybean (Glycine max L. cv. BR16 Conquista) on LT and ST no-tillage soils (field site REM2011). Values are means of 4 replicates ± SE. Means with different letters are significantly different (t- test, α = 0.05).

Growth Features	**LT**	**ST**
Germination% after 14 days a	25.00±15.00 (B)	45.00±5.00 (A)
#Average Number of leaves	3.59±0.21 (B)	6.04±0.82 (A)
Root fresh weight (g)	1.08±0.15 (A)	1.81±0.14 (B)
Total root length (cm)	81.08±6.09 (B)	380.53±37.70 (A)
Average root diameter (mm)	0.63±0.02 (A)	0.44±0.01(B)
Root length % of diameter 0-0.2mm	2.92±0.67 (B)	21.05±1.55 (A)
Root length % of diameter 0.2-0.4mm	23.83±3.62 (B)	42.93±0.49 (A)
Root length % of diameter 0.2-0.6mm	46.62±3.09 (A)	21.37±0.61 (B)
Root length % of diameter >0.6 mm	46.62±3.09 (A)	21.37±0.61 (B)

#-t-test is performed on transformed data (square root transformation).

Similar damage symptoms were also observed in a previous experiment with sunflower on short-term and long-term no-tillage soils collected from the field site SL 2008 (Bott, 2010).

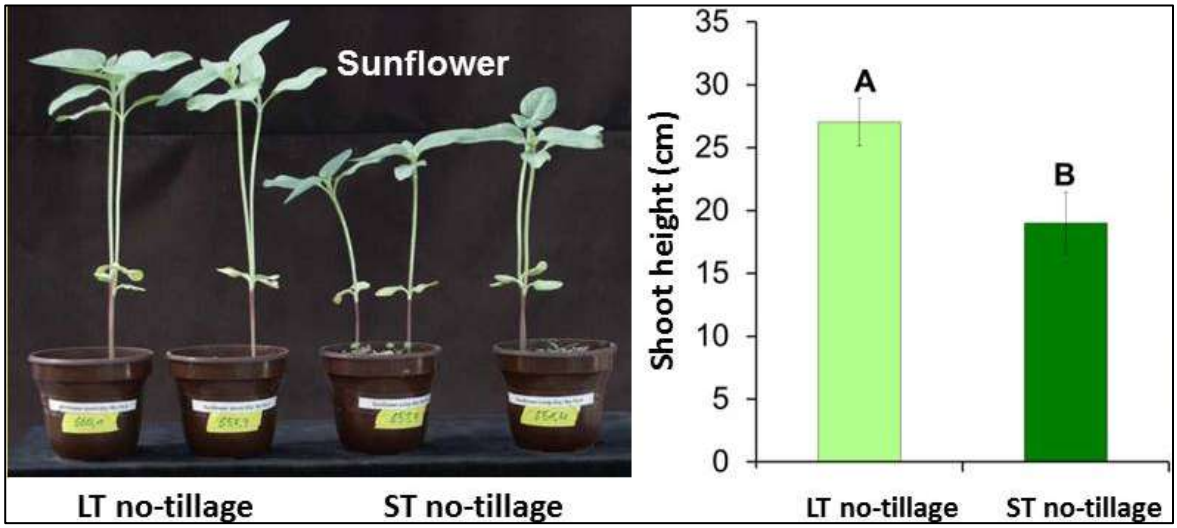

Figure 27: Shoot growth of Sunflower (Helianthus annuus) on LT and ST no-tillage soil (field site SL2008). Values are means of 4 replicates ± SE. Means with different letters are significantly different (t- test, α = 0.05) (Bott, 2010; personal communication).

Taken together, so far the rapid induction of damage symptoms during emergence and early growth in different plant species including wheat, and the remediation effects of biochar amendments point to an allelopathic potential of the soils collected from field sites with long-term no-tillage history. However, this scenario can hardly explain the field observation, showing that emergence and seedling development of winter wheat in autumn remains unaffected and damage symptoms usually appear at the begin of the growth period in early spring. If an autotoxicity potential would have accumulated in the respective soils as a consequence of a long-term wheat-dominated no-tillage management, damage symptoms should appear already during early growth as observed in the pot experiments.

Another interesting observation, hardly compatible with the assumption of a cumulative autotoxicity effect, is the finding that the toxicity potential of long-term no-tillage soils collected in early spring obviously disappears completely in summer soil samplings (Figure 28, Figure 29). Instead of cumulative enrichment over time, this would indicate a periodic degradation of toxic compounds in the LT no-tillage soils with the highest accumulation potential in early spring, reaching inhibitory levels for plant growth and subsequently followed by degradation during the further vegetation period.

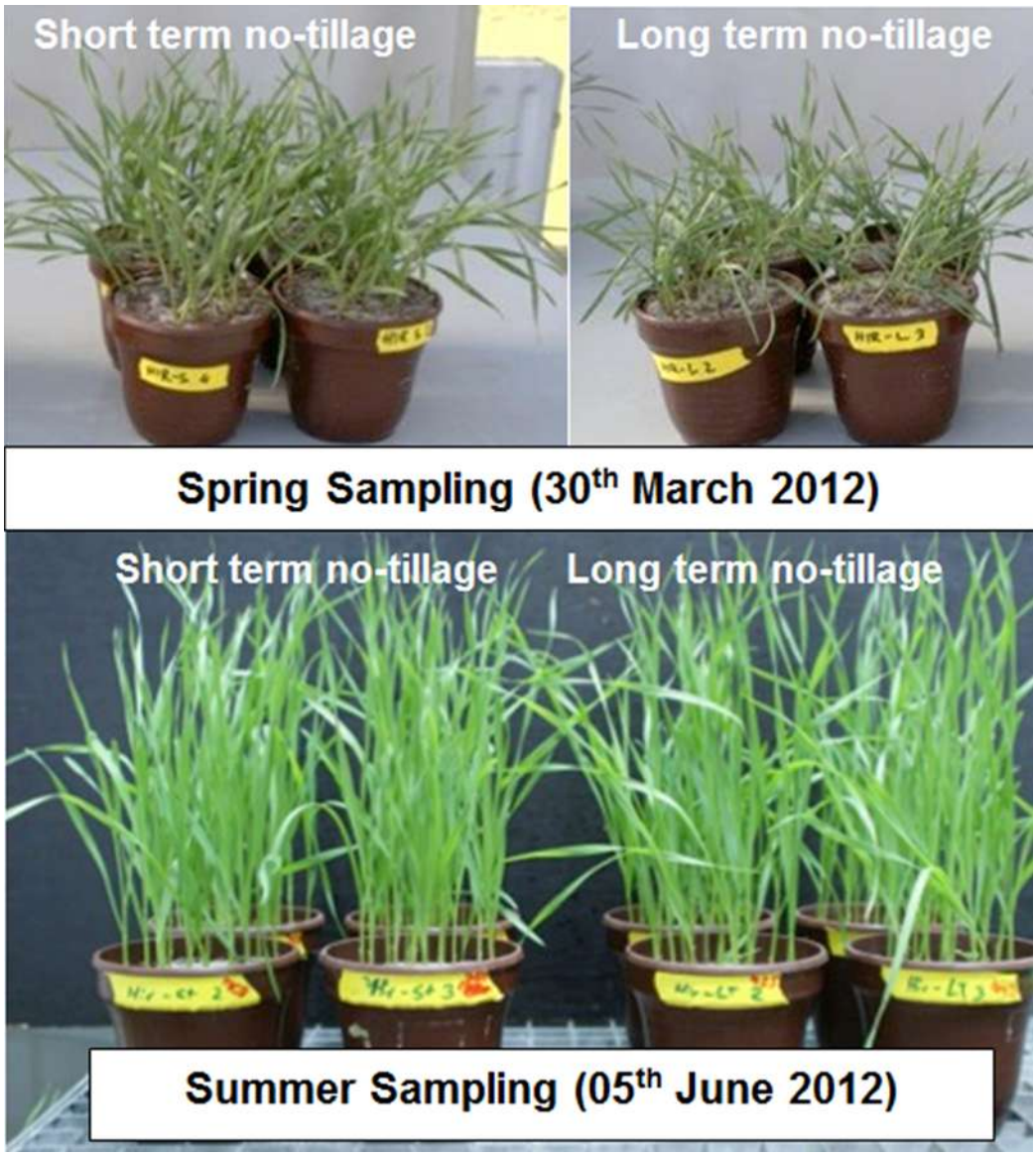

Figure 28: Changes in shoot growth of winter wheat (cv. Isengrain) grown on ST and LT no-tillage soils (field site HirG2012) collected in early spring and early summer.

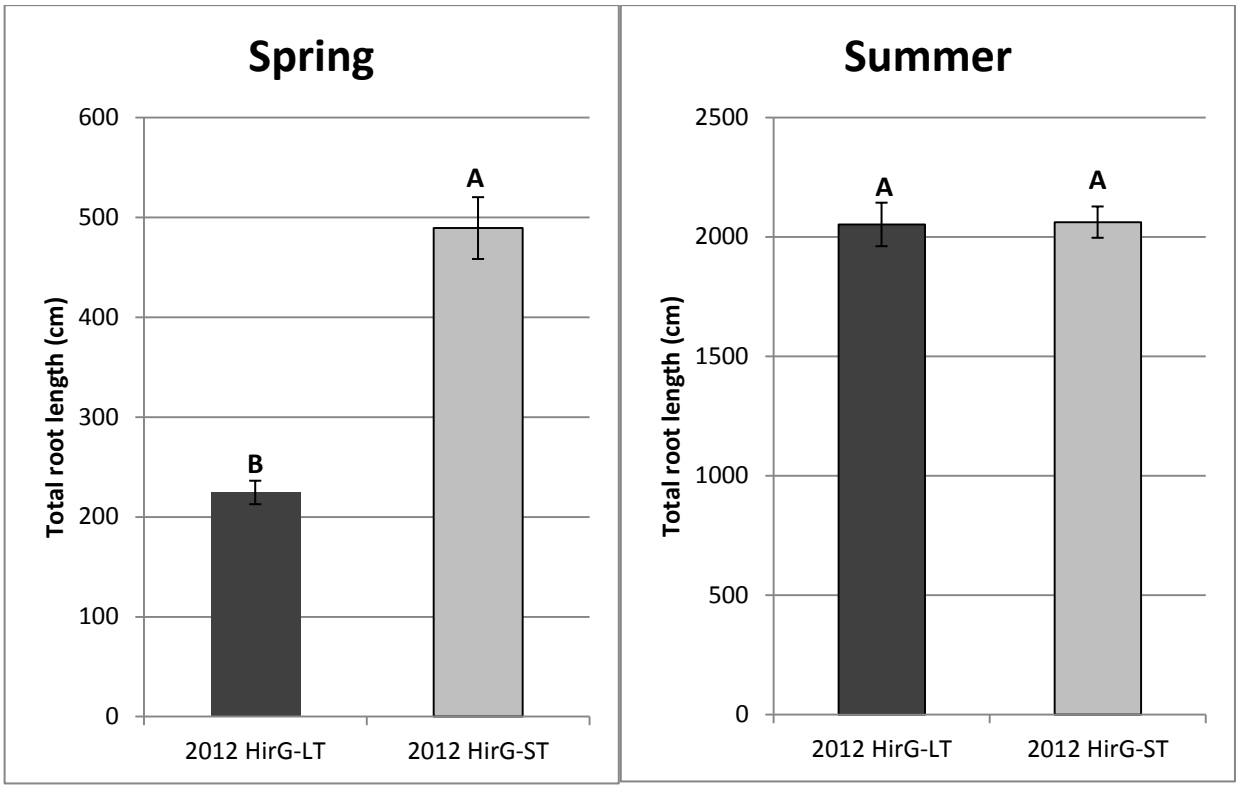

Figure 29: Changes in root length of winter wheat (cv. Isengrain) grown on ST and LT no-tillage soils (field site HirG2012) collected in early spring and early summer. Values are means of 4 replicates ± SE. Means with different letters are significantly different (t-test, α = 0.05).

3.5 Herbicide residues as potential causes for plant damage on long-term no-tillage soils

Some herbicides can show persistent soil activities over months and even years, providing an efficient long-term weed control but also bearing a risk of damaging sensitive subsequently grown crops. This is usually considered by specific waiting time recommendations for replanting, but the degradation speed of herbicide residues is influenced by many factors including soil properties and climatic conditions (Rueppel *et al*., 1977). Residual effects have been documented for different groups of herbicides including sulfonylureas, dinitroanilines, propyzamides and others (Hang *et al*., 2012; Agriculture Victoria, 2013) also used in the no-tillage cropping systems investigated in the present study. Therefore, residual levels of commonly used herbicides in soil pairs with short-term and long-term no-tillage history were determined to identify potential relationships with the observed crop damage symptoms.

3.5.1 Herbicide soil concentrations

Glyphosate was the only herbicide regularly used in all investigated plots, while sulfonylureas, pendimethalin, and propyzamide were applied more occasionally in various years and field sites.

*Table 13: Herbicide residues detected in soil samples from the field site HirF with short-term and long-term no-tillage history and corresponding symptoms of plant damage in the field and in pot experiments. Each soil sample was a pooled combination of 12 sub-samples. *Below detection limit.*

Herbicide in soil [mg kg^{-1}]	**2 years no-tillage**	**10 years no-tillage** (Moderate damage)	**10 years no-tillage** (Heavily damaged)
Glyphosate	< 0.05*	2.6	2.9-4.0
AMPA	0.2	1.6	1.2
Pendimethalin	0.1	0.2	0.8

Field site			
Pot culture			

Table 13 shows residual concentrations of frequently used herbicides on plots with long-term and short-term no-tillage history at the field site HirF and the respective symptoms of plant damage. For all investigated residues, higher levels were detected on long-term no-tillage plots, and the degree of plant damage was positively correlated with the soil

concentration of the herbicide residues, with the highest levels measured for glyphosate and its metabolite AMPA. However, due to rapid and intense immobilization of glyphosate in soils, the residues are generally regarded as non-phytotoxic (Gimsing *et al.*, 2004). To evaluate a potentially plant-available glyphosate fraction in the long-term no-tillage soil, water extraction was performed (100 g air- dried soil L^{-1}) with a soil sample pooled from 12 topsoil samplings at a depth of 15 cm, followed by filtration, centrifugation and vacuum -concentration. Assuming a soil moisture level of 20 %, the concentration of water-soluble and therefore, potentially plant-available residues re-calculated for the soil solution, comprised approximately 3 µg L^{-1} for glyphosate and 1.5 µg L^{-1} for AMPA, representing 0.023 % of the total soil residues.

To achieve a more comprehensive overview, an additional herbicide residue analysis was performed on six field sites with closely neighbored short-term and long-term no-tillage plots, including also samplings at different time points of the vegetation period (Table 14, Table 15). Glyphosate residues were detected on all investigated field sites with a consistent pattern of higher levels on plots with long-term no-tillage history as compared with the corresponding short-term no-tillage plots (Table 14). This may indicate a lower degradation potential for glyphosate and AMPA on LT no-tillage soils. Spring samplings showed higher glyphosate and AMPA concentrations than summer samplings (Table 14). This is in line with the decline of plant damage symptoms in summer samplings on soil collected from LT no-tillage plots as compared with soil samplings performed in early spring (Figure 28, Figure 29).

In contrast to Glyphosate and AMPA, the residues of Pendimethalin and Propyzamide did not show a consistent pattern related to the history of no-tillage management (Table 15).

Table 14: Glyphosate and AMPA residues detected in soils collected from different field sites with long-term and short-term no-tillage history.

Field site/year	**Glyphosate [mg kg^{-1}]**		**AMPA [mg kg^{-1}]**	
Hirrlingen	**(Roundup®)**		**(Roundup®)**	
	LT	**ST**	**LT**	**ST**
Steinbruch /09[1]	0.130	n.d.	0.506	0.298
Eichenberg /09[1]	n.d.	n.d.	0.402	0.152
Friedhof / 09[1]	0.094	n.d.	0.363	0.055
Friedhof / 10[2]	2.630	n.d.	1.620	0.160
Grassäcker /12[3]	0.034	n.d.	0.211	0.092
Schwarze Länder/12[3]	n.d.	n.d.	0.123	0.071

[1]Summer sampling, [2]Spring Sampling, [3]Reduced glyphosate dose, n.d. below the detection limit

Table 15: Pendimethalin and Propyzamide residues detected in soils collected from different field sites with long-term and short-term no-tillage history.

Field site/year	**Pendimethalin [mg kg^{-1}]**		**Propyzamide [mg kg^{-1}]**	
Hirrlingen	**(Product: Stomp)**		**(Product: Kerb)**	
	LT	**ST**	**LT**	**ST**
Steinbruch /09[1]	0.173	0.090	n.d.	n.d.
Eichenberg /09[1]	0.064	0.164	n.d.	n.d.
Friedhof / 09[1]	0.120	0.055	n.d.	n.d.
Grassäcker /12[2]	n.d.	n.d.	0.008	0.012
Schwarze Länder/12[2]	n.d.	n.d.	0.006	0.003

[1]Summer sampling, [2]Spring Sampling, [3]Reduced glyphosate dose, n.d. below the detection limit

3.5.2 Soil microbial activity

Microbial metabolization is the most important process for determining herbicide persistence in soils (Souza *et al.*, 1999) and is also regarded as the primary route of

glyphosate turnover (Tu *et al*., 2001). In the case of glyphosate, AMPA is produced as an intermediate product of microbial degradation, which is further metabolized to water, CO_2 and phosphate (Forlani *et al.*, 1999). Accordingly, the degradation rate of glyphosate is correlated with the rate of soil respiration (Franz *et al*., 1997). Therefore, higher concentrations of glyphosate residues on long-term no-tillage soils (Table 16) may be a consequence of lower soil microbial activity. To test this hypothesis, soil respiration was measured in soil samples collected from five different field sites characterized by closely neighbored plots with long-term and short-term no-tillage history. In four out of five tested soil samples, soil respiration was lower in LT than ST no-tillage plots, supporting the assumption that soil-microbial activity and thus glyphosate degradation was reduced by long-term no-tillage management.

Table 16: Soil respiration on five field sites with LT and ST no-tillage history. Measurements were performed in 12 pooled topsoil subsamples per plot. Values are means of 4 technical replicates.

Field Site	**LT** **Soil Respiration** (µg CO_2 g $Soil^{-1}$)	**ST** **Soil Respiration** (µg CO_2 g $Soil^{-1}$)
Gassäcker	7.54	5.62
Schwarze Länder	12.66	17.87
Beim Wald	8.34	21.37
Mittlere Weiherfichte	8.47	10.92
Sportplatz	8.09	9.85

3.5.3 Glyphosate residues in LT no-tillage soils as potential cause for plant damage

Based on the results obtained so far, delayed microbial degradation of glyphosate residues in LT no-tillage soils, reaching phytotoxic concentrations even in the water-soluble fraction, could be a major factor for induction of damage in plants exposed for longer

periods (autumn-spring) to these conditions. If this hypothesis holds true, and glyphosate toxicity is primarily responsible for plant damage on LT no-tillage soils, genetically modified, glyphosate-resistant (RR) plants should not be affected under these conditions.

3.5.3.1 Growth of glyphosate-resistant and non-resistant soybean on long-term no-tillage soils

To test a potential contribution of glyphosate toxicity to plant damage induced by LT no-tillage management, glyphosate-resistant (Roundup Ready®; RR) soybean (*Glycine max* cv. BSR Valiosa RR) and its parental, near isogenic, non-resistant (NR) line (*Glycine max* L. cv. BR16 Conquista) (Bott *et al*., 2008) were cultivated in a pot experiment on LT and ST no-tillage soils collected from the field site REM2011. All test plants grown on LT no-tillage soil showed the typical damage symptoms, comprising stunted shoot growth (Figure 30), reduced fine root production and root thickening (Table 17) on the soil collected from LT no-tillage plots. There were no significant differences between RR and NR soybean cultivars, suggesting that inhibition of the shikimate pathway as the primary cause for glyphosate toxicity (Duke and Hoagland, 1985; Panettieri *et al*., 2013) was not responsible for plant damage on LT no-tillage soils.

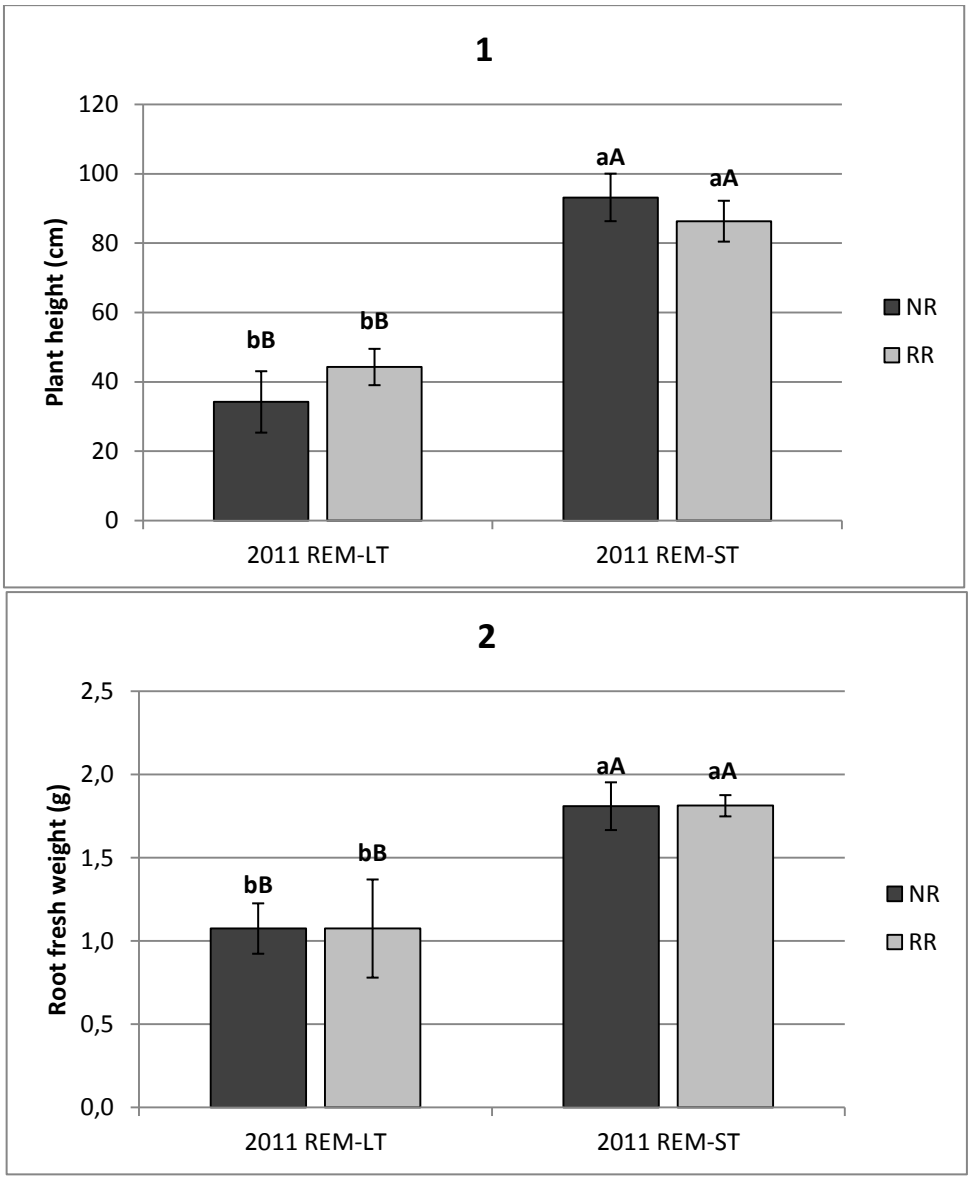

Figure 30: ***(1)*** *Plant height and* ***(2)*** *root fresh weight of glyphosate-resistant (*RR: *Glycine max* cv. BSR Valiosa*) and non-resistant (*NR: *Glycine max* L. cv. BR16 Conquista*) soybean after 4 weeks of growth on LT and ST no-tillage soil collected from the field site REM2011. Values are means of four replicates ± SE. Mean values with different letters are indicating significant differences where small letters compare soybean cultivars and big letters compare tillage duration. Two-way ANOVA ($p<0.05$) followed by Tukey's test ($\alpha = 5\%$).*

Table 17: Effect of tillage duration and variety on root growth characteristics of 24-days old soybean plants grown in soil from the field site REM2011. Values are means of four replicates ± SE. Different letters (A, B) indicate significant difference between treatments (Two-way ANOVA (p<0.05) followed by Tukey's test (α = 5%), P values are in bold italic. LT is long-term tillage, ST is long-term tillage, RR is Glyphosate resistant soybean cultivar (Glycine max cv. BSR Valiosa*), NR is Glyphosate conventional soybean (Glycine max L.* cv. BR16 Conquista*) variety, NS = not significant.*

Treatment	**Average root diameter** (mm)	**Total root length distribution in different diameter classes**			
		0.0-0.2 mm	**0.2-0.4 mm**	**0.4-0.6 mm**	**>0.6 mm**
		L S means tillage × cultivar			
LT NR	0.633	2.918	23.829	46.613	26.635
LT R	0.634	1.812	26.24	47.525	24.319
ST NR	0.439	21.05	42.928	21.366	14.656
ST R	0.452	19.504	44.174	21.053	15.450
SE	0.0164	1.026	2.807	2.109	2.038
Tillage Duration	***<0.001***	***<0.001***	***<0.001***[#]	***<0.001***	***<0.001***
LT	0.634 (A)	2.365 (B)	25.035 (B)	47.069 (A)	25.477 (A)
ST	0.446 (B)	20.277 (A)	43.551 (A)	21.210 (B)	15.053 (B)
Soybean Variety	***0.650***	***0.220***	***0.913***[#]	***0.889***	***0.715***
RR	0.543 (NS)	10.658 (NS)	35.207 (NS)	34.289 (NS)	19.885 (NS)
NR	0.536 (NS)	11.984 (NS)	33.378 (NS)	33.990 (NS)	20.646 (NS)
Tillage×Variety	***0.704***	***0.834***	***0.748***[#]	***0.777***	***0.460***

[#]Two-way ANOVA is performed on transformed data (square root transformation).

3.5.3.2 Shikimate accumulation

Glyphosate expresses herbicidal activity through inhibition of the shikimate pathway. This blockage of this metabolic pathway leads to increased intracellular accumulation of shikimate as a physiological indicator of glyphosate toxicity (Reddy *et al*., 2010). Therefore, shikimate concentrations were determined in the root tissue of winter wheat plants grown on LT and ST no-tillage soils collected from the field site REM2011 (see 3.2.3). Although plants grown on LT no-tillage soil showed typical damage symptoms

with stunted root growth and less fine root production (see 3.2.3.3) root concentrations of shikimate were even lower than in undamaged plants grown on soil with short-term no-tillage history (Figure 31).

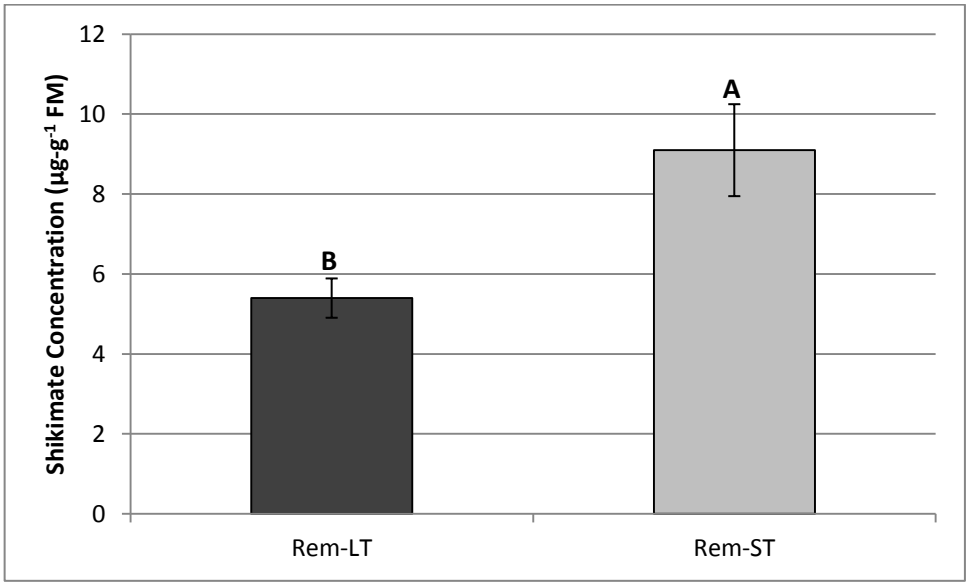

Figure 31: Shikimate concentrations detected in root tissue of winter wheat (cv. Isengrain) after 19 days of pot culture on LT and ST no-tillage soil. Values are means of 4 replicates ± SE. Means with different letters are indicating significant differences at α = 0.05% (t-test).

Similar to the induction of damage symptoms in glyphosate-resistant soybean plants grown on long-term no-tillage soils, the absence of shikimate accumulation in damaged winter wheat plants demonstrates that there is no relationship with glyphosate-induced inhibition of the shikimate pathway.

3.5.4 Dissection of phytotoxic effects induced by glyphosate and its degradation products in a soil-free system

Apart from glyphosate, also AMPA as a major degradation product in soils has a certain phytotoxic potential (Reddy *et al*., 2004) and synergistic effects of both compounds may also occur. Glyphosate and AMPA were not only detected as bound residues at the soil matrix but even in the water-soluble. Therefore, potentially plant-available phase in soil with long-term no-tillage history, still detectable even in spring samplings conducted six months after glyphosate application (see 3.5.1). Although only detectable in trace

concentrations (glyphosate approx.3 µg L^{-1}; AMPA approx. 1.5 µg L^{-1}), the damaged winter wheat plants sampled in spring have obviously been exposed to these water-soluble residues for at least six months, and even higher concentrations can be expected shortly after herbicide application in autumn.

3.5.4.1 Shoot growth of winter wheat in hydroponic culture

To investigate the effects of long-term plant exposure to trace concentrations of glyphosate residues detected in the water-soluble fraction of long-term no-tillage soil in the absence of any other potentially toxic compound, experiments were conducted with winter wheat in hydroponic culture. Glyphosate, AMPA, and a combination of both were applied to the growth medium in concentrations of 3 and 5 µg L^{-1} for glyphosate and 1.5 and 3 µg L^{-1} for AMPA. Growth media with herbicide amendments were replaced at intervals of 1-2 days over a growth period 4 and 6 weeks. To compensate for rapid inactivation of the herbicides by cation complexation in mineral nutrient solutions (Duke, 1988), nutrient solution was applied only once at intervals of three days and water was used as growth medium for the remaining time.

Already after three weeks exposure to trace concentrations of glyphosate and AMPA, visible chlorosis symptoms appeared in all herbicide-treated variants, comparable with leaf chlorosis also observed in early spring in winter wheat plants grown on soils with long-term no-tillage history (Figure 32). This was also confirmed in a second experiment by repeated measurements of SPAD values over a culture period of six weeks (Figure 33). However, shoot biomass production, plant height or leaf numbers were not affected at this stage (data not shown).

Figure 32: Winter wheat exposed to trace concentrations of Glyphosate (5 µg L^{-1}) and AMPA (3 µg L^{-1}) in hydroponics show chlorosis symptoms similar to plants grown under field conditions on the long-term no-tillage soil.

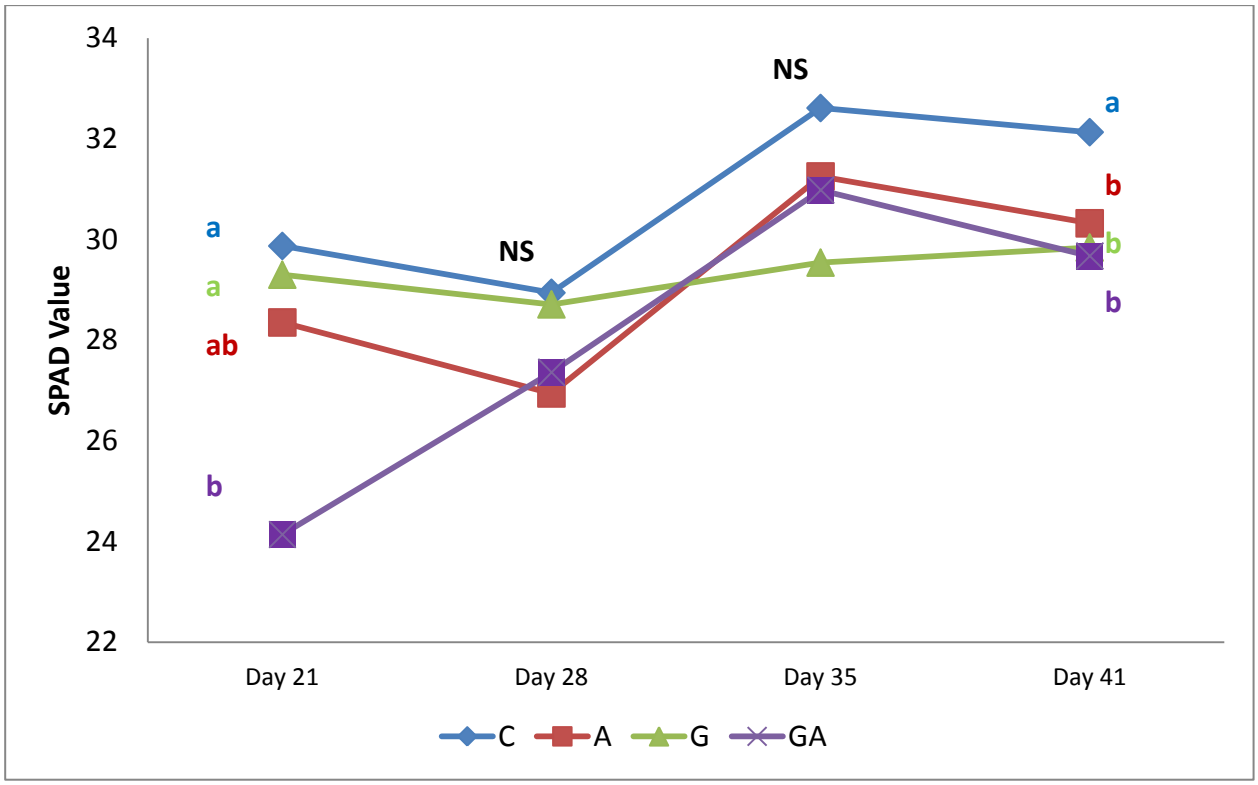

Figure 33: SPAD values of winter wheat (cv. Isengrain) measured during six weeks of growth in hydroponic culture. Untreated control (C), AMPA 3 µg L^{-1} (A), glyphosate 5 µg L^{-1} (G) and the combination of glyphosate and AMPA (GA). Values are means of 4 replicates per treatment. Means values with different letters are indicating significant differences (Tukey's test, α = 0.05). NS = not significant.

3.5.4.2 Root growth of winter wheat in hydroponic culture

Similar to winter wheat plants grown on LT no-tillage soils, also root growth was impaired in hydroponics by exposure to the glyphosate and AMPA trace concentrations detected in the soil solution. Reduced fine root development (Figure 34) was recorded after four weeks culture period in response to treatments with glyphosate (3 µg L^{-1}) and AMPA (1.5 µg L^{-1}).

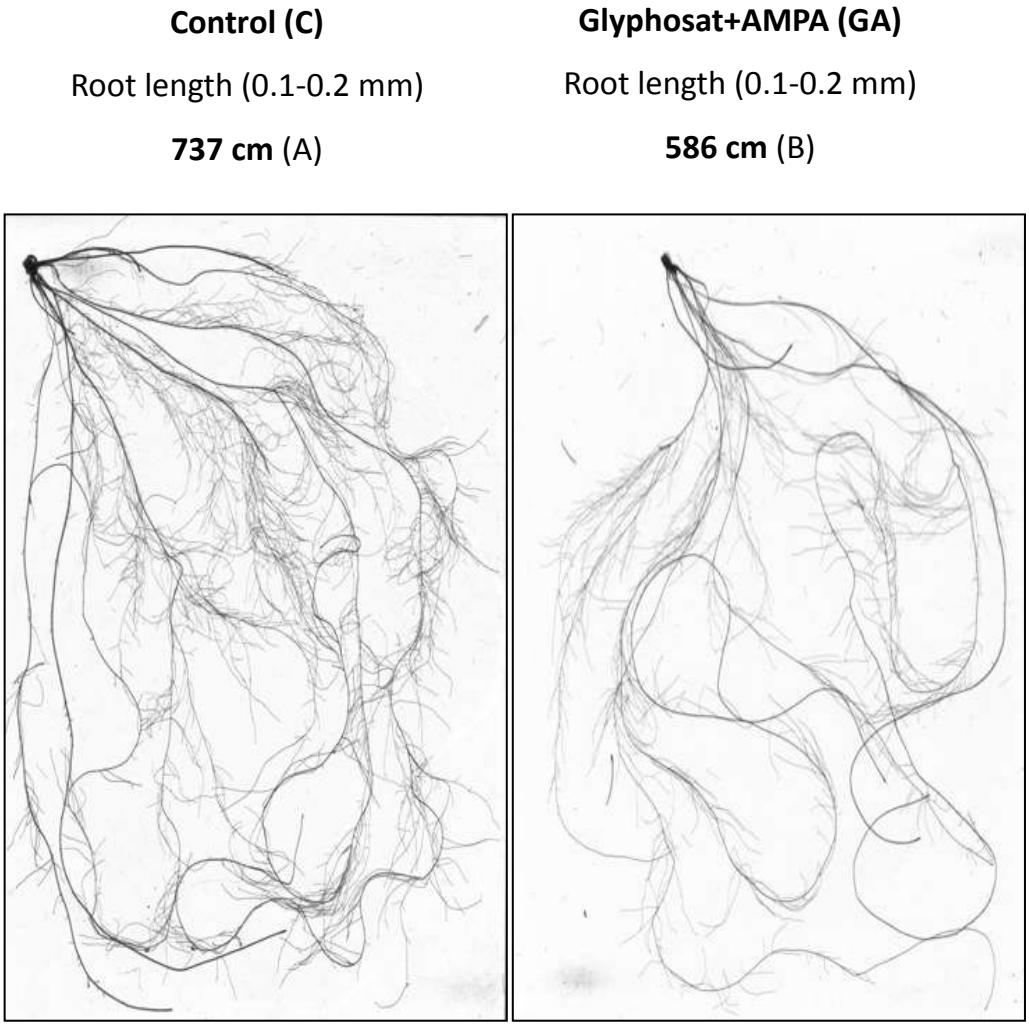

Figure 34: Root morphology and fine-root length of winter wheat (cv. Isengrain) grown for 4 weeks in hydroponic culture with and without amendments of glyphosate 3µgL^{-1} and AMPA 1.5µgL^{-1}. Values are means of 4 replicates per treatment. Means values with different letters are indicating significant differences (Tukey's test, α = 0.05).

This effect was even more expressed in the second experiment with a culture period of six weeks, and glyphosate and AMPA applications at concentrations of 5 and 3 µg L^{-1}, respectively (Figure 35).

Again, mainly fine root production was affected, but surprisingly the inhibitory effect was restricted mainly to the AMPA and glyphosate+AMPA treatments, while no significant root growth inhibition was observed after glyphosate application alone (Figure 35).

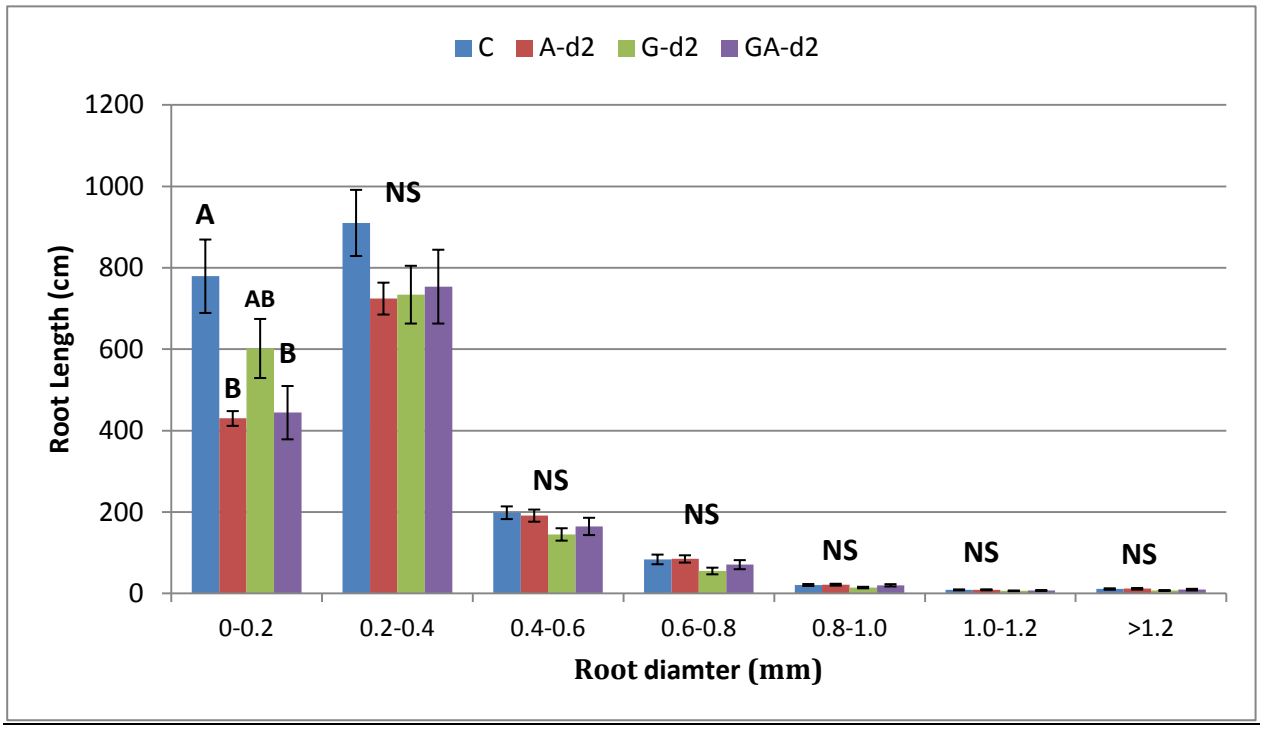

Figure 35: In seven-diameter classes root length of winter wheat (cv. Isengrain) grown in hydroponic culture after 6 weeks with d2 (AMPA 3µgL^{-1}, glyphosate 5µgL^{-1} and glyphosate+AMPA). Values are means ± SE of 4 replicates per treatment. Means values with different letters are indicating significant differences (Tukey's test, α = 0.05). NS = not significant.

3.5.4.3 Shikimate accumulation in the root tissue

In accordance with the observations in plants cultivated on soils with short-term and long-term no-tillage management (Figure 31), there was no indication for increased shikimate accumulation in the root tissue as a physiological indicator of glyphosate toxicity in the variants treated with glyphosate and glyphosate+AMPA (Table 18). Since root damage was observed particularly in the presence of AMPA, and the phytotoxic potential of AMPA is not associated with inhibition of the shikimate pathway and accumulation of shikimate (Reddy *et al*., 2004; Duke, 2011). These findings suggest that plant damage observed on long-term no-tillage soils is mainly a consequence of a phytotoxic AMPA effect rather than glyphosate toxicity. This effect is expressed after long-term plant exposure to the residues of glyphosate degradation and promoted by the delayed microbial turnover of glyphosate and AMPA in these soils (see 3.5.1, 3.5.2).

Table 18: Shikimate accumulation in the root tissue of winter wheat (cv. Isengrain) grown in hydroponic culture after 4 weeks with amendments of glyphosate (3µg L-1), AMPA (1.5µg L-1), glyphosate+AMPA (3+1.5 µgL-1) and an untreated control. Values are means ± SE of 4 replicates per treatment. NS = not significant (Tukey's test, α = 0.05).

Treatment	Shikimate Concentration
Control	2.4 ± 0.6 (NS)
Glyphosate	2.8 ± 0.3 (NS)
AMPA	2.4 ± 0.4 (NS)
Glyphosate+AMPA	2.4 ± 0.3 (NS)

3.5.4.4 Root vitality status

Impaired root growth of winter wheat exposed to AMPA toxicity in hydroponic culture was also associated with reduced metabolic activity, indicated by vital staining with triphenyltetrazolium chloride, which is converted to a red formazan by the activity of dehydrogenases in plant tissues, reflecting the activity status of the metabolism (Stūrīte *et al*., 2010). In accordance with the damage symptoms mainly affecting the growth of the fine lateral roots, particularly the zone of lateral root formation (6-8 cm behind the root tip) was affected by glyphosate+AMPA treatments, resulting in less color development (Figure 36 upper row). In undamaged roots, the central cylinder with the pericycle as the origin of lateral root formation showed the most intensive staining, which disappeared almost completly in the roots damaged by the long-term glyphosate+AMPA treatment (Figure 36 lower row).

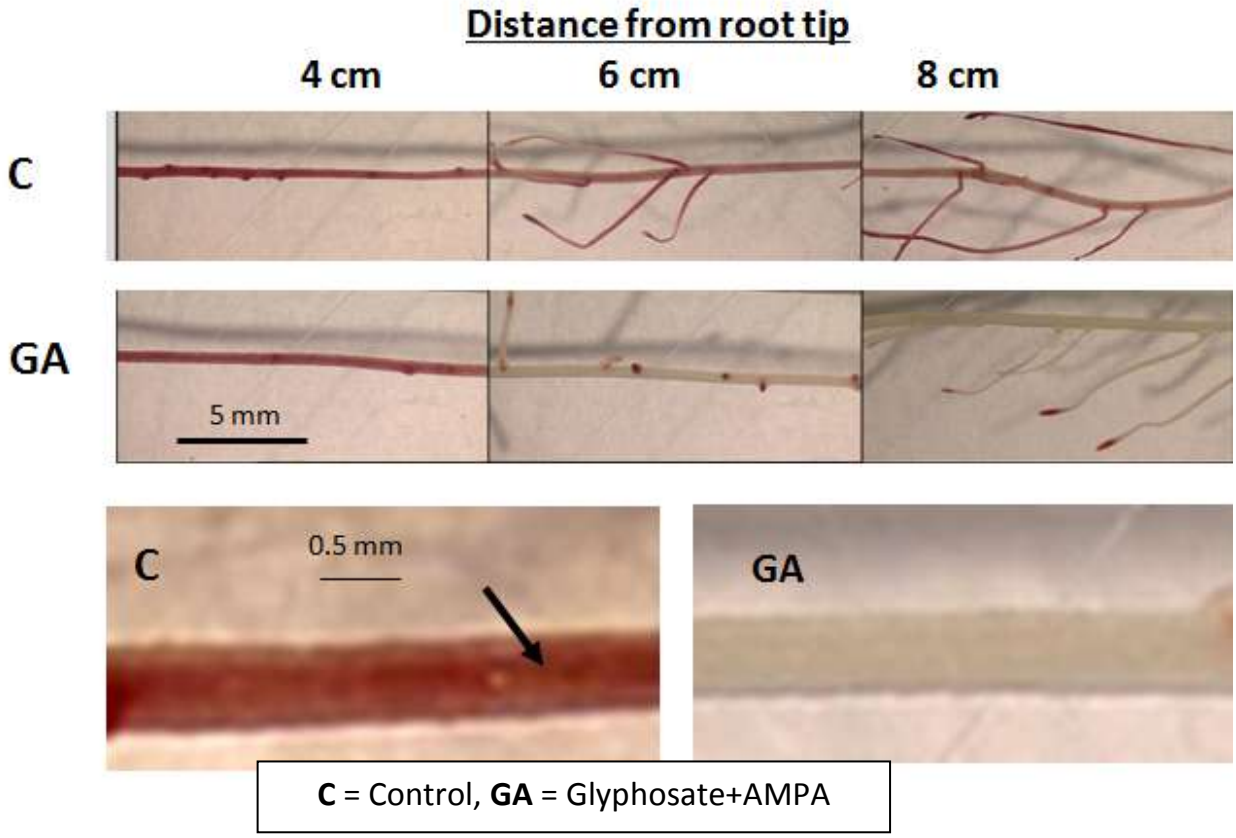

Figure 36: Triphenyltetrazoliumchloride (TTC) vital staining of apical and subapical root zones in seminal roots of winter wheat (cv. Isengrain) grown in hydroponic culture for 39 days with and without amendments of glyphosate 5µgL^{-1} and AMPA 3µgL^{-1}.

3.5.4.5 Root transcriptome analysis of winter wheat exposed to trace concentrations of glyphosate and AMPA in hydroponic culture

In contrast to glyphosate, much less is known about the mechanisms determining phyto-toxicity of AMPA (Duke, 2011; Gomes *et al*., 2014) and also the effects of long-term exposure to sub-toxic trace concentrations and synergisms between residues of different herbicides are poorly understood (Serra *et al*., 2013).

To collect more information on metabolic pathways and reactions affected by long-term exposure of wheat roots to trace concentrations of AMPA and glyphosate, a RNAseq transcriptome analysis was conducted with wheat (*Triticum aestivum*, cv. Isengrain) plants exposed to glyphosate (5µg L^{-1}); AMPA (3µg L^{-1}), and a mixture of glyphosate+AMPA in a hydroponic culture system (see 3.5.4.1). Three complementary DNA (cDNA) libraries were constructed from mRNA isolated from root systems of wheat

plants exposed to the AMPA, glyphosate, and glyphosate+AMPA treatments, respectively. Harvest was performed at 19 DAS, just prior to the appearance of visual damage symptoms to minimize the risk of analyzing secondary effects, triggered, e.g., by the AMPA-induced impairment of root growth (see 3.5.4.2) and root activity (see 3.5.4.4). After Illumina® sequencing, transcription profiles were compared to those of untreated control plants. The processed data (2.94 Gb in A, 1.31 Gb in G, 1.3 Gb in GA and 1.64 Gb in control) (see 2.9.3) was distributed into functional metabolic categories (bins), according to Mapman (Usadel *et al.*, 2009). Treatment-specific changes in bins were documented with the Pageman module of Mapman. The largest numbers of changes relative to the untreated control were recorded for the AMPA (total 160 bins) and the glyphosate+AMPA (total 130 bins) treatments but only 68 bins in the glyphosate variant with 78 up-regulations and 82 down-regulations for AMPA, 44 up-regulations and 86 down-regulations for glyphosate+AMPA and 49 up-regulations versus 19 down-regulations for glyphosate (Figure 37).

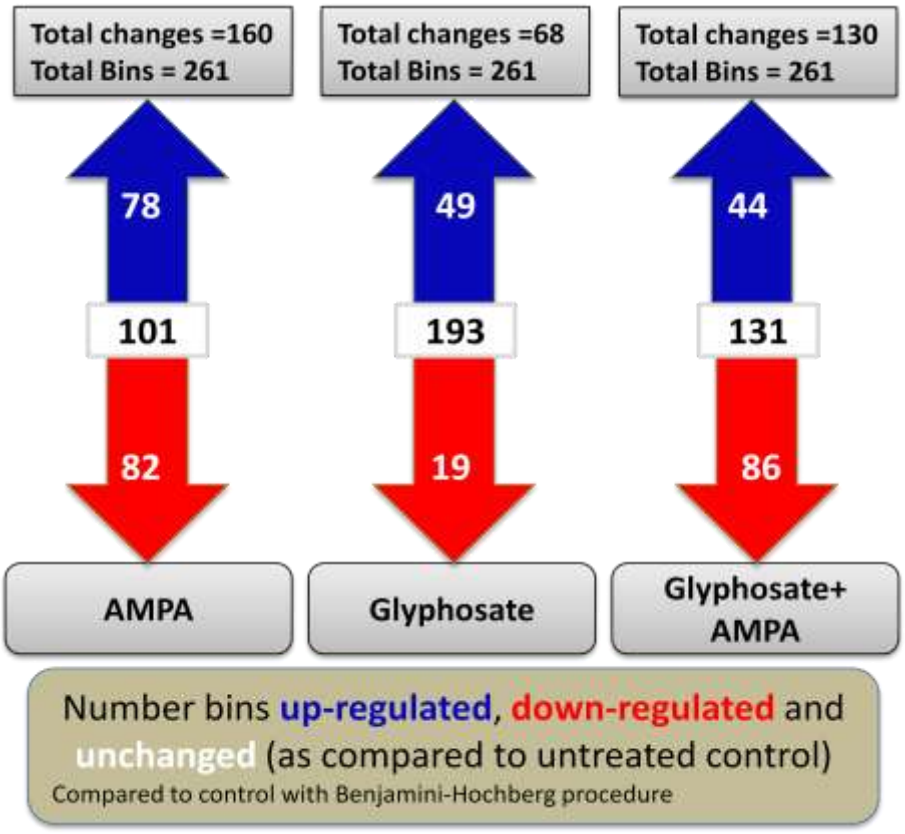

Figure 37: A Quantitative overview of transcriptional changes in gene expression in roots of winter wheat (cv. Isengrain) exposed for 19 days to trace concentrations of AMPA (3 µg L^{-1}), glyphosate (5 µg L^{-1}), and glyphosate+AMPA in a hydroponic culture system.

Since inhibitory effects on root growth were mainly restricted to the AMPA and glyphosate+AMPA treatments (Figure 35), particular emphasis was placed on transcriptional modifications simultaneously expressed in both treatments but not detectable in the glyphosate treatment. Interesting changes potentially related to root development and stress responses comprised alterations in hormone metabolism; up-regulation of cytokinin related genes, down-regulation of ethylene- and jasmonate-associated gene expression (Figure 38). Also expression of genes, related to stress responses and aquaporins, particularly plasma-membrane intrinsic proteins (PIPs) with essential functions in water transport and emergence and elongation of lateral roots (Péret *et al*., 2012), was down-regulated in the roots AMPA and glyphosate+AMPA treated plants. The same holds true for the synthesis of aromatic amino acids, simple phenolics and lignin, nitrilases, ß1-3 glucan hydrolases, peptide transport, and metal binding; while strong up-regulation was observed in genes involved in ribosome biogenesis. A complete overview on alterations in gene expression is given in the appendix.

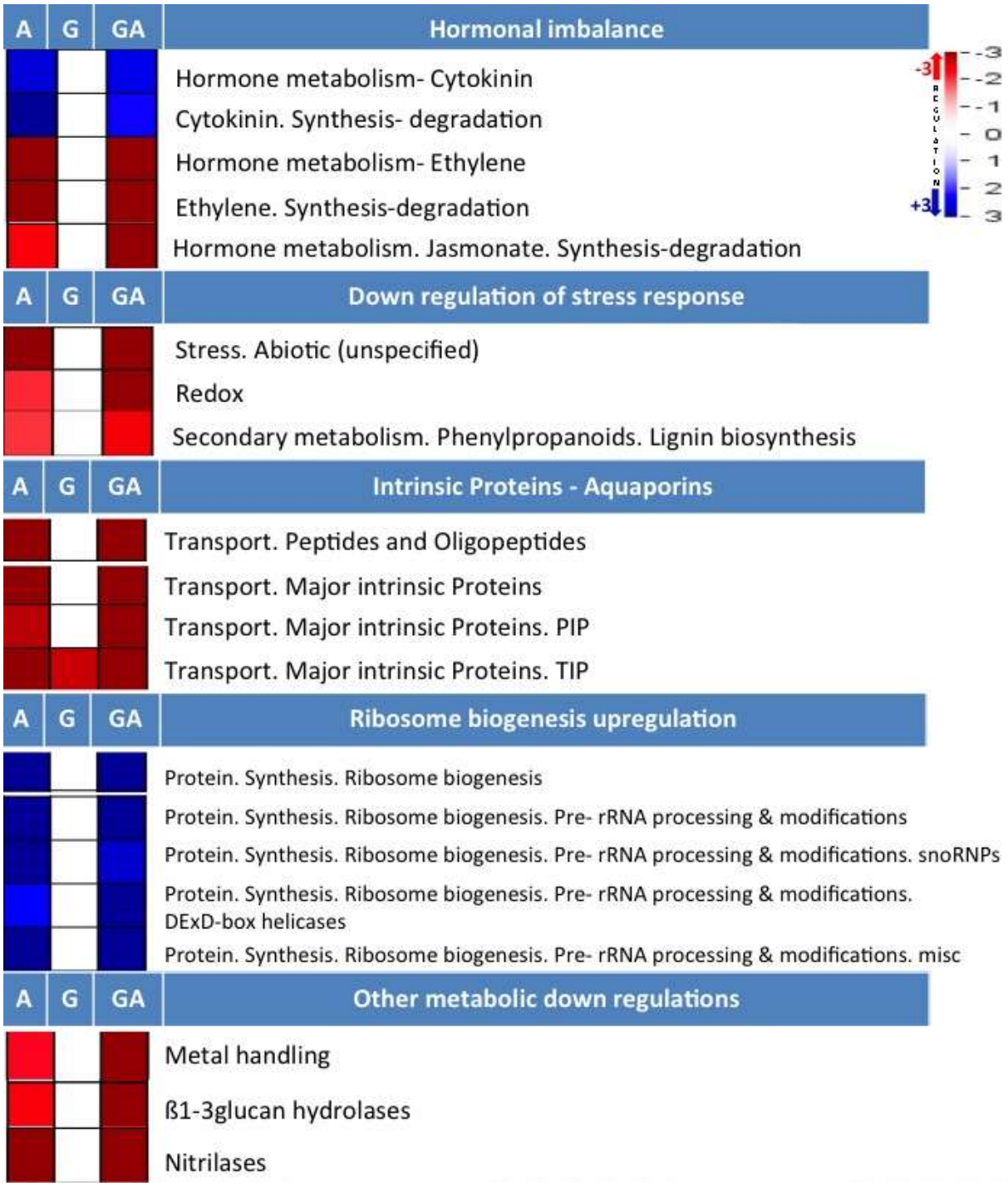

Figure 38: Overview on up-regulation (blue) and down-regulation (red) of gene expression in winter wheat (cv. Isengrain) roots with herbicide treatments inducing root growth inhibition (AMPA, glyphosate+AMPA).

3.6 Remediation Strategies

The characterization of factors, determining the observed plant growth suppression on LT no-tillage soils (see 3.2) may offer a perspective to identify adapted strategies for remediation or at least mitigation of damage symptoms.

3.6.1 Application of microbial bio-effectors

Since delayed microbial degradation of plant-available glyphosate soil residues in spring has been identified as the most likely critical factor for plant damage on the investigated long-term no-tillage field sites, plant co-inoculation with glyphosate-degrading microorganisms may offer a protective strategy to promote degradation of glyphosate residues in the rhizosphere (Kryuchkova *et al*., 2014). For many soil microorganisms including bacteria and fungi, degradation potential for glyphosate is well documented. The most widespread commercial microbial plant-inoculants are members of the bacterial genera *Pseudomonas*, *Bacillus*, *Rhizobium* and the fungal genera *Trichoderma* and *Penicillium*, mainly sold as biocontrol agents and plant growth-promoting microorganisms (Calvo *et al*., 2014). However, also glyphosate degradation has been reported as a widespread feature in these microbial groups (Jacob *et al*., 1988; Arfarita *et al*., 2013). Moreover, impairment of root growth has been identified as one of the major restrictions on the growth of plants exposed to herbicide residues on LT no-tillage soils (Figure 19, Figure 20) and stimulation of root growth is a major mode of action in microbial plant-growth promotion (Calvo *et al*., 2014). Therefore, the potential of selected commercially available microbial bio-effectors to mitigate symptoms of plant damage in winter wheat, cultivated on LT no-tillage soils was investigated in pot and field experiments. Table 19 summarizes the investigated commercial bio-effectors, their active microbial strains and the expected effects on plant growth according to the specifications of the manufacturers.

Table 19: Tested bio-effector products with their active biological agents and expected activities.

Bio-effector Product	Producer	Type	Expected Activity
PRORADIX® *Pseudomonas sp.* DMSZ 13134	Sourcon-padena GmbH (Tübingen, Germany)	Bacteria (Gram -)	- Stimulates root growth - Supports mycorrhiza - Pathogen suppression
TRICHOSTAR® *Trichoderma harzianum*	GERLACH Natürliche Düngemittel GmbH (Hannover, Germany)	Fungi	- Nutrient mobilization - Growth stimulation - Pathogen suppression
RHIZOVITAL 42® *Bacillus amyloliquefaciens FZB42*	ABiTEP GmbH (Berlin, Germany)	Bacteria (Gram+)	- Nutrient mobilization - Root growth stimulation - Pathogen suppression - Supports mycorrhiza

3.6.1.1 Starter application of microbial bio-effectors (pot experiment)

The respective microbial bio-effectors were applied by fertigation as starter applications before sowing in the dosage recommended by the manufacturer to winter wheat (*Triticum aestivum* cv. Isengrain) cultivated in pots (see 2.6.2) on LT and ST soil samples collected from Hirrlingen Friedhof (Table 6).

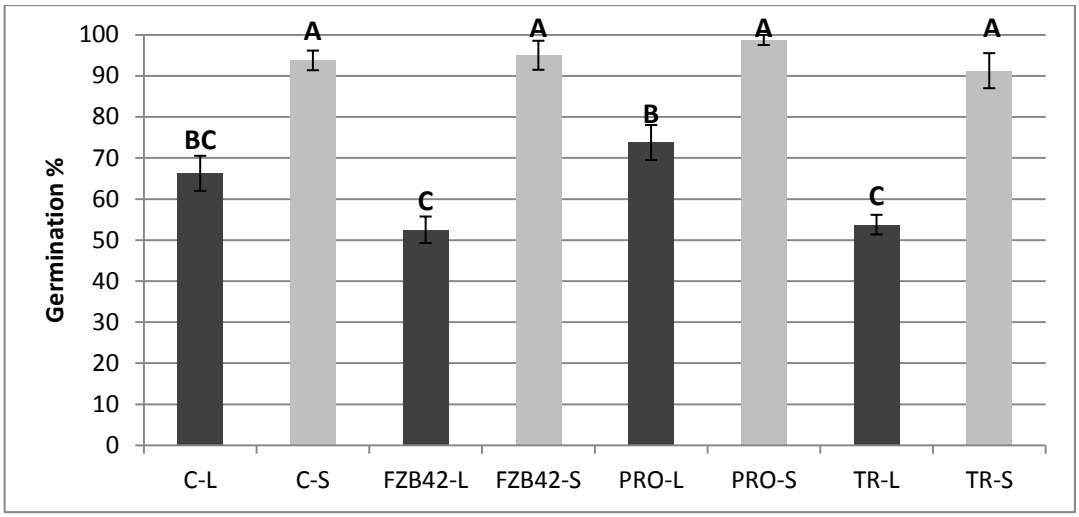

Figure 39: Germination of winter wheat (cv. Isengrain) at 2 weeks after sowing on short-term (ST) and long-term (LT) no-tillage soil collected from the field site "Hirrlingen Friedhof" with and without (C) application of microbial bio-effectors: Rhizovital 42® (FZB), Proradix® (PRO) and Trichostar® (TR). Values are means ± SE of 4 replicates per treatment. Means values with different letters are indicating significant differences (Tukey's test, α = 0.05).

Germination of winter wheat was significantly reduced on LT no-tillage soil. No mitigation effect was induced by application of the various *Bacillus*-, *Pseudomonas*-, and *Trichoderma*-based microbial bio-effectors.

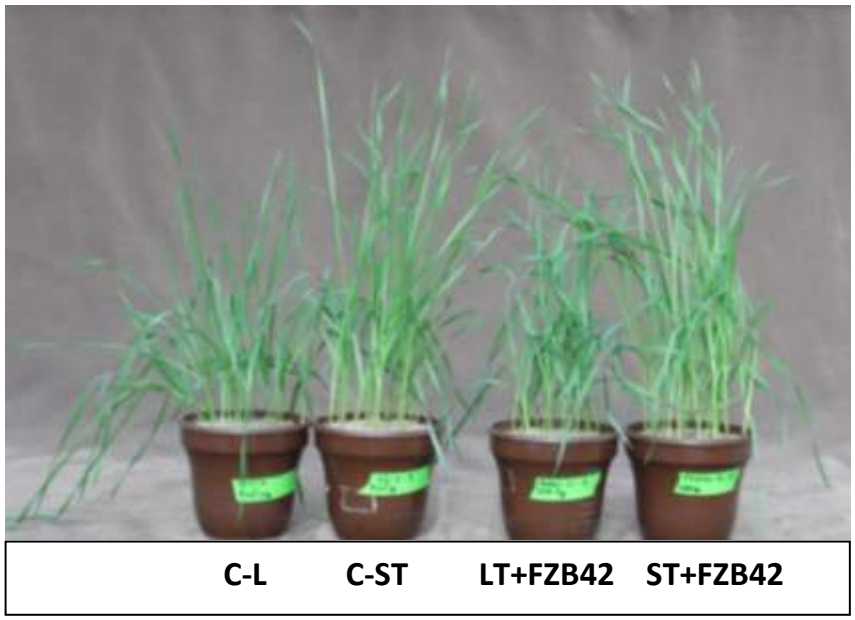

Figure 40: Habitus of winter wheat (cv. Isengrain) at 4 weeks after sowing on short-term (ST) and long-term (LT) no-tillage soil collected from the field site "Hirrlingen Friedhof" with and without (C) application of the microbial bio-effector Rhizovital 42® (Bacillus amyloliquefaciens FZB42*).*

Stressed plants cultivated on LT soil, showed no significant treatment differences were for shoot and root biomass production at four weeks after sowing in response to application of bioeffectors. However, growth-promoting effects of the microbial inoculants were observed on ST soil. Shoot biomass production increased by 20-30% (Figure 41A) and root fresh weight increased by 9-36% (Figure 41B) as compared to the untreated control.

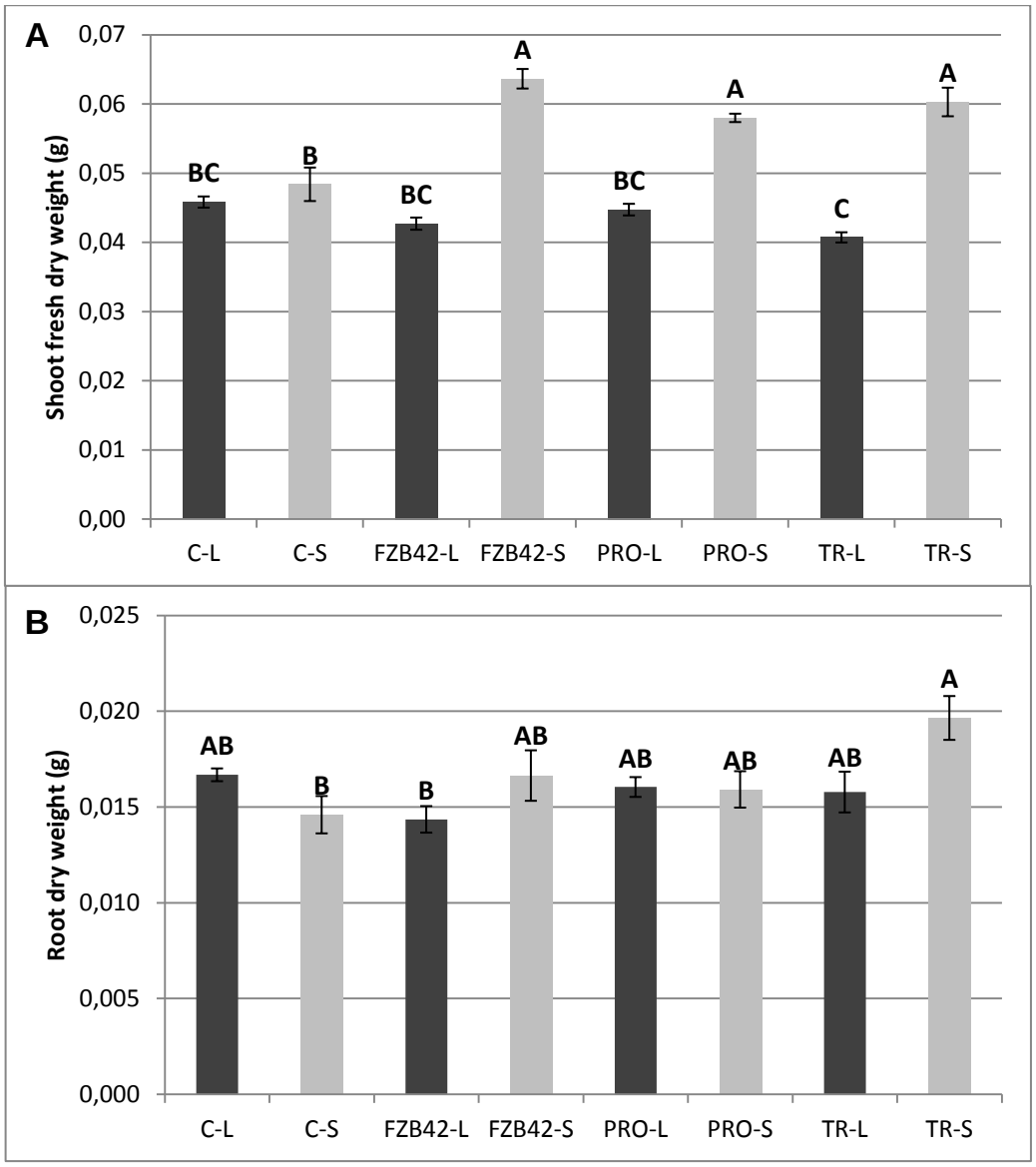

Figure 41: ***(A)*** *Shoot and* ***(B)*** *root dry matter of winter wheat (cv. Isengrain) at 2 weeks after sowing on short-term (ST) and long-term (LT) no-tillage soil collected from the field site "Hirrlingen Friedhof" with and without (C) application of microbial bio-effectors: Rhizovital 42® (FZB), Proradix® (PRO) and Trichostar® (TR). Values are means ± SE of 4 replicates per treatment. Means values with different letters are indicating significant differences (Tukey's test, α = 0.05).*

3.6.1.2 Spring application of microbial bio-effectors under field conditions

Apart from starter applications in the pot experiment, the selected microbial bio-effectors were tested also under field conditions with spring applications (begin of May) on LT no-tillage field sites, after expression of plant damage symptoms in Hirrlingen, (Schwarze Länder) and Wendelsheim (Sülcher Wegle, Remmingsheimer Weg), (see 2.2, Table 6). Proradix®, Rhizovital® and Trichostar® were applied in three replicates on 1 m^2 plots by fertigation according to the instructions of the manufacturers. Control plots were treated with distilled water.

Visual scoring of plant damage after 8 weeks revealed strong suppression of plant growth on all investigated field sites due to early summer drought (Figure 42). No protective effects could be recorded in any of the bio-effector treatments. An exemplary overview of field performance of plants with and without bio-effector treatments is given in Figure 42 for the field site Wendelsheim, Remingsheimer Weg.

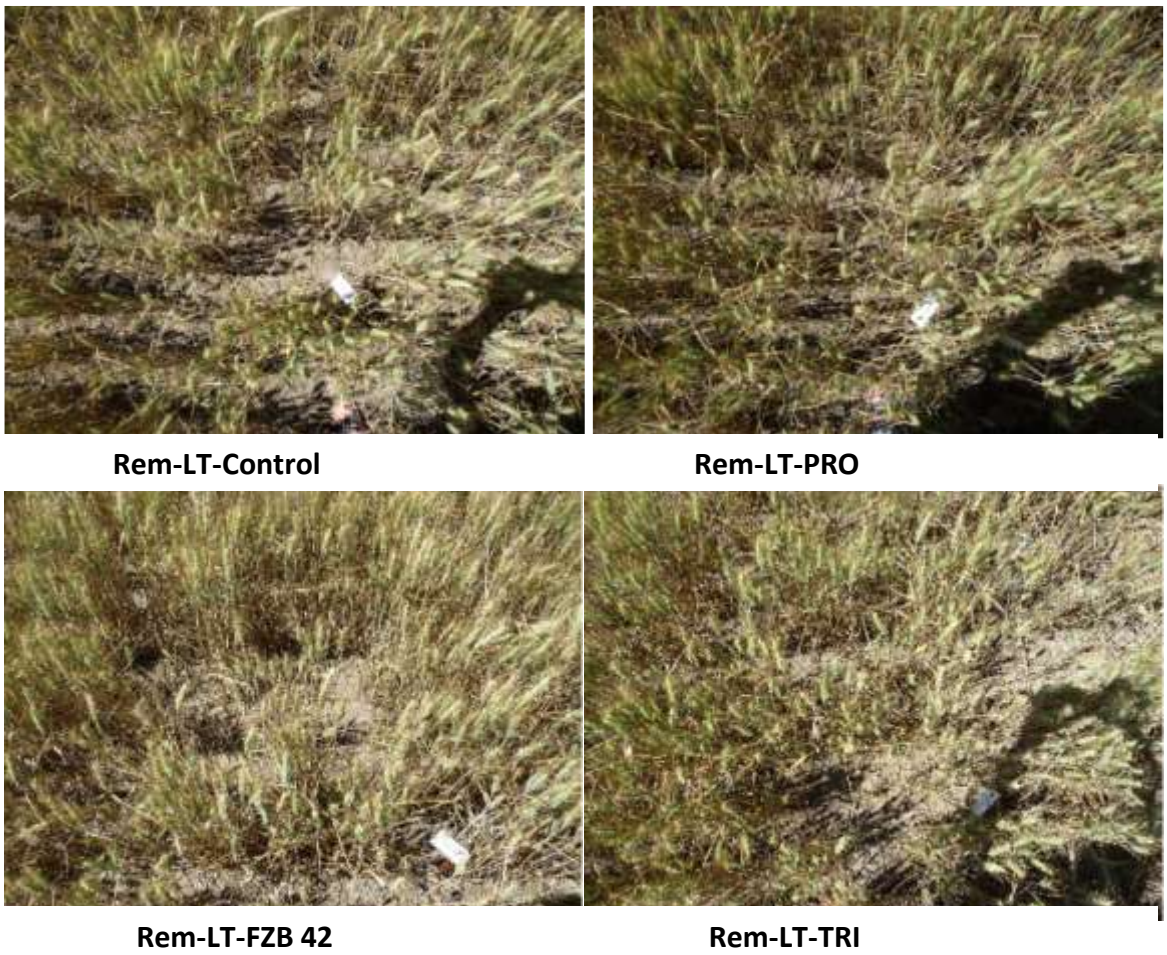

Figure 42: Field performance of winter wheat a8 weeks after spring application (May 4th) of microbial bio-effectors (Proradix® PRO, Rhizovital 42® FZB 42, Trichostar® TRI) and a water control at the long-term (LT) no-tillage field site Wendelsheim, Remingsheimer Weg.

3.6.2 Detoxification of herbicide residues by immobilization

Based on the observation that biochar amendments to LT no-tillage soil could mitigate plant damage of winter wheat in greenhouse culture (see 3.4, Figure 26), it was hypothesized that the observed mitigation effect was due to detoxification of plant-available glyphosate residues in soil by immobilization. As previously reported, other herbicides and organic contaminants were adsorbed by activated carbon or biochar (Bes and Mench, 2008; Loganathan *et al*., 2009; Kookana, 2010).

3.6.2.1 Glyphosate detoxification potential of biochar in a peat culture substrate-sand mixture

A peat culture substrate (TKS®, Table 8) sand mixture (TKSS 50/50% v/v) was used as a plant growth medium with very low adsorption potential for glyphosate to induce a maximum level of toxicity. Roundup Ultramax® was used as a glyphosate source in an overdose application rate of 8 L ha^{-1} and was homogeneously mixed in the 7 cm top layer of the culture substrate before sowing of winter wheat in a pot experiment (see 2.6.4C) to simulate soil surface contamination under field conditions. Pyrolysis biochar (Table 7) was applied at concentrations 0, 1.0 and 5.0 % (v/v).

As expected, the herbicide treatment (TG) exerted strong inhibitory effects on germination and plant growth of winter wheat grown on the TKSS substrate In the TG treatment all plants showed abnormal development with stunted growth, strong chlorosis and impaired root growth (Figure 43A). This damage was reduced, and plant growth was improved by application of 5% (v/v) biochar (TGB5) but not by the lower application rate of 1% (TGB1) (Figure 43B) while control plants not exposed to glyphosate showed normal development (C, TB1, TB5).

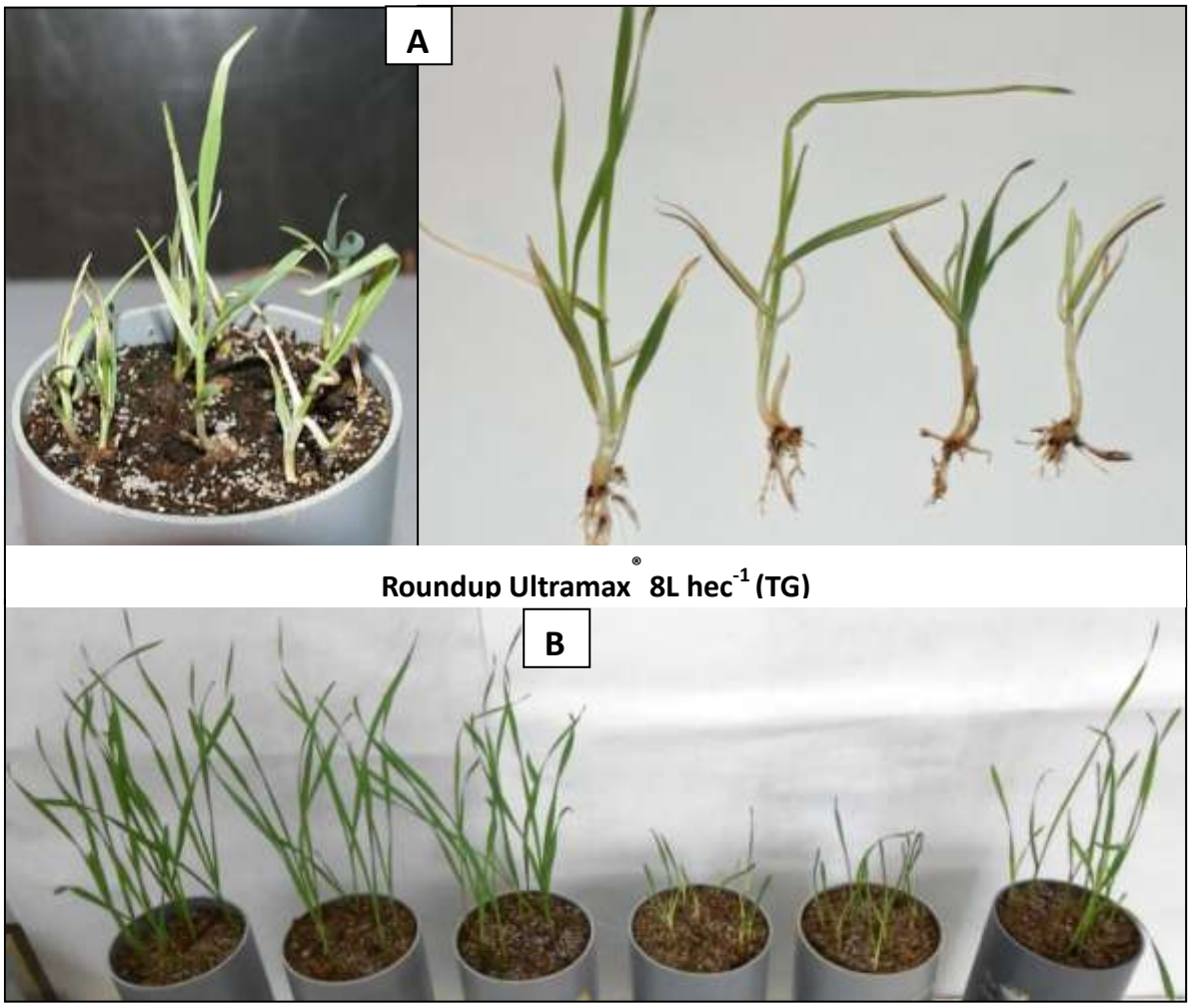

Figure 43: ***(A)*** *Habitus of winter wheat (cv. Isengrain) at two weeks after sowing, grown on peat culture substrate/sand mixture (TKSS 50/50 v/v) contaminated with glyphosate (TG) applied as Roundup® Ultramax (8 L ha^{-1}).* ***(B)*** *Mitigation of glyphosate-induced growth inhibition (TG) by biochar amendments (TGB1, TGB5) as compared with control variants without glyphosate application (C, TB1, TB5).*

Accordingly, shoot biomass production after a culture period of two weeks was reduced by approximately 70 % in response to glyphosate application, which was partially reverted by 5 % biochar application, but not in the 1 % biochar treatment. Also, root growth was strongly affected by glyphosate application. However, no quantitative analysis was performed in this case due to strong adsorption of peat particles to the root system which could not be separated mechanically.

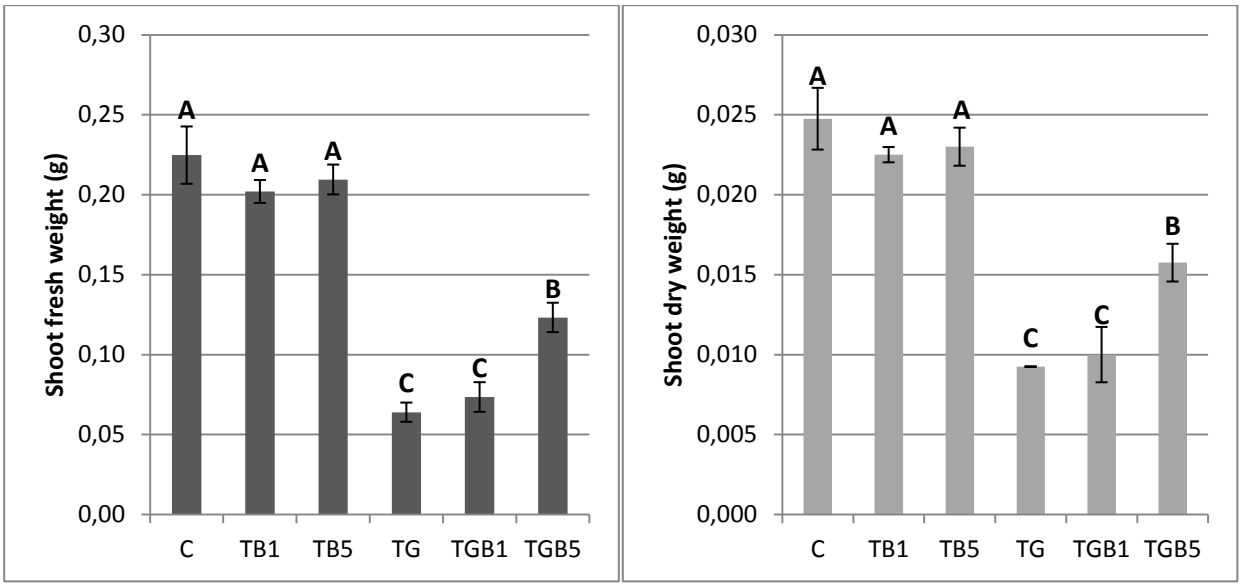

Figure 44: Shoot biomass of winter wheat (cv. Isengrain) a two weeks after sowing, grown on peat culture substrate/sand mixture (TKSS 50/50 v/v) with (TG, TGB1, TGB5) and without (C, TB1, TB5) application of glyphosate (Roundup Ultramax® 8 L ha-1) and biochar amendments of 1 % (TB1, TGB1) and 5 % v/v (TB5, TGB5). Values are means of 4 replicates per treatment. Means values with different letters are indicating significant differences (Tukey's test, α = 0.05).

3.6.2.2 Glyphosate detoxification potential of biochar in soil culture

To investigate the glyphosate detoxification potential of biochar demonstrated in the artificial peat culture substrate-sand mixture also under soil conditions, Roundup Ultramax as a glyphosate source was homogeneously mixed in a pot experiment with HirG-ST field soil at a high dosage of 4 L ha^{-1} with biochar amendments of 0, 5, 10 and 20 % (v/v)

After sowing of winter wheat (cv. Isengrain), biochar treatments increased the speed of seedling emergence with the fastest emergence rate with a biochar dose of 10 % and 20% (v/v) Figure 45.

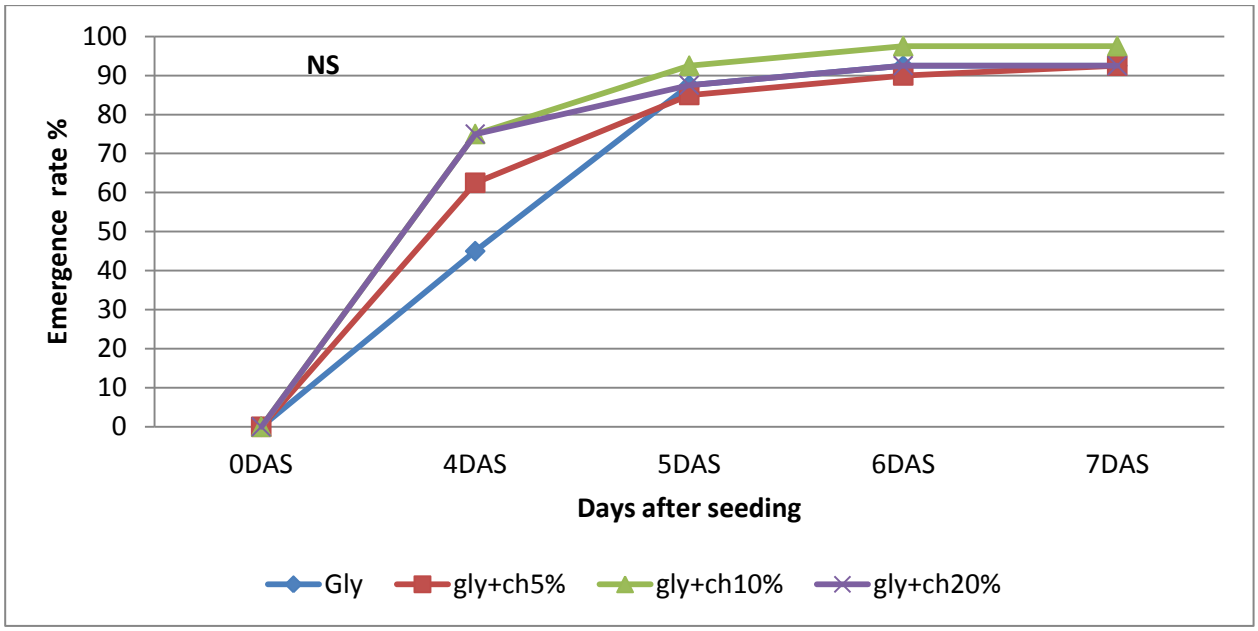

Figure 45: Emergence percentage of winter wheat (cv. Isengrain) during first week pot culture with glyphosate, contaminated soil and homogeneously applied biochar treatments. Values are means of 4 replicates per treatment. NS = not significant. (Tukey's test, α = 0.05).

After a culture period of three weeks, 5 % (v/v) biochar application significantly improved root development (biomass, root length) of winter wheat grown on the glyphosate contaminated soil but this effect disappeared at higher application rates of biochar (Figure 46, Table 20). Shoot biomass production was not significantly increased by the biochar treatments.

Glyphosate **Glyphosate+Biochar 5%** **Glyphosate+Biochar 10%** **Glyphosate+Biochar 20%**

Figure 46: Habitus of winter wheat (cv. Isengrain) at three weeks after sowing in glyphosate-contaminated soil (Roundup Ultramax® 6 L ha^{-1}) with and without biochar amendments (5%, 10% and 20% (v/v) homogeneously mixed with the soil.

Table 20: Shoot and root growth of winter wheat (cv. Isengrain) at three weeks after sowing in glyphosate contaminated soil (Roundup Ultramax® 6 L ha^{-1}) with and without biochar amendments (5%, 10% and 20% (v/v) homogeneously mixed with the soil. Values are means of 4 replicates per treatment. Means values with different letters are indicating significant differences (Tukey's test, α = 0.05).

Growth Features	Glyphosate (Roundup®)	Glyphosate +Biochar 5%	Glyphosate +Biochar 10%	Glyphosate +Biochar 20%
Shoot fresh weight (g)	0.24 ± 0.01 (AB)	0.28 ± 0.01 (A)	0.29 ± 0.02 (A)	0.23 ± 0.01 (B)
Root fresh weight (g)	0.11 ± 0.01 (B)	0.21 ± 0.03 (A)	0.15 ± 0.01 (AB)	0.14 ± 0.01 (AB)
Root length (g)	265.9 ± 10.4 (B)	406.3 ± 50.25 (A)	295.2 ± 20.52 (AB)	282.2 ± 12.20 (B)

3.6.2.3 Application of biochar and Roundup® to the topsoil

Due to rapid soil adsorption, most of the applied glyphosate will remain in the uppermost soil layers (2-5cm). This holds particularly true for minimal- or no-tillage systems (Alletto *et al.*, 2010; Bott *et al.*, 2011) with minimal soil disturbance. To simulate this situation a pot experiment was conducted by mixing Roundup Ultramax® at rate of 6 L ha^{-1} (G) and/or biochar 5% (GB5), 10% (GB10) and 20% (GB20) only into the uppermost soil layer of approximately 5cm rest of the 5cm and control (C) was untreated soil.

After a culture period of three weeks, particularly fine root production was affected by glyphosate application with a trend for mitigation induced by all biochar treatments although the differences were not significant. Similarly, trends of mitigation were observed in seedling emergence and total root length with best results in 5% (GB5) application (Table 21).

Table 21: Shoot and root growth of winter wheat (cv. Isengrain) at three weeks after sowing in glyphosate contaminated topsoil layer (5cm) (Roundup Ultramax® 6 L ha^{-1}) with and without biochar amendments (5%, 10% and 20% (v/v) mixed with the topsoil layer soil. Values are means of 4 replicates per treatment. Means values with different letters are indicating significant differences (Tukey's test, α = 0.05). NS = not significant.

Growth Features	Control	Glyphosate (Roundup®)	Glyphosate +Biochar 5%	Glyphosate +Biochar 10%	Glyphosate +OBiochar 20%
Emergence	90.0 ± 5.77 (NS)	85.0 ± 6.45 (NS)	100.0 ± 0.00 (NS)	92.50 ± 2.5 (NS)	87.50 ± 6.3 (NS)
Shoot fresh weight (g)	0.298 ± 0.02 (NS)	0.322 ± 0.01 (NS)	0.299 ± 0.01 (NS)	0.295 ± 0.0 (NS)	0.315 ± 0.0 (NS)
Root fresh weight (g)	0.196 ± 0.02 (NS)	0.237 ± 0.03 (NS)	0.201±0.01 (NS)	0.202 ± 0.0 (NS)	0.209 ± 0.0 (NS)
Total Root length (cm)	4453.7 ± 361.6 (NS)	2717.2 ± 112.9 (NS)	3753.6 ± 691.6 (NS)	3332.5 ± 243.4 (NS)	3363.00 ± 368.0 (NS)
Fine root length (cm) 0-0.2mm diameter	154.4 ± 7.95 (A)	84.5 ± 6.52(B)	107.5 ± 25.73 (AB)	100.4 ± 8.54 (AB)	105.70 ± 16.0 AB

4 Discussion

During the last decades, no-tillage and reduced-tillage cropping systems have been increasingly adopted by many countries, mainly to counteract soil erosion and offering numerous additional benefits that conventional tillage could not match (Uri, 2000; Borie *et al*., 2002; Wang *et al*., 2006). The expected advantages comprise reduced costs for energy and fewer labour requirements due to fewer field operations (Tebrügge, 2001), beneficial effects on topsoil structure, organic matter retention, soil fauna and flora, water holding capacity, resistance against temperature extremes, and finally soil fertility (Baker *et al*., 2007), associated also with reduced greenhouse gas emissions (FAO, 2015).

However, in contrast to the promising expectations, experiences with long-term no-tillage management systems in Southwest Germany demonstrated that the beneficial effects could not be maintained over longer time periods. Starting with occasional observations on plant damage and yield losses on long-term no-tillage field plots (10 years and more) in comparison with directly neighbored plots brought into no-tillage management just two years before, e.g., by grassland conversion, there was increasing evidence for yet unexplained constraints for no-tillage cropping.

Therefore, the aim of this study was a characterization of critical factors, determining the unexpected limitations and to define potential options for mitigation. Since direct investigations with long-term field experiments were not feasible for the schedule available within a Ph.D. project, a "field to lab approach" was employed, starting with the selection of suitable field sites with closely neighbored long-term and short-term no-tillage plots and similar pre-cropping history. Finally, five field sites were selected for more detailed investigations out of a group of potentially suitable locations (Table 6). After characterization of type, intensity and timing of appearance of common plant damage symptoms, reproducibility of the effects were tested in pot experiments with soil samples collected from the respective field sites under controlled conditions, to facilitate the identification of factors triggering the observed symptoms of plant damage. After characterization of potentially critical factors, further investigations were conducted in soil free systems to isolate the impact of single stress factors and to identify the

underlying mechanisms. Also, potential mitigation strategies were first tested in pot experiments and partially followed by pilot experiments under field conditions.

4.1 Plant nutrient availability on long-term and short-term no-tillage field sites

Alteration of soil conditions by tillage can significantly affect soil productivity and sustainability through influences on depth distribution, soil organic matter (SOM), microbial activity, and nutrient dynamics (Doran and Smith, 1987; Follett and Peterson, 1988; Mahboubi *et al*., 1993). Numerous reports in the past decade have found greater organic carbon and microbial activity in the soil surface layer of no-tillage soil as compared to conventional tillage as a response to crop residue accumulation at the soil surface (Dalal *et al*., 1991; Bauer and Black, 1994; Franzluebbers *et al*., 1995). This effect was also detectable on the investigated long-term no-tillage field sites in this study, generally showing higher organic carbon percentage than the corresponding short-term no-tillage plots (Table 10) but unexpectedly this was associated with growth suppression of winter wheat on the respective soils (Figure 12, Figure 13). Soil nutrient analysis revealed no apparent nutrient deficiencies or toxicities of P, K, Mg, Zn, Mn and Fe and no systematic nutrient patterns in three investigated soil pairs, characteristic for long-term versus short-term no-tillage field plots (Table 10). Also, nitrogen was not a limiting nutritional factor since it was regularly applied in the recommended dosage on all investigated field sites. Soil pH as an important factor, determining nutrient availability in soils, varied between 6.6 and 7.2 (Table 10) in the optimum range for wheat cultivation (Lufa, 2012) and similar to reports in earlier studies (Lal *et al*., 1994; Pikul and Aase, 1995), soil pH was not influenced by long-term no-tillage management. The corresponding analysis of the plant nutritional status also provided no indications for nutrient deficiencies or toxicities except P, which was usually sufficient (Bergmann, 1988) only in winter wheat plants grown on the short-term no-tillage plots but declined below the deficiency threshold on long-term no-tillage soils (Figure 21). This was associated with limited root growth and particularly reduced the formation of fine roots (Figure 20). Since soil P availabilities ranged between moderate and high levels (LTZ Augustenberg, 2011) and P concentrations on long-term no-tillage plots were not

particularly low, the low P status of the respective plants must be rather attributed to limitations in P acquisition due to root growth inhibition, than to limited soil P availability related to the tillage management.

4.2 Pathogen pressure on long-term no-tillage soils

Tillage can control plant diseases by breaking fungal hyphae networks in the soil and controlling weeds, which serve as host-bridge (Roget *et al*., 1987). In no-tillage systems, root diseases are representing one of the major problems, as disease pressure increases due to increased crop residues at the soil surface, particularly under cool and wet soil conditions in early spring (Paulitz *et al*., 2002). The concentration of plant debris in the top 10-15 cm soil can promote the over wintering and survival of various pathogens waiting for the next crop. The organic residues provide energy to pathogens before and during the infection period. This energy source is important for the interactions between host and pathogen, pathogen survival (Boosalis *et al*., 1981), germination (Tousson *et al*., 1963) and capability to cause infection (Garrett, 1976). Roots confined to, or growing near the soil surface may be prone to pathogen attack. Pathogen inoculum concentrations in no-tillage systems can be much greater than in conventionally plowed soils (Khan, 1975; McFadden and Sutton, 1975). Particularly high inoculum concentrations have been observed in the case of monocropping because of attraction and accumulation of host-crop specific pathogens. Disease severity was also higher under zero tillage with short non-host crops rotations (Gossen and Derksen, 2003). Accordingly, crop rotations can break soil pathogen cycles and reduce weed pressure (Karlen *et al*., 1994).

Many of the conditions promoting disease pressure in no-tillage farming as described above, also applied to the no-tillage systems investigated in the present study: during the years preceding the investigated period, winter wheat (75 %) and winter rape (25%) were the predominant crops in short crop rotations, or even with several years of winter wheat mono-cropping for economic reasons in some cases. Similar to root-rot diseases dominant in no-tillage systems (Paulitz *et al*., 2002), the observed symptoms of plant damage on long-term no-tillage plots were reflected in impaired root growth and fine root development (Figure 12) and usually appeared in early spring providing the most

favorable weather conditions for pathogen development (Paulitz *et al*., 2002). In some years even increased levels of barley yellow dwarf virosis had been observed in later spring.

For further assessment of potential pathogen effects, the possibility to reproduce the damage symptoms under laboratory conditions (Figure 14) was of substantial significance, since it opened the perspective to employ gamma ray soil sterilization with minimal side effects on physicochemical soil properties (Stroetmann *et al*., 1994). To investigate the impact of potential pathogen effects on plant growth on long-term no-tillage soils in controlled environments. Except the field site SW2011, soil sterilization had no mitigation effect on the expression of plant damage symptoms on long-term no-tillage soils in all investigated soil samples (Figure 22, Figure 25), suggesting that increased pathogen pressure was not the primary cause for the observed growth suppressions in winter wheat. Only on the soil collected from the long-term no-tillage SW2011 field site, germination of winter wheat seedlings was significantly increased by soil sterilization, suggesting the presence of a damping-off disease in this particular case. However, due to the absence of comparable effects in other soils, the SW2011 field site was no longer included in further investigations.

4.3 Allelopathic interactions in long-term no-tillage soils

In no-tillage systems, crop residues are left on the field contributing to increased accumulation and stabilization of organic carbon in the topsoil layer (Dalal *et al*., 1991; Bauer and Black, 1994; Franzluebbers *et al*., 1995). However, during degradation, plant residues can also release toxic compounds, so called allelochemicals with detrimental effects on growth of other plants (Patrick and Koch, 1958; Kimber, 1973; Rahman *et al*., 2005) or even on the plant species providing the respective crop residues (auto-allelopathy; Lodhi *et al*., 1987; Protic *et al*., 1980). Especially in monoculture agroecosystems, the risk of allelopathic effects is high, due to the input of the same type of toxic compounds over longer time periods into the same soil volume, causing cumulative effects. According to Chapman (1966), root excretions, plant residues, and microbial colonization of plant residues can all contribute to the accumulation of growth

inhibitors in soils. Particularly in wheat, both, allelopathic and auto allelopathic effects are well-documented (Schreiner and Reed, 1907; Guenzi and McCalla, 1962; Rahman *et al*., 2005, Fragasso *et al*., 2013), leading to inhibition of germination, and impaired root and shoot development. Both, root exudates and decaying plant residues of wheat exhibit allelopathic potential and various low-molecular weight phenolics, as well as hydroxamic acids, short chain fatty acids, naphtoic and azaleic acids, carboxylic acid methyl esters, triterpenoids and even microbial antibiotics such as patulin have been identified as potentially active compounds (Waller *et al*., 1987; Fragasso *et al*., 2013).

Also on the long-term no-tillage field sites investigated in the present study, wheat-dominated crop rotations or even wheat monoculture may have promoted long-term accumulation of allelochemicals, increasing the risk of auto-allelopathic effects. A potential accumulation of phytotoxins in the respective soils was further supported by the finding that detrimental effects on plant growth were rapidly eliminated already during the germination period by application of biochar, which exhibits binding potential for various organic compounds with phytotoxic properties (Loganathan *et al*., 2009; Kookana, 2010). This view was also supported by the experiments with soil sterilization, showing no mitigation effects on growth inhibition of wheat plants, cultivated on long-term no-tillage soils (Figure 24), thereby excluding pathogens as a major cause for plant damage. A general phytotoxic potential of the respective soils was further confirmed by reproduction of inhibitory effects in pot experiments with various plant species including wheat (Figure 19), soybean (Table 12) and sunflower (Figure 27).

However, some observations are not easily compatible with the concept of auto-allelopathic effects by accumulation of phytotoxins, released from crop residues or as root exudates of wheat on the investigated long-term no-tillage soils: (i) in the pot experiments, reproducing the symptoms of plant-damage in the field (Figure 14), crop residues had been largely removed by soil sieving prior to the start of the experiments. (ii) Typical for allelopathic effects, plant damage symptoms detected in the pot experiments appeared rapidly after sowing (Putnam, 1985) and affected already germination or early growth of the seedlings (Figure 24). However, under field conditions, germination and early development of winter wheat remained completely unaffected in most cases, and

damage symptoms preferentially appeared with the start of the re-growth period in spring, up to six months after sowing. (iii) Moreover, if wheat root exudates are acting as allelochemicals with auto-toxicity effects, plant growth on long-term no-tillage soils should be increasingly affected during the growth period due to the increase in root development and root density in the topsoil, but this was not the case. By contrast, the phytotoxic potential of long-term no-tillage soil completely disappeared when soil sampling for the biotests in pot experiments was not conducted in early spring but later during the vegetation period at the beginning of summer (Figure 28). This demonstrates that no further accumulation but rather a degradation of phytotoxins occurred during the growth period of winter wheat on long-term no-tillage field sites.

4.4 Herbicide residues in long-term no-tillage soils

In no-tillage management, weed control is one of the major challenges due to the absence of mechanical weed removal. Alternative methods, using special mechanical techniques, electricity, and integration of cover crops are still under development and frequently require the additional input of labor and machinery, at least partially counteracting the benefits of no-tillage management. Therefore, application of herbicides is still the most widely used method for weed control in no-tillage systems. Some herbicides can stay active in soils for extended time periods of weeks months or even years depending on environmental factors such temperature, soil moisture, and microbial activity. This can be an advantage for long-term weed control, but severely delayed degradation can also cause problems by damaging sensitive crops grown subsequently on the herbicide-treated field sites (Hang *et al*., 2012; Agriculture Victoria, 2013). The damage potential of herbicide residues in soils depends on persistence and bioavailability of the residues. For many herbicides degradation and/or bioavailability are restricted in the environment through sorption, hydrolysis, volatilization, transport, and accumulation of bound residues (Sims and Cupples, 1999). In soils or on plant surfaces, herbicides can be degraded to some extent by photochemical reactions, but microbial degradation is the major the degradation process in agricultural soils (Cox *et al*., 1996).

Also on the investigated long-term no-tillage field sites various herbicides with known residual activity, such as sulfonylureas, dinitroanilines (pendimethalin), propyzamides (Hang *et al.*, 2012; Agriculture Victoria, 2013) were applied at least occasionally. By contrast, glyphosate as the most widely used herbicide particularly in reduced tillage systems was regularly applied on all investigated field sites. This is characteristic for winter wheat/winter rape-dominated cropping systems, where glyphosate application has been documented in 87% of the rape cropping area and in 23 % of the wheat cropping systems mainly for stubble management and pre-sowing application, comprising 38 % of the total glyphosate use in Germany (Dickeduisberg *et al.,* 2012). However, in contrast to many other herbicides, residual effects of glyphosate applications are not widely documented in the literature. A risk of contact contamination of crops with glyphosate-treated weed straw residues shortly after the application is documented even in the application instructions (Monsanto UK, 2016). However, Tesfamariam *et al.* (2009) and Bott (2010) showed in elegant experiments with the removal of areal plant parts of glyphosate-treated weeds, that even root residues bear a risk of contact contamination for the subsequent crop, showing growth depressions and shikimate accumulation in the root tissue as physiological indicator for glyphosate toxicity. Accordingly, genetically modified glyphosate-resistant soybean plants were not affected (Bott, 2010). Since pathogen damage (see 3.3) or allelopathic effects (see 3.4) could not provide satisfactory explanations for the observed symptoms of plant damage on the investigated long-term no-tillage field sites, residual effects of long-term herbicide applications need to be also taken into consideration.

An overview of herbicide residues in six closely neighboured long-term and short-term no- tillage field plots revealed a consistent pattern with higher residual soil levels of glyphosate and its main metabolite AMPA at the long-term no-tillage sites (Table 14). The absolute concentrations were highly variable, but the highest levels were recorded for soil samplings conducted in early spring with a sharp decline in summer samplings. This was in line with the observation that the plant damage potential of a long-term no-tillage was high in spring samplings but completely declined in soil samples collected at the same site in summer (Figure 28). Moreover, the degree of plant damage recorded in the field on a short-term and long-term no-tillage field site in spring was reflected in the soil

concentration of glyphosate and AMPA residues, reaching values of 2.6-4.0 mg kg^{-1} soil even six months after the last glyphosate application (Table 13); - a concentration range reported for field soils shortly (15 d) after application of high doses (4-6 kg ha^{-1}) of glyphosate (Franz *et al.*, 1997).

Table 22: Glyphosate persistence in soil (Field Data) (modified after Franz et al., 1997).

Soil type	Rate (kg ha^{-1})	Observation Time (days)	Soil Residues	
			Glyphosate	AMPA
Irrigation Ditchbanks				
A	5.6	158	0.37 µg g^{-1}	0.74 µg g^{-1}
B	5.6	172	0.33 µg g^{-1}	0.82 µg g^{-1}
Forest Soils				
Clay loam	2.0	92	0.08 kg ha^{-1}	0.09 kg ha^{-1}
Mull/brown soil	2.0	92	0.06 kg ha^{-1}	0.02 kg ha^{-1}
Brown soil-weak	2.0	92	0.22 kg ha^{-1}	0.11 kg ha^{-1}
Podsol mull/brown	2.0	92	0.15 kg ha^{-1}	0.08 kg ha^{-1}
Soil-weak podsol (Weakly formed iron)	2.0	98	0.27 kg ha^{-1}	0.03 kg ha^{-1}
Podsol	4.0	98	0.38 kg ha^{-1}	0.05 kg ha^{-1}
Iron podsol	2.0	104	0.05 kg ha^{-1}	0.02 kg ha^{-1}
Iron podsol	4.0	104	0.13 kg ha^{-1}	0.05 kg ha^{-1}
Agriculture Soil				
Loam	2.6	249	0.9 µg g^{-1}	0.3 µg g^{-1}
Fine silt	2.6	249	1.0 µg g^{-1}	0.2 µg g^{-1}
Sandy loam	4.0	103	1.1 µg g^{-1}	-
Clay loam	2.0	15	0.8 µg g^{-1}	-
Clay loam	4.0	15	1.5 µg g^{-1}	-
Clay loam	**6.0**	**15**	**2.4 µg g^{-1}**	-

No comparable relationships were detected for the residues of other herbicides such as Pendimethalin or Propyzamide (Table 15). Although, glyphosate persistence in soils can be highly variable (Franz *et al.*, 1997) depending on soil properties, applied rate and observation time (Table 22), the observed levels of glyphosate residues recorded in spring

on long-term no-tillage field sites, expressing plant damage (Figure 11, Figure 12), are unusually high (Table 13, Table 22), suggesting a reduced degradation potential in these soils.

In soils, the primary route of glyphosate degradation is microbial, although some photodegradation and chemical degradation may occur (Tu *et al.*, 2001). Microbial glyphosate degradation in soils usually follows a biphasic pattern with rapid decomposition rates associated with increased microbial activity shortly after glyphosate application, which are rapidly slowing down due to soil adsorption of glyphosate, reducing the availability for soil microorganisms with degradation potential (Sprankle *et al.*, 1975; Nomura and Hilton, 1977, Araújo *et al.*, 2003). It is well documented that in reduced or no-tillage systems, the alterations in soil-physical and chemical conditions can induce changes in structure and activity of soil microbial communities (Helgason *et al.*, 2009). However, this was mainly characterized by increases in soil microbial populations, activity (Staley, 1999) and microbial biomass (Kandeler *et al.,* 1999, Balota *et al.,* 2003) with increased abundance of fungi, bacteria, arbuscular mycorrhizal fungi and actinobacteria (Mathew *et al.*, 2012; Feng *et al.*, 2003; Helgason *et al.,* 2009; Pankhurst *et al.,* 2002) and a higher diversity of bacterial communities (Ceja-Navarro *et al.,* 2010). Also increased the abundance of pathogens has been reported (see 3.3), particularly under mono-cropping or short non-host crop rotations (Khan, 1975; McFadden and Sutton, 1975; Gossen and Derksen, 2003). These changes can be attributed to crop residues left in the topsoil, which provide organic matter and also to reduced soil disturbance, preventing disruption of microbial consortia and soil aggregates (González-Chávez *et al.*, 2010). Accordingly, also in this study, the investigated long-term no-tillage soils showed increased levels of organic matter (Table 10), but surprisingly, the degradation potential for glyphosate soil residues obviously declined.

In face of this unexpected result, soil respiration as indicator for microbial activity was determined in samples collected from five closely neighbored pairs of field plots with long-term and short-term no-tillage history (Table 16), since the degradation of glyphosate is related to microbial activity, and its degradation rate is correlated with the rate of soil respiration (Franz *et al.,* 1997). In accordance with increased levels of

glyphosate residues in long-term no-tillage soils, in four out of five cases, soil respiration was reduced by long-term no-tillage management (Table 16), suggesting a lower microbial activity, contrary to the experiences of other studies reported in the literature. In contrast to our results, Zablotowicz *et al.* (2009) found no differences in glyphosate degradation, comparing topsoil samples from long-term conventional tillage and no-tillage soybean field sites. However, there are also studies showing a decline in glyphosate degradation potential in soils repeatedly supplied with glyphosate (Andréa *et al*., 2003). Apart from tillage management, also glyphosate itself has distinct effects on soil microorganisms, reflected by the well documented increase in microbial activity shortly after glyphosate application due to stimulation of microorganisms with glyphosate degradation potential (Araújo *et al*., 2003). For certain pathogenic fungi, both, stimulatory (*Fusarium*) and inhibitory effects (*Puccinia*) have been reported (reviewed by Duke *et al*. (2012). Also for certain strains of plant growth-promoting bacteria (*Bradyrhizobia*, Mn-reducers) inhibitory effects of glyphosate are documented while other strains remained unaffected (Duke *et al*., 2012) and contradictory reports are also available for arbuscular mycorrhizal fungi (Duke *et al*., 2012; Zaller *et al*., 2014). Glyphosate has antimicrobial properties since many soil microorganisms express a glyphosate-sensitive shikimate pathway similar to higher plants and accordingly antibiotic applications of glyphosate have been patented (Abraham, 2010). Although it has been claimed that the glyphosate concentrations required for antibiotic effects are far above the levels commonly detected in field soils (Monsanto, 2013). Toxic effects on soil microorganisms have been reported at glyphosate soil concentrations around 1 mg kg^{-1} (Roslycky, 1982), which is comparable to the soil concentrations detected in this study on long-term no-tillage field sites, associated with plant damage (Table 13).

The availability of modern high throughput sequencing technologies offers the opportunity to the responses of the whole soil microbiome to agricultural management practices and first studies have already employed these methods to investigate potential effects of long-term glyphosate use on rhizosphere microbial communities with a common outcome that Actinobacteria populations declined (Barriuso *et al*., 2010; 2011; Molli *et al*., 2016). However, under real field conditions, it is difficult to separate

glyphosate effects from effects related to the tillage management since both factors are acting simultaneously with a potential to impact on soil microbial communities.

4.5 Phytotoxicity of herbicide residues in long-term no-tillage soils

Even high soil residues of herbicides are not necessarily correlated with a high risk of phytotoxicity. In the case of glyphosate, strong and rapid anion exchange adsorption to clay minerals, iron, and aluminum oxides as well as adsorption to organic matter by formation of hydrogen bonds has been reported (Aubin & Smith, 1992; Haney *et al*., 2000; Veiga *et al*., 2001), which strongly limits the bioavailability as a major principle of glyphosate detoxification in soils but also increases the persistence of bound residues (Veiga *et al*., 2001).

Consequently, the water-soluble and therefore, a potentially plant-available fraction of glyphosate residues (Zablotowicz *et al*., 2009) was extracted from soil samples collected in spring on from a long-term no-tillage field site with high potential for plant damage (Hirrlingen, Friedhof, Table 13). Calculated back to an assumed soil moisture level of 20 % (w/w) favorable for plant growth, soil solution concentrations of 3.1 µg L^{-1} of glyphosate and 1.5 µg L^{-1} of AMPA have been determined. Since these concentrations were detected in spring, six months after the last glyphosate application, it can be assumed that the damaged wheat plants on long-term no-tillage fields were continuously exposed during the culture period to these low concentrations of glyphosate and AMPA and even somewhat higher levels can be expected shortly after herbicide application. However, the observed concentrations are by far lower than the toxicity threshold of approximately 2 mg L^{-1} (approx. 10 µM), reported for root exposure of winter wheat to glyphosate between 1 and 10 d in hydroponics (Mülleder, 2009; Bott, 2010). This raises the question whether long-term exposure to sub-toxic concentrations of plant-available glyphosate soil-residues can induce phytotoxicity by cumulative effects. To test this hypothesis, hydroponic culture experiments were conducted with winter wheat, with continuous root exposure to glyphosate in concentrations of 3–5 µg L^{-1} and the main metabolite AMPA (1.5-2.5 µg L^{-1}) supplied during 3-6 weeks to the liquid growth medium. To simulate the situation in the soil also a combination of glyphosate and AMPA

was applied. The growth medium was replaced daily to account for microbial degradation of the herbicides. Under field conditions, microbial degradation of herbicide residues in the soluble phase will be compensated by continuous desorption from the solid phase to reach the solubility equilibrium. To minimize the effects of glyphosate inactivation by complexation with the high concentrations of divalent cations (Sprankle, 1975) supplied in the nutrient solution, mineral nutrients were supplied only every second day and during the remaining time, the herbicides were applied in pure aqueous solution.

During the first week of the culture period, a trend for increased shoot biomass production and root growth was observed in the glyphosate variant (data not shown), which may represent the well documented hormesis effect of plant exposure to subtoxic levels of glyphosate with stimulatory effects on plant growth (Schabenberger *et al*., 1999; Duke *et al*., 2006, Cedergreen, 2008) although the underlying mechanisms currently are not well understood. However, the growth stimulatory effects disappeared after three weeks and turned into negative responses indicated by chlorosis development (Figure 32) and inhibition of fine root development, with an initial reduction by 20 % after three weeks (Figure 34) and about 50 % reduction after six weeks of the culture period (Figure 35) similar to the damage symptoms observed in the pot experiments and under field conditions (Figure 19, Figure 12). Accordingly, also Cedergreen (2008) reported the only short-term expression of hormesis effects of glyphosate, which were not sustained over longer time periods. Surprisingly, significant inhibitory effects on fine root production were observed only in the treatments with AMPA and the combination of glyphosate and AMPA but not with glyphosate alone (Figure 35). This finding suggests that unexpectedly, long-term exposure to AMPA and not to glyphosate was responsible for root growth depression in winter wheat. Accordingly, no shikimate accumulation was detected in the root tissue (Table 18) since in contrast to glyphosate, AMPA toxicity is not associated with inhibition of the shikimate pathway and accumulation of shikimate (Reddy *et al*., 2004; Duke, 2011) and the same observation was also made in soil culture in pot experiments with wheat plants damaged on soils collected from long-term no-tillage field sites (Figure 31). The identification of long-term exposure to AMPA as major stress factor inducing the observed plant damage symptoms on long-term no-tillage soils is also in line with the finding that transgenic, glyphosate-resistant soybean was similarly

affected as compared with a non-resistant cultivar, since tolerance to glyphosate in the genetically modified soybean plants is not associated with tolerance also to AMPA toxicity (Reddy *et al*., 2004).

A histological evaluation of AMPA-induced damage symptoms in roots of winter wheat, using vitality staining with triphenyltetrazolium chloride as physiological indicator for metabolic activity (Stūrīte *et al*., 2010), revealed no inhibition in the root tips and the 0-3 cm subapical root zones of seminal roots in winter wheat, treated with a mixture of AMPA and glyphosate but a substantial decline in vitality in the more basal zone of lateral root emergence, particularly expressed in the central cylinder as origin of lateral root initiation (Figure 36). Assuming a cumulative effect as a prerequisite for the induction of AMPA toxicity, this pattern would make sense: in the young meristematic and actively growing parts of the root may not be able to accumulate AMPA up to toxic concentrations due to permanent formation of new cells by the activity of the meristem. However, the more basal, older parts of the root are exposed to the initially sub-toxic concentrations of glyphosate and AMPA over longer time periods, which may finally affect the information of new laterals in these root zones due to cumulative effects. This may be of particular importance on the long-term no-tillage field sites in spring when new root formation usually starts in overwintering crops just in the topsoil with the highest levels of herbicide residues (Aletto *et al*., 2010) to replace root decay during the winter period (Chen *et al*., 1983). Cumulative effects are possible since, in contrast to soil microorganisms, plants are usually not able to degrade glyphosate and AMPA (Reddy *et al*., 2004, Duke, 2011).

4.6 Physiological basis of plant damages induced by glyphosate residues in long-term no-tillage soils

Glyphosate in soil solutions is prone to rapid microbial turnover yielding AMPA as major degradation product (Franz *et al*., 1997; Van Eerd *et al*., 2003). Reddy *et al*. (2004) proposed a similar degradation mechanism of glyphosate in soybean plants and concluded co-occurrence of glyphosate and AMPA in plant tissues, although, among crops, glyphosate degradation to AMPA seems to be mainly expressed in soybean. Glyphosate

penetrates into plant tissues and reaches to active metabolic tissues. Similarly, organs with high rates of metabolic activity and growth are important sinks of glyphosate and AMPA (Feng *et al*., 2003). Toxic effects of glyphosate have repeatedly been reported even in glyphosate-resistant (GR) plants. These effects comprise induction of chlorosis and limitation of photosynthesis, disturbances in mineral nutrition and oxidative stress (Zobiole *et al*., 2009, 2012). Also in our experiments with GR soybean grown on herbicide contaminated long-term no-tillage soil, the strong damage was observed. Ready *et al*. (2014) explained the damage in GR plants as AMPA toxicity, which is generally less severe than glyphosate toxicity but the underlying mechanisms are still poorly understood. Due to the high structural similarity of AMPA as well as glyphosate with glycine and alanine may induce chlorosis, also observed in our study (Figure 33) due to competitive interactions during synthesis of these amino acids required for chlorophyll formation (Gomes *et al*., 2014). Accordingly, Serra *et al*. (2013) reported 87% and 64 % reduction of glycine and glutamic acid, respectively in *Arabidopsis thaliana* after 72 h exposure to low concentrations of AMPA (300 µg L^{-1}). Recently, Samsel and Seneff (2016) also discussed the risk that the structural similarity of glyphosate with glycine, which also applies for AMPA, could lead to the replacement of glycine in proteins, associated with impairment of protein (enzyme) functions.

In the study by Serra *et al*. (2013), glyphosate and AMPA were applied separated and in combination to Arabidopsis thaliana. Glyphosate alone and in combination with AMPA significantly inhibited root growth, while AMPA alone did not affect. By contrast, we noticed root growth inhibition in AMPA and glyphosate & AMPA treatments but no significant effects induced by single application of glyphosate. However, in our study mainly the production of fine lateral roots was affected, while Serra *et al*. (2013) investigated only primary root elongation and applied higher herbicide concentrations (300 µg L^{-1} vs 1.5-5 µg L^{-1}) for a shorter period (72 h vs 3–6 weeks) with a different plant species. Lee *et al*., (1983) reported perturbances of indole acetic acid (IAA) metabolism as a key regulator of root growth. Both, IAA conjugation and oxidation were increased, associated with reduced levels of free IAA and growth inhibition in tobacco callus, treated with sub-lethal concentrations of glyphosate or AMPA. However, so far little attention

has been given to the effects of AMPA in plant hormone metabolism and biosynthetic pathways (Gomes *et al*., 2014).

Based on the very limited information available in the literature on physiological effects of sub-lethal doses of glyphosate and particularly AMPA on higher plants, a RNAseq transcriptome study was initiated with winter wheat exposed in hydroponic culture to concentrations of glyphosate (G), AMPA (A) and a combination of both (GA) determined for the soil solution on long-term no-tillage field sites (see 4.5). Root material for analysis of gene expression was harvested at 19 days after sowing (DAS) just prior to the appearance of visible damage symptoms. Transcription profiles were compared to those of untreated control plants. After data processing and distribution into functional metabolic categories (bins) according to Mapman (Usadel *et al*., 2009), the largest number of changes in gene expression relative to the untreated control was recorded for the AMPA (total 160 bins) and the glyphosate+AMPA (total 130 bins) treatments but only 68 bins in the glyphosate variant. Since significant effects on root growth inhibition were recorded only in the AMPA and AMPA+glyphosate variants (Figure 35), particular emphasis was placed on bins showing alterations in gene expression for both, AMPA and AMPA+glyphosate treatments but not in the glyphosate variant (Figure 38).

4.6.1 Hormonal balances

In this category, up-regulation of cytokinin-related bins and down-regulation of bins related with ethylene metabolism (Figure 38) suggested disturbances in hormonal balances. Both ethylene and cytokinins are involved in lateral root (LR) formation. The gaseous hormone ethylene is reported to promote the development of lateral root primordia (LRP) and has stimulatory effects on LR growth (Clark *et al*., 1999; Ivanchenko *et al*., 2008) by regulating auxin transport and signaling (Stepanova and Alonso, 2009). The root growth-promoting effect of ethylene is higher in regions nearer the growing tips (Ivanchenko *et al*., 2008; Negi *et al*., 2008). Ethylene affects LR development in dose dependent manner (Moriwaki *et al*., 2011): in low concentration, it promotes LR initiation. In higher doses, it inhibits LRP initiation but promotes the emergence of existing LRPs (Ivanchenko *et al*., 2008). Similar to ethylene, also cytokinin

action shows a biphasic pattern with respect to root development. Higher concentration of cytokinins can act as auxin antagonists and suppress LR formation in various plant species, such as *Arabidopsis*, rice (*Oryza sativa*), alfalfa (*Medicago sativa*) and poplar (*Populus alba*) (Bellini *et al*., 2014). Cytokinins exert their inhibitory effects on lateral root formation by interference with cytokinin signaling and affecting PIN-mediated auxin transport (reviewed by Fukaki & Tasaka, 2009). However, the reported inhibition of auxin transport seems also to play a role in the induction of lateral root formation at low cytokinin concentrations by mediating the formation of auxin gradients required for initiation of LRPs (Jung and McCouch, 2013). Moreover, almost every aspect of root apical meristem activity is controlled by auxin/cytokinin interactions (Schaller *et al*., 2015). Based on the documented importance of ethylene and cytokinins for lateral root growth, it is feasible to assume that the observed changes in gene expression of ethylene-, and cytokinin-related genes by long-term exposure to trace concentrations of AMPA (and glyphosate+AMPA) are a likely cause for the observed disruptions of lateral root development, which needs to be further investigated to clarify the underlying mechanisms.

4.6.2 Aquaporins

Major intrinsic proteins (PIPs, TIPs, Aquaporins) represent another group of genes strongly down-regulated in AMPA-, and AMPA+glyphosate treated wheat roots (Figure 38). Aquaporins are membrane channels, facilitating water movement across cell membranes. They belong to the large family of MAJOR INTRINSIC PROTEINS (MIPs) and were identified in plants in 1987 (Fortin *et al*., 1987). Lateral roots are derived from secondary meristems (LRPs), formed in the central cylinder and LR emergence requires mechanical force to drive out the developing roots (Vilches-Barro and Maizel, 2015). This driving force is provided by increased turgor pressure mediated by water movement through aquaporins. In most cases, aquaporins are localized to the plasma membrane (Plasma Membrane-intrinsic proteins, PIPs) but the Tonoplast Intrinsic Proteins (TIPs) are targeted to the tonoplast (Maurel *et al*., 2008; Li *et al*., 2014). In Arabidopsis, the expression pattern of AtTIP1; 1 promoter is correlated with cell enlargement in roots, hypocotyls, leaves and flower stems (Ludevid *et al*., 1992). PIPs play an important role in

LR emergence (Péret *et al*., 2012). Plant roots use auxin for the regulation of aquaporins, and this fine-tuning of water flow speeds up LR emergence (Vermeer *et al*., 2014). Péret *et al*. (2012) demonstrated auxin-regulated water exchange between the stele, the LRP, and the overlaying tissues by controlling aquaporin expression. This happens with the most highly expressed aquaporin genes, PIP2;1 by auxin-dependent reduction in expression in cortical cells. On the other hand at the base of the LRP and underlying stele, the PIP2;8 is activated, leading to repressed water uptake in overlaying tissues but water transport is directed from the overlaying tissues into the primordium. This type of coordinated regulation of aquaporins is required for the proper emergence of LRs (Vilches-Barro and Alexis Maizel, 2015) and the aquaporin genes involved in this process are promising candidates to be investigated by for quantitative expression analysis in AMPA-treated wheat roots by RT-qPCR. The finding that AMPA affects the expression of ethylene and cytokinin-related genes may offer a link also to the expression patterns of aquaporins since both hormones are involved in regulating the formation of auxin gradients in the root tissue responsible for the coordinated expression of aquaporin genes triggering lateral root formation (see 3.5.4.5).

4.6.3 Stress defense

The third group of genes significantly down-regulated by AMPA and AMPA+glyphosate treatments was represented by genes involved in stress defense (abiotic stress, jasmonate, redox-related, phenolics, and also aquaporins). In contrast to glyphosate, where induction of oxidative stress is well-documented, based on experiments investigating oxidative stress markers it is hypothesized that this is not the case for AMPA (reviewed by Gomes *et al*., 2014). Also in our study, no AMPA-induced up-regulation of oxidative stress-related genes could be detected. However, the coordinated down regulation of various stress-related genes after long-term exposure to AMPA observed in the present study (Figure 38) may indicate a general decline in stress tolerance implicating a higher sensitivity to various abiotic and biotic stress factors, which was accordingly characteristic also for the plants grown on long-term no-tillage field sites characterized by high levels of herbicide residues (see 3.5.4.5).

Taken together, the transcriptome analysis provided valuable information on candidate genes and physiological processes to be confirmed by RT-qPCR and to be addressed in more detailed studies to clarify the underlying mechanisms. For a complete picture also covering alterations at the post-transcriptional level, as a next step a proteome and metabolome analysis would be recommended for wheat plants exposed to long-term exposure of AMPA and glyphosate+AMPA, preferentially conducted during the expression of first visible damage symptoms. Particular emphasis should be placed on hormonal changes during initiation and emergence of lateral roots.

4.7 Mitigation Strategies

The present study suggests that delayed degradation of glyphosate soil residues seems to be a primary factor for induction of crop damage, observed on the investigated long-term no-tillage field sites. Pant damage appears to be mediated by yet unknown cumulative effects of long-term root-exposure to sub-toxic levels of herbicide residues, with the microbial degradation product AMPA but surprisingly not glyphosate as a major toxic compound. The major damage symptoms were characterized by inhibition of fine root production, limiting acquisition of water and nutrients, finally responsible for stunted growth and weak plant development. Since inhibition of glyphosate degradation was mainly restricted to the winter period and early spring and disappeared with higher soil temperatures during summer, fortunately, long-term accumulation of herbicide residues was not a problem. This situation may offer the opportunity to find protective measures for the plants, restricted to the critical re-growth stage in early spring. However, the long-term goal must be focused on the restoration of the herbicide degradation potential of the respective soils.

4.7.1 Inoculation with plant growth-promoting microorganisms

Since impairment of root growth and nutrient acquisition were identified as major stress factors for plants grown on the selected long-term no-tillage field sites (Figure 19, Table 11), approaches to overcome or at least mitigate the inhibition of root growth and to increase nutrient availability, could offer a measure to improve plant performance during

the critical period in early spring. Artificial inoculation of crops with selected strains of symbiotic or associative microorganisms, expressing plant growth-promoting properties could provide a potential strategy in this direction. Root growth promotion by production of auxins (Steenhoudt and Vanderleyden, 2000) or preventing accumulation of excessive levels of ethylene with inhibitory effects on root growth (Li *et al*., 2000), as well as mobilization of phosphate and other sparingly- available nutrients (Rodríguez *et al*., 2006; Bashan and De-Bashan, 2010) are discussed as major modes of action of the respective microbial inoculants. Selected strains of the bacterial and fungal genera *Pseudomonas, Bacillus, Rhizobium, Azospirillum, Burkholderia*, *Trichoderma,* and *Penicillium,* as well as various arbuscular mycorrhizal fungi are among the most widely used commercially available inoculants with the ability to colonize plant roots as rhizosphere microorganisms or even as endophytes. Moreover, various strains of *Pseudomonas, Bacillus, Rhizobium, Trichoderma,* and *Penicillium* exhibit glyphosate degrading potential (Jacob *et al*. 1988, Arfarita *et al*. 2013). This could offer the possibility to restore glyphosate degradation on long-term no-tillage field sites at least in the rhizosphere of the target plants. Biocontrol potential against root pathogens, also frequently reported for these inoculants (Fröhlich *et al*., 2011), could be an additional beneficial feature. Although, after application to natural soils, the inoculant populations decline more or less rapidly due to competition between inoculants and indigenous microbial populations of the substrate (van Veen *et al*., 1997), even a transient expression of the beneficial effects over a limited period would be helpful to protect the plants on the long-term no-tillage soils during the critical time period in spring. Recently, several studies have reported the isolation of microbial strains with a particularly high potential for glyphosate degradation (Eman *et al*., 2013; Kryuchkova *et al*., 2014). However, a mitigation strategy with potential for practical application would require inoculants in sufficient amounts to be applied under field conditions. Therefore, a range of commercial formulations, containing spores of *Bacillus amyloliquefaciens* (RhizoVital® 42 TB, ABiTEP, Berlin, Germany), *Trichoderma harzianum* (Trichostar® Trichoderma T58, GERLACH Natürliche Düngemittel GmbH & Co. KG Hannover) and a dry formulation of *Pseudomonas* sp. DSMZ 13134 (Proradix®, Sourcon Padena, Tübingen-Germany), were tested in a pilot study.

In winter wheat, grown in a pot experiment on soils collected from long-term and short-term no-tillage field sites, plant growth suppression was induced on the long-term no-tillage soil (Figure 13). Interestingly all tested microbial inoculants stimulated plant growth, demonstrating the principal effectiveness of the plant growth-promoting microorganisms but unfortunately, this effect was restricted to the short-term no-tillage soil (Figure 41). The same inoculants were also tested under field conditions with spring applications on three long-term no-tillage field sites, expressing symptoms of plant damage in winter wheat. However, also under field conditions, no beneficial effects of the plant growth promoting microorganisms were recorded (Figure 42). These observations are in line with general findings on high variability in performance of microbial inoculants, depending on a wide range of environmental conditions (Crowley and Kramer, 2007). In the field experiment, early summer drought most probably affected the survival and the colonization efficiency of the inoculated microorganisms, as previously reported also in field experiments with Proradix® used for inoculation of barley (Fröhlich *et al*, 2011). Since rhizosphere microorganisms depend on carbohydrate supply via root exudation of the host plant, every stress factor affecting root growth and activity will also impair interactions of the host plant with the microbial inoculants and finally root colonization and the expression of plant growth-promoting effects. This scenario applies, both, for the drought-stress induced suppression of plant growth in the field experiments and also for the pot experiment with strong impairment of root growth on the long term no-tillage soils. Moreover, within selected microbial species, not all strains exhibit tolerance to herbicide residues, such as glyphosate as demonstrated, e.g., for *Rhizobia* (Duke *et al*., 2012). Therefore, it is by far not sure that the selected inoculants represented glyphosate-tolerant strains.

4.7.2 Detoxification potential of biochar amendments

Facing the lack of protective effects of the investigated microbial inoculants against plant damage on long-term no-tillage soils (see 3.6.1), the application of biochar was tested as a "non-biotic alternative", supposed to be less sensitive to variable environmental conditions. Adsorption onto activated carbon is the best available method used, e.g., in filter technologies for removal of agrochemicals and other synthetic organic chemicals

including glyphosate from drinking water (Nourouzi *et al*., 2010). Activated carbon is charcoal, which is usually treated with oxygen to increase its micro-porosity and surface area. The enhancement of surface area of charcoal by thermal and chemical treatments is referred as "activation" (Ahmad *et al*., 2014). However, a large-scale application of activated charcoal in agricultural practice would not provide an economical solution.

Similar to activated carbon, the use of so-called biochar has gained increased attention for removal of organic contaminants from water and soil (Zhang *et al*., 2013). Soil amendment of biochar is a historical practice observed in indigenous cultures in Australia, Africa, South America and Asia (Joseph *et al*., 2013). The origin of biochar is connected to the Amazon region, where the dark earth was created through slash and char techniques locally known as Terra Preta de Indio (Lehmann and Joseph, 2009). In this soil, enriched in black carbon-like biochar, increased bacterial diversity was reported (Kim *et al*., 2007; O'Neill *et al*., 2009). According to the "International Biochar Initiative", it represents a solid material obtained from the thermochemical conversion of biomass in an oxygen-limited environment'' (IBI, 2012). Lehmann and Joseph (2009) defined biochar as ''a carbon-rich product obtained when biomass such as wood, manure or leaves is heated in a closed container with little or unavailable air''. Biochar is a solid co-product of pyrolysis of biomass with potential as a soil amendment, which gains increasing interest for further examination from scientific and commercial perspectives (Jaiswal *et al*., 2014).

Biochar produced by burning of wood, wheat, and rice residues showed 400-2500 times more effectiveness in adsorption of pesticides as compared to the soil (Yang and Sheng, 2003; Yu *et al*., 2006; Xu *et al*., 2008). Wang *et al*., (2010) and Jones *et al*., (2011) reported that biochar reduced herbicide leaching. Hanger *et al*., (2013) showed that birch wood-derived biochar could reduce glyphosate leaching but did not affect glyphosate degradation in soil. There are contradicting reports concerning the role of biochar in degradation of chemical pesticides. Yang *et al*., (2006) and Jones *et al*., (2011) reported higher persistence of pesticides like simazine and diuron in biochar-amended soils, probably as a consequence of reduced bio-availability by immobilization. By contrast, Zhang *et al*., (2005) reported accelerated the degradation of benzonitrile in the presence of

biochar and explained it as result of increased soil nutrient content through biochar application, which can stimulate soil-microbial activity.

Other benefits attributed to biochar application comprise improvement of soil structure (Verheijen *et al*., 2010), increased soil water holding capacity (Glaser *et al*., 2002) and decreased nutrient leaching (Sohi *et al*., 2009). In some biochars, high ash continent contributes to input of plant nutrients like potassium, calcium and magnesium (Deenik *et al*., 2011; Rajkovich *et al*., 2012) and also effects on disease suppression, such as foliar gray mold and powdery mildew in tomato, sweet pepper and mite damage in sweet pepper (Elad *et al*., 2010) have been reported.

Taken together, the amendment of biochar may offer a perspective to reduce the concentration of bio-available herbicide residues and other toxins on long-term no-tillage sites during the critical growth phase in spring and adapt it to the reduced degradation potential on the respective soils. Degradation of herbicide residues may even be increased by beneficial effects on soil microbial activity, repeatedly observed after biochar soil amendments (Kolb *et al*., 2009; Kolton *et al*., 2011; Rutigliano *et al*., 2014). A plant-strengthening effect may further arise from an additional input of nutrients and potential suppression of pathogens. Therefore, the effects of biochar amendments on the growth of winter wheat were tested in model experiments on soils collected from long-term and short-term no-tillage field sites and on soils and substrates intentionally contaminated with glyphosate.

On long-term no-tillage soil with growth-suppressive potential on winter wheat, application of a pyrolysis biochar produced from woody substrate obtained from landscape conservation work (Pyreg GmbH, Doerth, Germany), completely restored normal plant development at an application rate of 5% (v/v). The beneficial effects were first detectable already during the first week after sowing (Figure 26), suggesting adsorption of a toxic soil contamination as the primary cause for the protective effect. Improved nutrient supply or stimulation of microbial activity for degradation of toxic compounds would most probably require longer time periods for expression of symptoms.

To evaluate the potential of the selected biochar preparation for detoxification of glyphosate residues, two additional winter wheat pot experiments were conducted:

On a peat culture substrate - sand mixture (1:1) characterized by an extremely low adsorption potential for glyphosate, with artificial contamination using a commercial glyphosate formulation (Roundup Ultramax®) at high application levels (8 L ha^{-1}) to create a worst-case scenario, and biochar applications at different concentrations (0, 1 and 5 % v/v).

A soil experiment conducted on a short-term no-tillage soil showing no potential for plant damage, artificially contaminated with Roundup Ultramax® (6 L ha^{-1}) and supplied with biochar concentrations of 0, 5, 10 and 20% (v/v).

In both cases, glyphosate application induced plant damage (Figure 26, Figure 43), as expected most strongly expressed on the peat culture substrate/sand mixture (Figure 43) with the lowest adsorption potential for glyphosate. Also in both cases, glyphosate damage was rapidly mitigated by biochar amendments with an optimum concentration of 5% (v/v) detectable already during seedling emergence. Lower concentrations were inefficient, and higher concentrations started to induce inhibitory effects again (Table 20, Table 21). These findings demonstrated that the selected biochar was able (i) to detoxify glyphosate residues in soils even at high contamination rates, and (ii) to protect plants also from the toxic effects on the investigated long-term no-tillage soils. However, in accordance with literature reports (Graber *et al*., 2010) high doses of biochar (≥ 10% v/v) exhibited growth inhibitory effects, since biochar contains different types and amounts of organic compounds, which can be phytotoxic at certain levels (Graber *et al*., 2010; Spokas *et al*., 2011; Kloss *et al*., 2012; Rogovska *et al*., 2012).

Based on the optimum biochar concentration of 5% (v/v) and a specific volume of 4.3 mL g^{-1} for the selected biochar product, the field application rate would translate into approximately 35 t ha^{-1}, incorporated into the 10 cm topsoil layer. This is in the upper range of biochar soil amendments reported in other field studies (Kammann *et al*., 2016). Under real field conditions, the required amounts may be lower, since the optimum biochar concentration of 5 % (v/v) was determined under worst-case conditions, shortly

after application of extremely high Roundup® doses of 6-8 L ha^{-1}, while the application rates on the investigated field sites ranged around 2 L ha^{-1}. However, in no-tillage systems, incorporation of soil amendments may be problematic due to mechanical disturbance of the topsoil layers. Nevertheless, under practical conditions, biochar applications in the seeding row may be sufficient to create a protective effect for the seedlings, since the highest contamination with glyphosate residues is expected in the uppermost soil layers (Alletto *et al*. 2010). This would further reduce also the biochar requirements, as an important economic factor in the face of current prices between € 300 to € 1,000 per ton (Kammann *et al*., 2016). Therefore, an additional pot experiment was conducted with glyphosate and biochar amendments restricted to the 5 cm topsoil layer. However, under these conditions, only a trend for improved fine root production was detected in the biochar treatments (Table 21).

As a next step, field-testing of biochar application would be indispensable but was unfortunately not possible within the available time frame of the thesis. However, it is also evident that even a successful biochar application could only provide a symptom-oriented, short-term mitigation strategy since the causes of the observed re-growth problems on the investigated long-term no-tillage field sites are not addressed with this approach. Moreover, the potential of the applied biochar for adsorption and inactivation of soil glyphosate residues may also cause problems, since similar inactivation effects can also be expected for other organic compounds, such as seed fungicides, insecticides and other herbicides with soil activity (Kookana *et al*., 2011; Nag *et al*., 2011) or secondary metabolites involved in plant-microbial signaling.

4.7.3 Long-term remediation strategies

As a consequence of increasing problems with yield depressions on the investigated long-term no-tillage field sites, meanwhile also changes in management practices have been introduced, partly by conversion into ecological farming. Other farmers still perform no-tillage cropping, including regular glyphosate application but established more variable crop rotations including winter wheat, oilseed rape, maize and soybean and started to use cover crops, such as mustard, pea and *Crotalaria* (Schiebel, 2015). In all cases, a distinct

recovery from re-plant damage has been observed during the last two years with beneficial effects also on crop health, such as reduced *Fusarium* disease. These observations may be explained by re-establishment of a more variable soil microflora, induced by the increased crop diversity, recruiting individual crop specific rhizosphere microbiomes (Mendes *et al*., 2013). Since many soil microorganisms of different phylogenetic origin can degrade glyphosate (Jacob *et al*., 1988; Arfarita *et al*., 2013), a higher soil microbial diversity is likely to promote the degradation of glyphosate soil residues and counteracts selective accumulation of crop-specific pathogens (Karlen *et al*., 1994, Gossen and Derksen, 2003). Therefore, the observed re-plant damage effects on long term no-tillage field sites, associated with delayed degradation of herbicide residues may be rather attributed to a reduction in microbial diversity, as a consequence of narrow crop rotations and monoculture, than to direct effects of long-term glyphosate use.

5 Concluding remarks

Well-documented challenges of no-tillage farming systems, comprise risks for promotion of soil pathogens (Khan, 1975; McFadden and Sutton, 1975) and allelopathic effects, (Patrick and Koch, 1958; Kimber, 1973; Rahman *et al*., 2005), particularly under conditions of limited crop rotations (Friedrich and Kassam, 2012; Ratnadass et al., 2012). The present study suggests that this can also apply for phytotoxic effects of glyphosate soil residues, as a consequence of delayed microbial degradation. The latest observations indicate that a lack of crop diversity seems to be more important in this context than potential direct effects of glyphosate on microbial communities. This is in line with the findings of a recent review covering more than 300 studies on the impact of herbicide application on soil functions (Rose *et al*., 2016). With some exceptions (e.g., repeated application of sulfonylureas), by far the majority of the studies reported only limited or only transient effects on beneficial soil functions relevant for agricultural practice after herbicide applications in recommended doses. However, this should not be generalized, since obviously, the behavior of herbicide residues in soils can be modified by management practices (e.g., crop rotation, cover crops) and may lead to unexpected residual effects, even when herbicide application is performed according to the recommendations. Of particular importance is the finding that under these conditions, even sub-toxic concentrations and even metabolites of minor toxicity, such as AMPA can affect crop performance by cumulative effects. The mechanisms are still largely unknown and require further investigations at the molecular and physiological level. The same holds true for potential synergisms or antagonisms (Serra *et al*., 2013) since frequently combinations of herbicides, fungicides, and insecticides are applied during the culture period. Also, the impact of soil properties and climatic factors needs to be addressed more in detail.

The importance of crop diversity management for the sustainability of no-tillage systems is further illustrated by a recent meta-analysis, summarizing the effects of crop rotation and cover crops on yield formation in no-tillage systems obtained from 610 field experiments in 63 countries (Pittelkow *et al*., 2015). As also observed in the present study, the results of the meta-analysis demonstrate continuously increasing yield losses in

no-tillage systems lacking crop rotations and/or cover crops (Figure 11, Figure 13), with increased pathogen pressure, accumulation of allelochemicals and herbicide residues as potential causes.

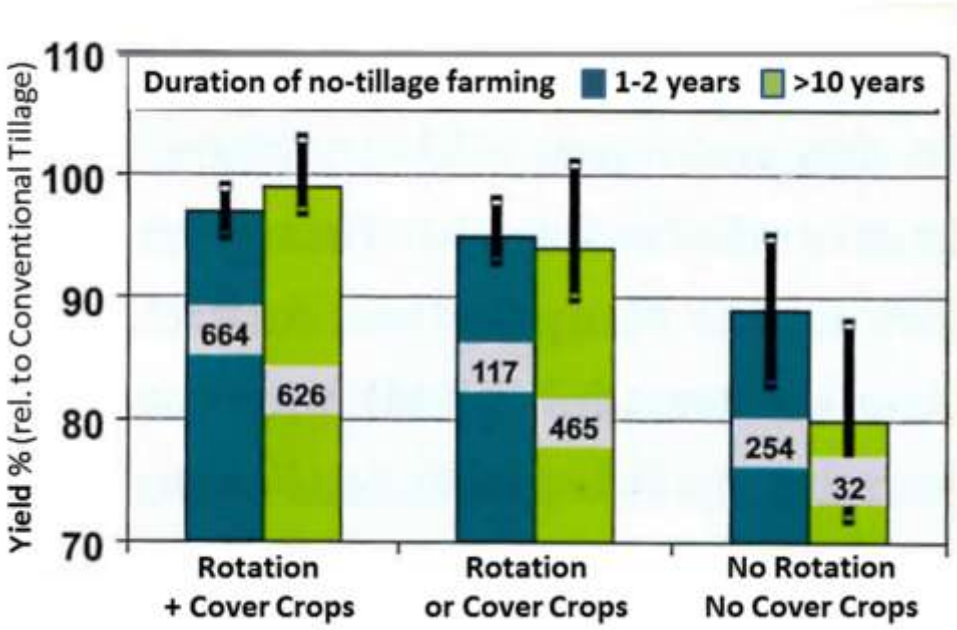

Figure 47: Meta-analysis of yields losses, depending on the time of no-tillage cropping and the integration of crop rotations and cover crops. Numbers within bars indicate the number of observations (modified after Pittelkow et al., 2015 and Finckh et al., 2016).

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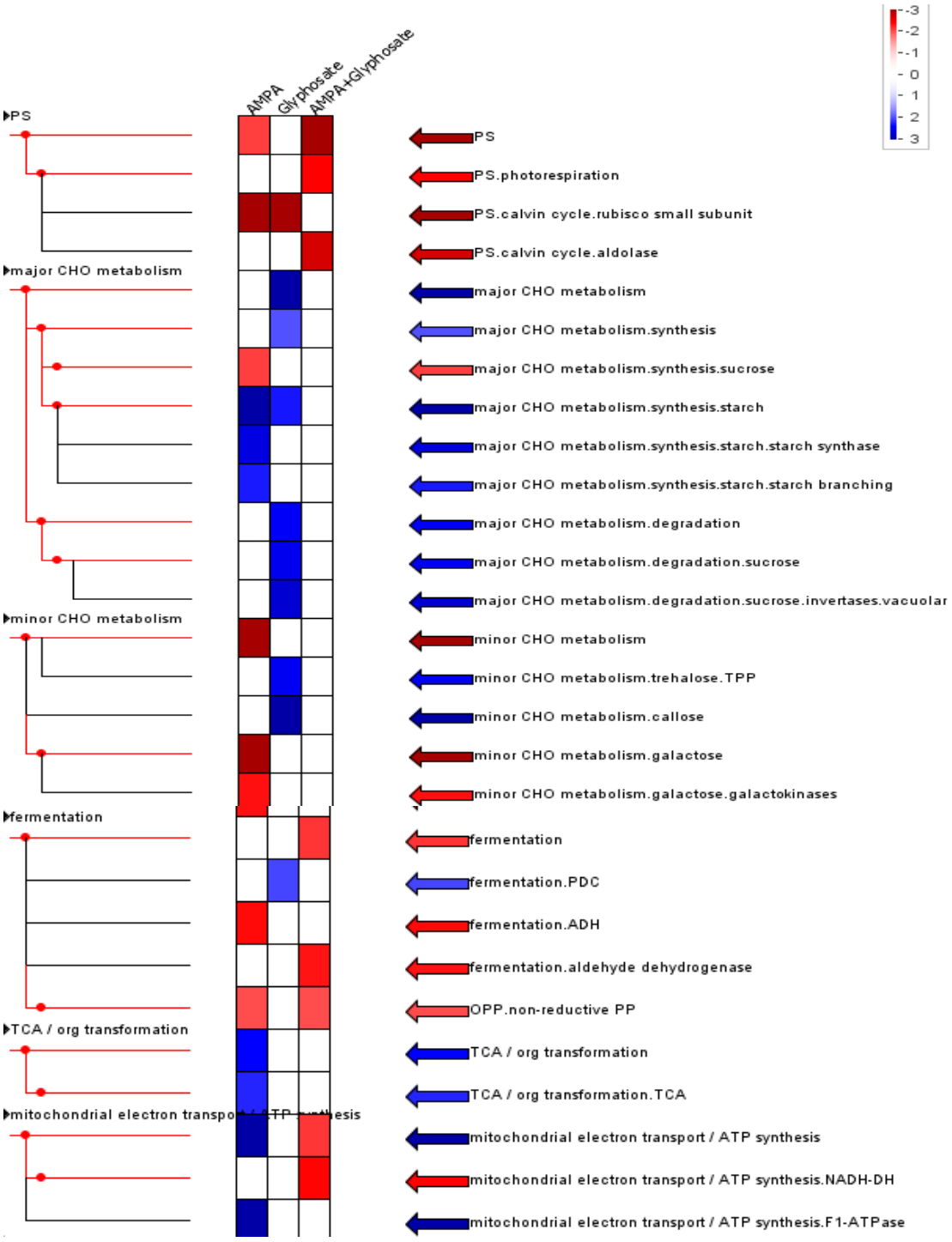
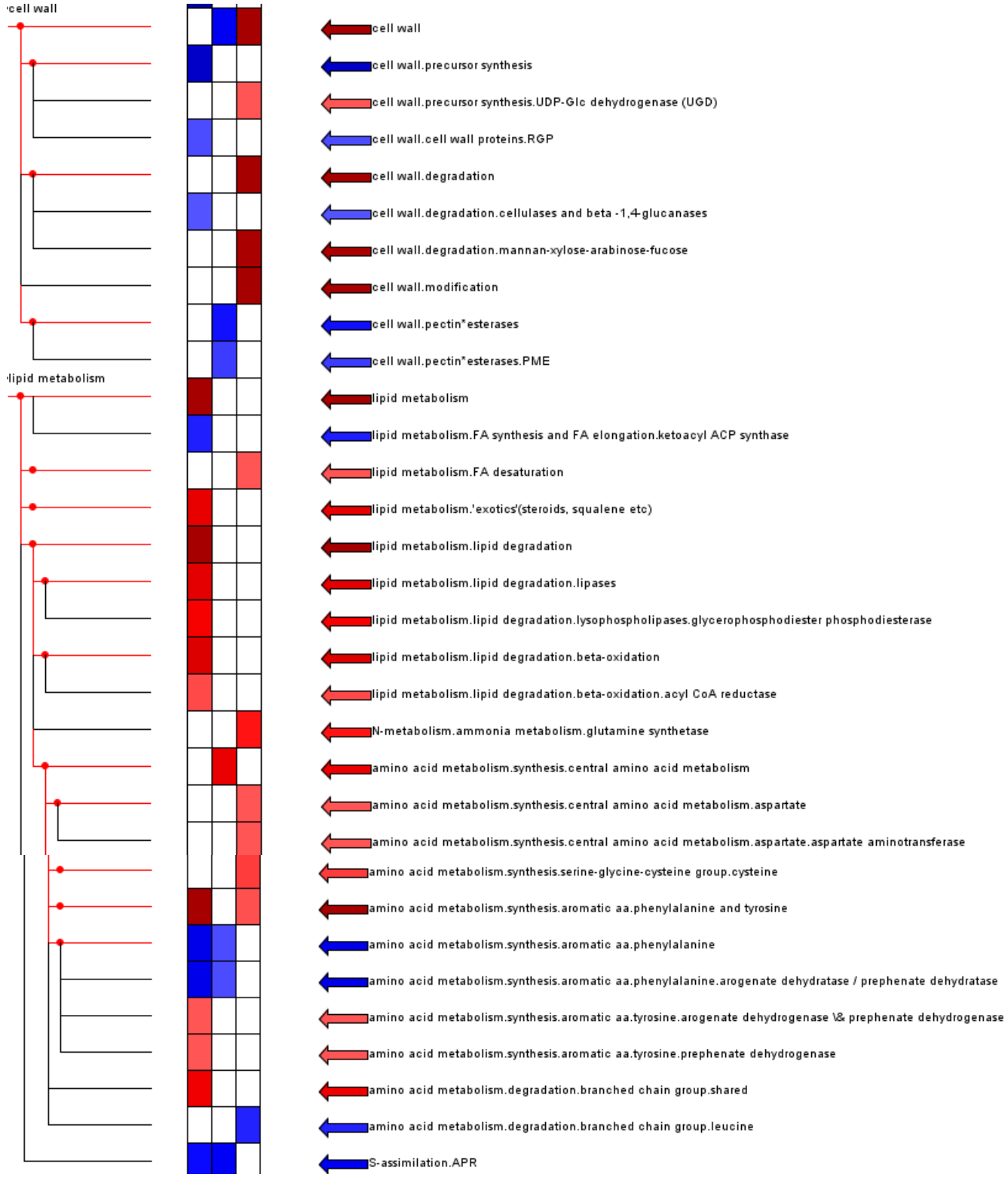
7 Appendix

Complete overview (Pageman) on alterations in gene expression

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cell wall
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cell wall.degradation.cellulases and beta -1,4-glucanases
cell wall.degradation.mannan-xylose-arabinose-fucose
cell wall.modification
cell wall.pectin*esterases
cell wall.pectin*esterases.PME
lipid metabolism
lipid metabolism.FA synthesis and FA elongation.ketoacyl ACP synthase
lipid metabolism.FA desaturation
lipid metabolism.'exotics'(steroids, squalene etc)
lipid metabolism.lipid degradation
lipid metabolism.lipid degradation.lipases
lipid metabolism.lipid degradation.lysophospholipases.glycerophosphodiester phosphodiesterase
lipid metabolism.lipid degradation.beta-oxidation
lipid metabolism.lipid degradation.beta-oxidation.acyl CoA reductase
N-metabolism.ammonia metabolism.glutamine synthetase
amino acid metabolism.synthesis.central amino acid metabolism
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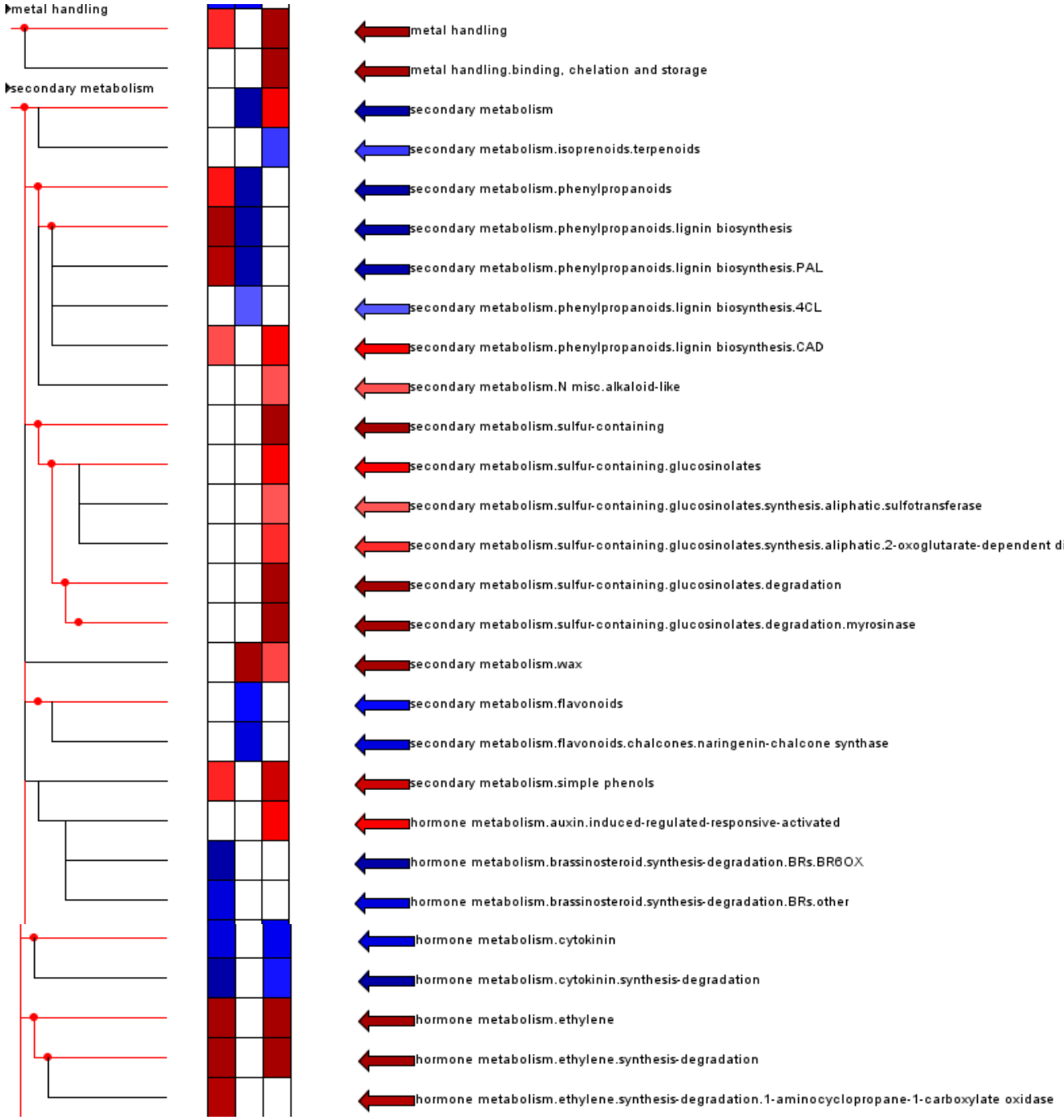
S-assimilation.APR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metal handling
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secondary metabolism
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secondary metabolism.phenylpropanoids.lignin biosynthesis.PAL
secondary metabolism.phenylpropanoids.lignin biosynthesis.4CL
secondary metabolism.phenylpropanoids.lignin biosynthesis.CAD
secondary metabolism.N misc.alkaloid-like
secondary metabolism.sulfur-containing
secondary metabolism.sulfur-containing.glucosinolates
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secondary metabolism.flavonoids
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secondary metabolism.simple phenols
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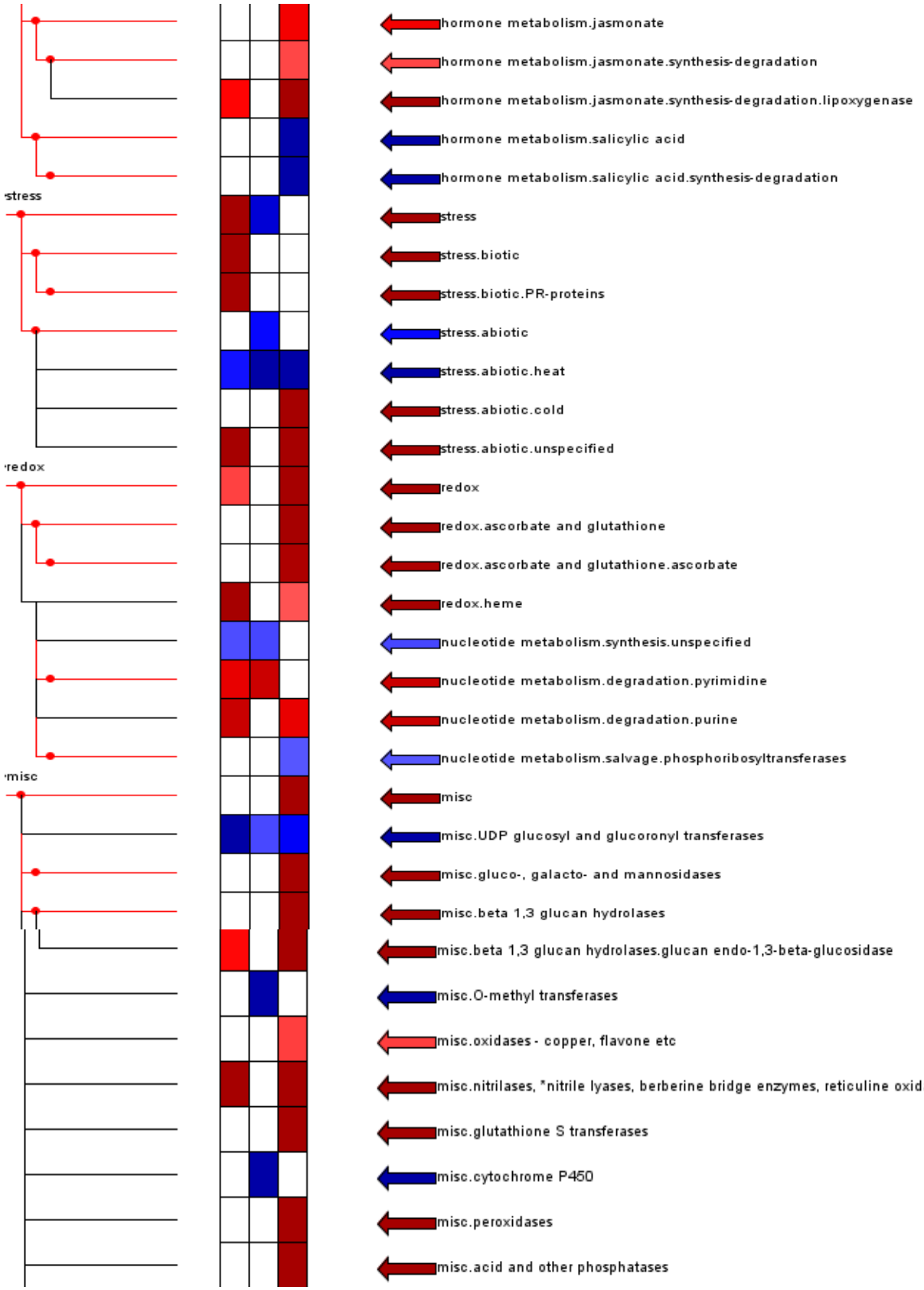
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stress.biotic.PR-proteins
stress.abiotic
stress.abiotic.heat
stress.abiotic.cold
stress.abiotic.unspecified
redox
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redox.ascorbate and glutathione.ascorbate
redox.heme
nucleotide metabolism.synthesis.unspecified
nucleotide metabolism.degradation.pyrimidine
nucleotide metabolism.degradation.purine
nucleotide metabolism.salvage.phosphoribosyltransferases
misc
misc.UDP glucosyl and glucoronyl transferases
misc.gluco-, galacto- and mannosidases
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misc.beta 1,3 glucan hydrolases.glucan endo-1,3-beta-glucosidase
misc.O-methyl transferases
misc.oxidases - copper, flavone etc
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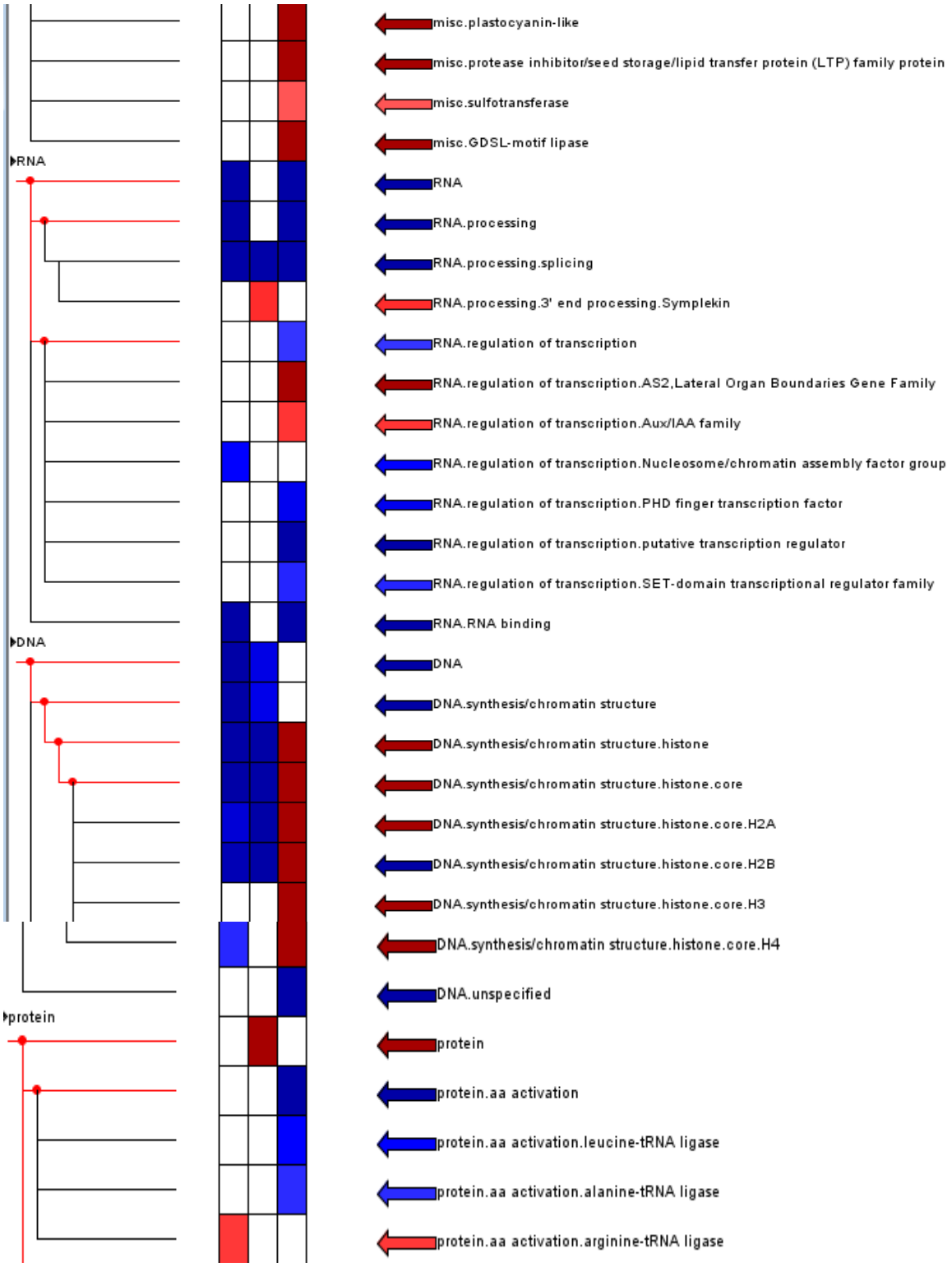
misc.acid and other phosphatases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RNA.processing.splicing
RNA.processing.3' end processing.Symplekin
RNA.regulation of transcription
RNA.regulation of transcription.AS2,Lateral Organ Boundaries Gene Family
RNA.regulation of transcription.Aux/IAA family
RNA.regulation of transcription.Nucleosome/chromatin assembly factor group
RNA.regulation of transcription.PHD finger transcription factor
RNA.regulation of transcription.putative transcription regulator
RNA.regulation of transcription.SET-domain transcriptional regulator family
RNA.RNA binding
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DNA.synthesis/chromatin structure
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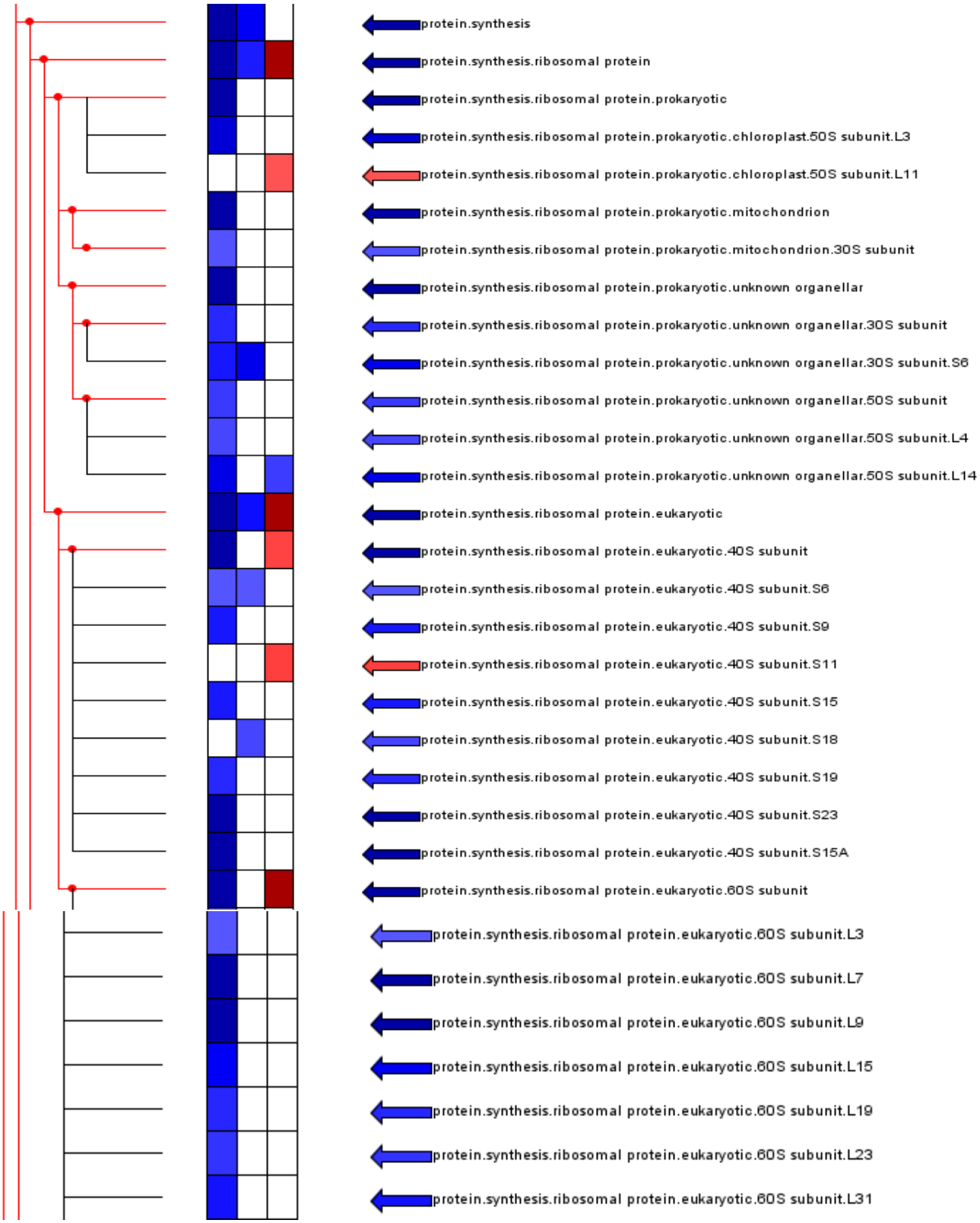
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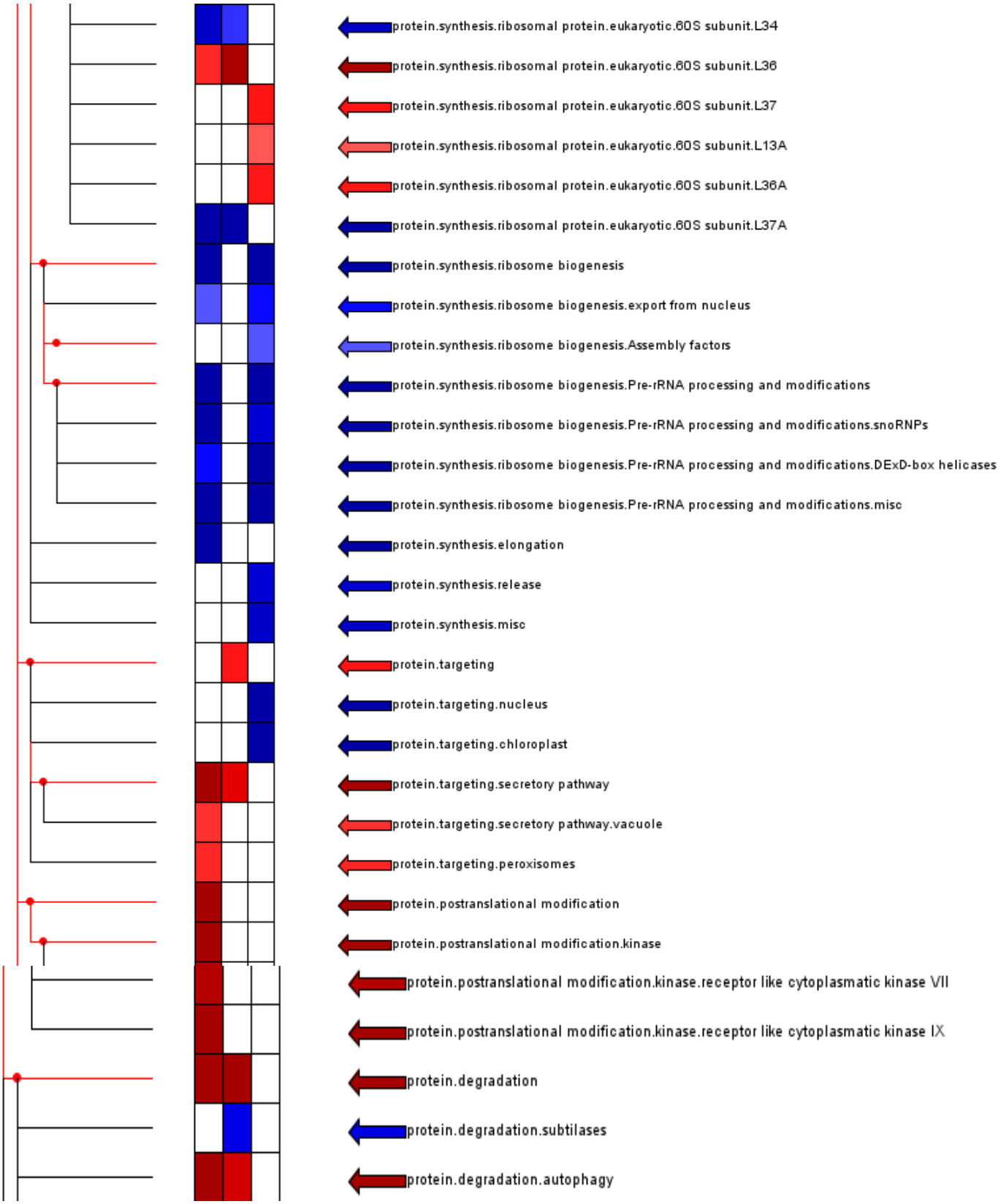
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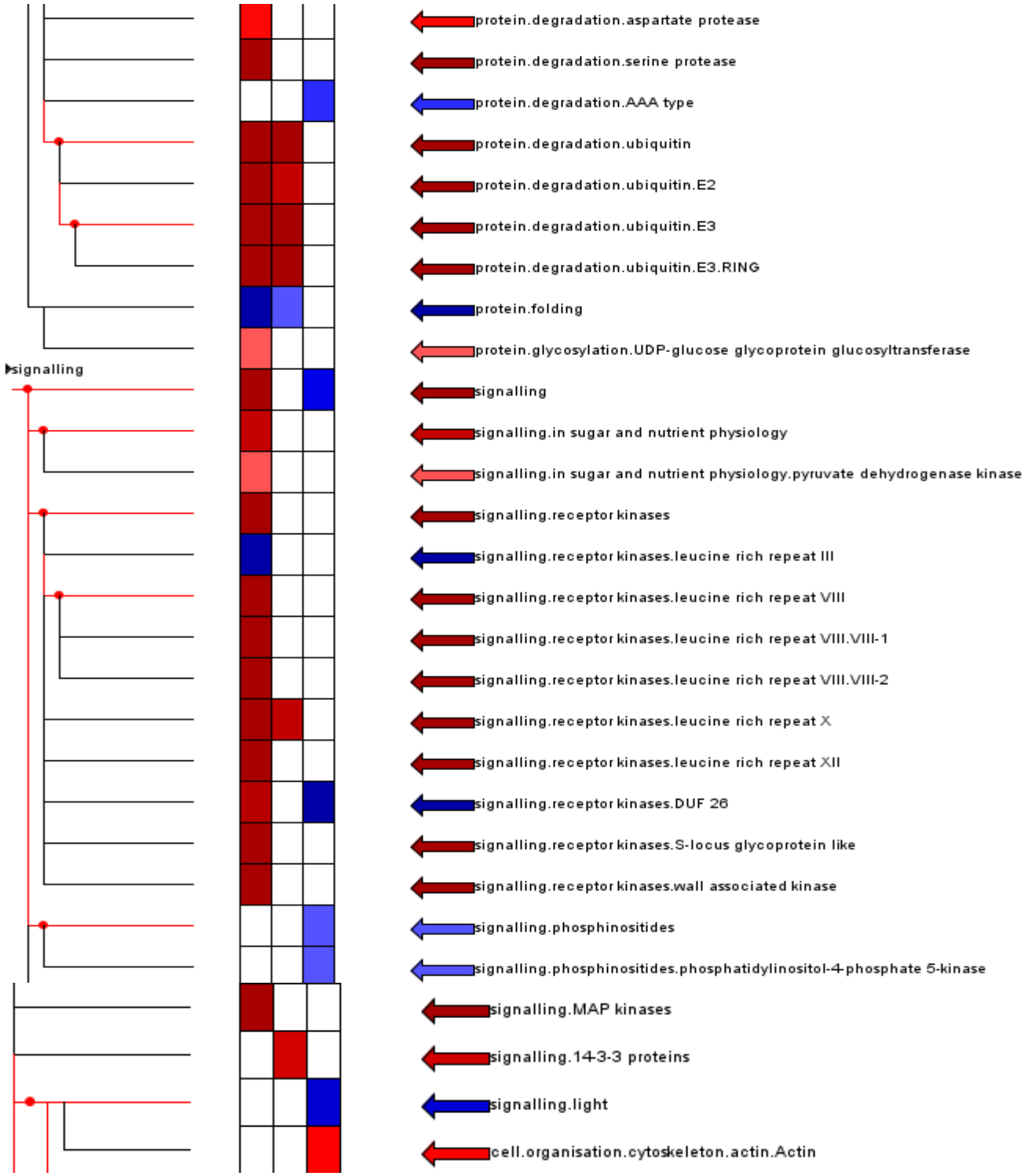
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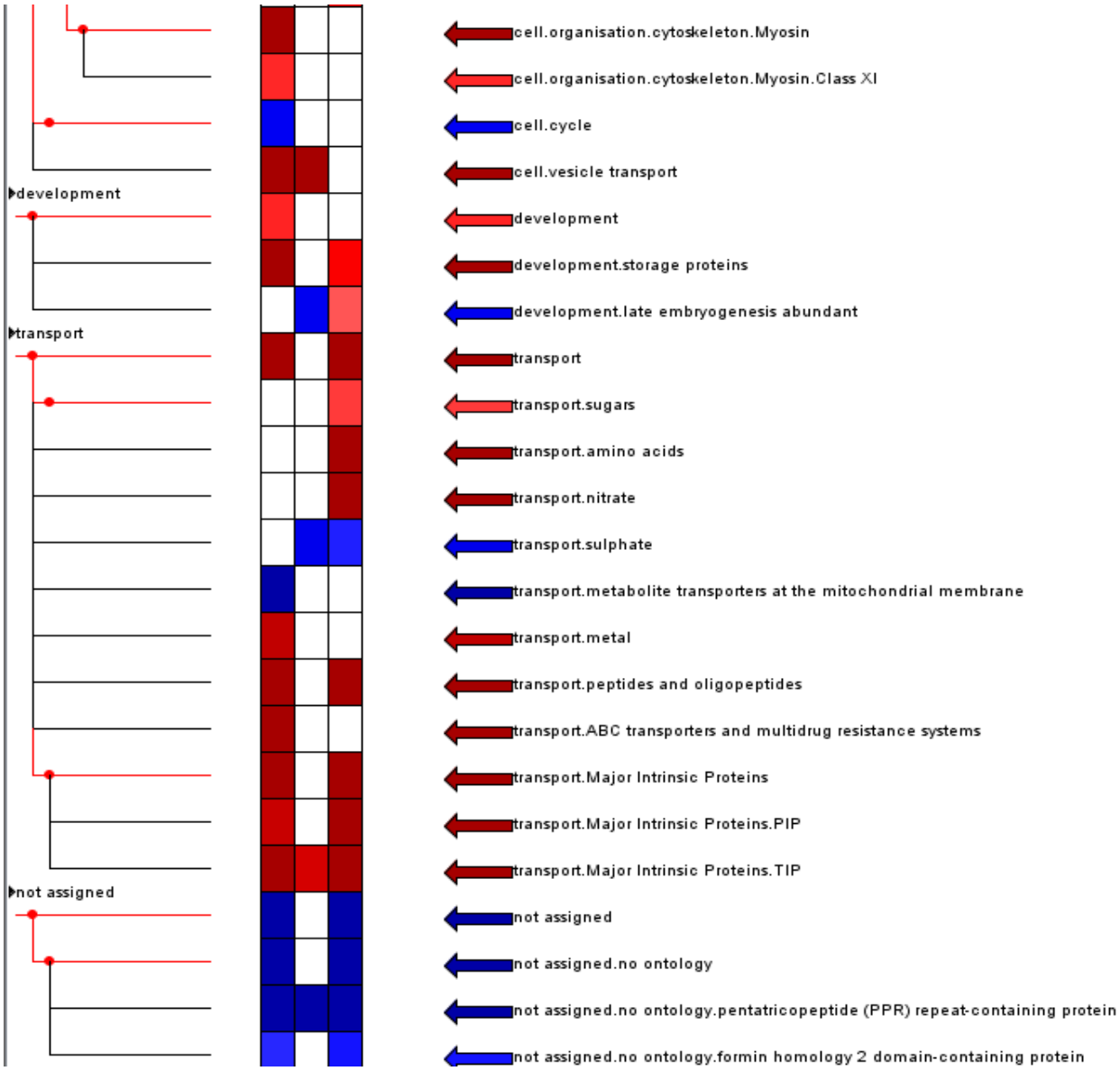
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8 Curriculum vitae

AFZAL

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phyto_pk@yahoo.co.uk

EDUCATION & QUALIFICATIONS

2007-2009: M.Sc. (Hons.) Agriculture (Plant Pathology) University of Agriculture, Faisalabad (UAF), Pakistan

2003-2007: B.Sc. (Hons.) Agriculture, (Plant Pathology) University of Agriculture, Faisalabad, Pakistan

2001-2003: F. Sc. (Pre-Medical)

Govt. Degree College, Khanewal

2001: Matriculation (Science Subjects)

St. Joseph's High School, Khanewal

TECHNICAL SKILLS

- Plant RNA extraction and sequencing
- Root morphology measurements using WhiRhizo™ software
- Hydroponic cultivation system
- Plant tissue culture and Oster mushroom cultivation

SUPERVISING EXPERIENCE

2011-2013: Supervision of 8 Bachelors and 2 Masters students in glyphosate project (Institute of Crop Sciences (340h), University of Hohenheim, Stuttgart).

2008-2009: Co-supervision of 4 Masters students in plant disease diagnosis lab (University of Agriculture, Faisalabad).

ACHIEVEMENTS

- Best poster presentation award, ISRR Conference, Dundee, Scotland, 2012
- Scholarship award for Ph.D., KAAD Germany, 2011
- Selected for merit scholarship, Department of Plant Pathology, UAF, 2008

COMPUTER SKILLS

PagMan, MapMan, WinRhizo™, SigmaPlot®, SPSS®
M.S® Word, Excel & Power Point, Computer Installation& Hardware

LANGUAGE

-English (Fluent) -Urdu (Fluent) -Punjabi (Fluent) -German (Basic)

PUBLICATIONS & PARTICIPATIONS

Martha M, Sebastan H, Günter N, Afzal, Wolfram R. (2017). Glyphosate, a chelating agent – relevant for ecological risk assessment? *Submitted to Environmental Science and Pollution Research.*

Afzal A., Müller D, Jocher F., Tesfamariam T., Bott S., Römheld V., Neumann G. (2016). Long-term exposure to sub-toxic levels of the glyphosate metabolite AMPA can explain plant damage in no-tillage winter wheat production systems with long-term glyphosate use in southwest Germany. *Proceedings German Plant Nutrition International Conference 2016,* Stuttgart-Hohenheim, Germany, page 71.

Heinrichs R., Afzal A., Machado, J.L., Müller D., Schmid P., Kühn, B., Bott S., Römheld V., Neumann G. (2013). Long-term no-tillage winter wheat production affected by delayed degradation of herbicide residues in soils. *XVII international plant nutrition colloquium & boron satellite meeting*, page 231

Afzal A, Müller D, Jocher F, Tesfamariam T, Bott S, Römheld V, Neumann G. (2012). Limitations of no-tillage winter wheat production with long-term glyphosate use in South-West Germany. *Proceedings 8th symposium of International Society of Root Research 2012,* Dundee, Scotland, page 85.

Neumann G., Afzal A., Bott S., Tesfamariam T., Römheld V. (2012). Was passiert an der Wurtzel. *DLG-Mettellungen 2012 (2),* 26-29.

Afzal A. (2009). Incidence of Mango Sudden Death Syndrome caused by *C eratocystis fimbriata*, its physiology and epidemiology. Master's thesis. University of Agriculture, Faisalabad.

Poster Presentation in 3 international conferences.

Participation in 15 international seminars.

WORKING EXPERIENCE

University of Hohenheim Stuttgart, August 2014- till date

- Bioeffectors project

University of Hohenheim Stuttgart, December 2010- July 2014

- Glyphosate project
- Research on biochat and bioeffectors

University of Agriculture Faisalabad, Oct 2008- Oct 2009

- Plant disease diagnosis**,** collection and preserves of fungus cultures.
- Research on tree decline and biocontrol.

Mango Research Station Shujabad, Jan 2007- July 2007

- Research on Mango sudden death syndrome and biocontrol
- Biocontrol through plant extracts and rootstalk resistance

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