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Herbicide Resistance

Molecular and Physiological Characterization of the Glyphosate Resistant Weeds *Amaranthus* ssp. and *Sorghum* ssp.

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Herbicide Resistance. Molecular and Physiological Characterization of the Glyphosate Resistant Weeds *Amaranthus* ssp. and *Sorghum* ssp..

Herbicides are an important tool for agricultural production to control weeds, avoid soil erosion, and maintain high yields. Highly competitive weeds like *Amaranthus* ssp. and *Sorghum halepense* have to be controlled in key crops like corn, cotton and soybean. During the past 30 years glyphosate has proven to be the most cost effective and environmentally benign herbicide controlling these weeds without significant resistance development. Since the introduction of glyphosate tolerant crop varieties and the almost exclusive use of glyphosate to control weeds, the development of glyphosate resistance has recently increased dramatically and is today in the Southeast of U.S.A. the most important threat to cropping systems. In order to maintain a sustainable agricultural production, weed resistance mechanisms and their spread in weed populations have to be better characterized and understood.
The response of several ***Amaranthus palmeri*** and ***Amaranthus tuberculatus*** populations, collected across the South- and Mideast of the U.S.A., to the herbicide glyphosate was studied and for most of them resistance was detected or confirmed by performing a dose response curve in the greenhouse. Neither a reduction of glyphosate uptake or translocation was observed in the glyphosate resistant plants, nor was a mutation in glyphosate target enzyme EPSPS at the amino acid position G101, T101 or P106 detected. The analysis of the *EPSPS* gene copy number revealed that almost all glyphosate resistant populations possessed variable but high *EPSPS* gene copy number which was also correlated with its expression both at the RNA and protein levels and with the resistance level observed in the greenhouse. In resistant *A. tuberculatus* the *EPSPS* gene amplification and the expressed resistance factors found were lower than in *A. palmeri*. Nevertheless, it is possible to conclude that the *EPSPS* gene amplification is the main glyphosate resistance mechanism in the *A. tuberculatus* populations analyzed. In *A. palmeri EPSPS* gene amplification is the most common and most important resistance mechanism found so far as shown by its widespread geographical occurrence through the U.S.A. and by the high resistance factors conferred, but it is not the only resistance mechanism developed by this weed species. RAPD analysis of several glyphosate sensitive and resistant *A. palmeri* populations reveals a stronger relationship based on the response to glyphosate than based on geographical separation. This suggests that the glyphosate resistant individuals have a common ancestor plant or population. These data are discussed related to plant migration, in particular plant seed dispersal, which seems often to be underestimated. These findings stress the importance of farm and field hygiene for weed management to prevent field infection with nearly uncontrollable weeds and lastly to protect efficient crop production.
A ***Sorghum halepense*** (Johnsongrass) population collected in AR, U.S.A. was found resistant to glyphosate and to APP ACCase inhibitors. The resistances were confirmed in greenhouse experiments. The resistance of mature plants to glyphosate was moderate, with a resistance factor of 3.6. The *EPSPS* gene sequence was analyzed for the known mutation sites G101, T102 and P106, but no changes were detected, suggesting that resistance to glyphosate was not caused by these point mutations. A heterozygous target site mutation, W2027C, on the *ACCase* gene sequence was found to cause the resistance to the ACCase inhibitors fluazifop-p-butyl and decreases the sensitivity to quizalofop and clethodim. In this tetraploid species, plants possessing 2 mutated *ACCase* alleles, out of a total of 4, were shown to be less affected by APP ACCase inhibitor treatments than individuals possessing a single mutated *ACCase* allele. Other known *ACCase* gene mutations conferring resistance were not detected in this population. ALS, HPPD and glutamine synthetase inhibitors, further options for Johnsongrass control, were checked and gave good initial control under greenhouse conditions. To our knowledge, this is the first *S. halepense* population with a reported multiple herbicide resistance showing a specific target site mutation conferring resistance to ACCase herbicides. Moreover it is one of the rare evidence showing that the herbicide resistance observed is directly correlated to the number of mutated alleles.

Herbizid Resistenz. Molekularbiologische und Physiologische Charakterisierung der glyphosat-resistenten Unkrautarten *Amaranthus* ssp. und *Sorghum* ssp.

Herbizide sind ein wichtiges Hilfsmittel in der landwirtschaftlichen Produktion um Unkräuter zu kontrollieren, Bodenerosion zu vermindern und hohe Erträge zu sichern. Gerade in wirtschaftlich wichtigen Kulturen wie Mais, Baumwolle und Sojabohne müssen daher konkurrenzstarke Unkräuter wie *Amaranthus* ssp. und *Sorghum halepense* wirkungsvoll bekämpft werden. Seit der Einführung von glyphosattoleranten Kulturpflanzen und des nahezu flächendeckenden Einsatzes des herbiziden Wirkstoffs Glyphosat, hat die Zahl der glyphosatresistenten Unkräuter allerdings rapide zugenommen und ist inzwischen zu einem der größten Probleme in der Landwirtschaft in weiten Teilen der U.S.A. geworden. Um die landwirtschaftliche Produktion in diesen Regionen langfristig zu sichern ist es daher wichtig die Resistenzmechanismen und die Ausbreitung von resistenten Unkräutern besser zu erforschen und zu verstehen.
Verschiedene ***Amaranthus palmeri*** und ***Amaranthus tuberculatus*** Populationen aus dem südlichen und mittleren Osten der U.S.A. wurden auf ihre Glyphosatresistenz hin untersucht. Diese konnte in Dosis-Wirkungsbeziehungen in den meisten Populationen bestätigt oder erstmalig nachgewiesen werden. Unterschiede in der Glyphosataufnahme oder Verlagerung innerhalb der Pflanze sowie *EPSPS* Target-Site-Mutationen (TSM) konnten als Grund für die Resistenz nicht nachgewiesen werden. In nahezu allen glyphosatresistenten Pflanzen wurde aber, korreliert mit dem Grad der Resistenz, eine höhere *EPSPS* Genkopienanzahl im Genom gefunden. Dies korrelierte in zwei *A. palmeri* und drei *A. tuberculatus* Populationen auch mit dem EPSPS-Gehalt in Transcriptom und Proteom. Im Vergleich zwischen den Arten waren die ermittelten Resistenzfaktoren sowie die *EPSPS* Genamplifikation in *A. tuberculatus* niedriger als in *A. palmeri*, dennoch ist anzunehmen, dass in beiden Arten die Glyphosatresistenz auf *EPSPS* Genamplifikation beruht. Der Nachweis des gleichen Resistenzmechanismuses in unterschiedlichen *A.palmeri* Populationen zeigt dass dies der wichtigste und am weitesten weitverbreitete Resistenzmechanismus in dieser Unkrautart ist. Eine schwach resistente Population ohne *EPSPS* Genamplifikation zeigt aber, dass sich weitere Resistenzmechanismen entwickelt haben. RAPD Verwandschaftsanalysen belegen zudem eine hohe genetische Variabilität innerhalb der Populationen und in *A. palmeri* zudem eine Abhängigkeit der Verwandschaft von der Glyphosatresistenz. Die verschiedenen glyphosatresistenten *A. palmeri* Populationen scheinen sich daher aus einem gemeinsamen Vorfahren entwickelt zu haben. Diese Ergebnisse zeigen, dass auch im Ackerbau die Verbreitung von Unkrautvermehrungsgut vermieden werden muss, um einer Besiedlung durch nicht mehr kontrollierbare und herbizidresistente Unkräuter vorzubeugen und damit eine effiziente landwirtschaftliche Produktion für die Zukunft zu sichern.
Eine ***Sorghum halepense*** Population aus AR, U.S.A. wurde als Glyphosat und APP ACCase inhibitor resistent beschrieben, welches wir unter Gewächshausbedingungen bestätigen konnten. Für Glyphosat wurde so ein Resistenzfaktor von 3,6 ermittelt. In der *EPSPS* Gensequenz wurden daraufhin die resistenzauslösenden Mutationsstellen G101, T102 und P106 sequenziert. Eine Veränderung wurde nicht gefunden, so dass die Glyphosatresistenz nicht durch TSM in der *EPSPS* bedingt ist. In der *ACCase* Gensequenz wurde eine TSM in W2027C gefunden, die die Resistenz gegenüber dem ACCase Inhibitor Fluazifop-p-butyl und die verringerte Wirksamkeit von Quizalofop and Clethodim erklärt. Im Vergleich war die APP ACCase Resistenz der tetraploiden Pflanzen bei 2 mutierten *ACCase* Allelen stärker als bei Pflanzen mit nur einem oder ohne W2027 mutiertem *ACCase* Allel im Genom. Weitere TSM in der *ACCase* Gensequenz konnten in dieser Population nicht nachgewiesen werden. Auch ALS, HPPD und Glutamin Syntethase Inhibitoren wurden auf ihre Wirksamkeit im Gewächshaus geprüft und zeigten keine Anzeichen einer verringerten Wirksamkeit. Nach unserem Wissenstand ist dies die erste multiple Herbizid resistente *S. halepense* Population und die erste in der eine bestimmte TSM für die Resistenz gegenüber ACCase Inhibitoren verantwortlich ist. Zudem ist dies eines der wenigen Beispiele für den Zusammenhang zwischen der Anzahl der mutierten *ACCase* Allele und den daraus resultierenden Unterschieden in der Herbizidresistenz *in planta*.

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1 List of Abbreviations

AA	aminoacid
ae	acid equivalent
a.i.	active ingredient
AMOVA	analysis of molecular variance
ANOVA	analysis of variance
ATP	adenosintriphosphat
BBCH	growth stages of mono- and dicotyledonus plants. Cooperative development of Biologische **B**undesanstalt, **B**undessortenamt and **Ch**emical industry.
BLAST	"basic local alignment search tool"
bp	base pair
Bq	becquerel, decays per second
C	celsius
cDNA	copy-desoxyribonukleinsäure
Ci	curie, 1 Ci = 3,77 * 10^{10} Bq
DAT	days after treatment
DIG	dioxigentin labeling
DNA	desoxyribonukleinsäure
ssDNA	"single stranded DNA"
dsDNA	"double stranded DNA"
dpm	decays per minute
E	einstein, 1 E = 1 mol Quanten (Photonen)
$ED_{(x)}$	effective Dose rate, necessary herbicide concentration to provoke x % injury of the total observed
EDTA	ethylendiamintetraacedicacid
e.g.	in example
EPSPS	5-enolpyruvylshikimic acid-3-phosphate synthase (EC 2.5.1.19)
EtBr	ethidiumbromid
Fig.	figure
g	gram, 1 g = 10^{-3} kg
kg	kilogram
GMO	genetic modified organism
h	hours
ha	hectar, 1 ha = 10 000 m^2
HAT	hours after treatment
i.e.	in particular

kb	kilobases, 1000 bp
keV	kilo-electron-volt
kPA	kilo pascal
L	liter
μl	mikroliter, 1 μl = 10^{-6} l
ml	milliliter, 1 ml = 10^{-3} l
Lux	lx = lm * m^{-2}
M	molar
m	meter
m^2	square meter
nm	nanometer, 1 nm = 10^{-9} m
MoA	Mode of Action
NCBI	"National Center for Biotechnology Information"
NIS	non ionic surfactant
n.s.	not significant
P_i	inorganic phosphate
PCR	polymerase chain reaction
qPCR	quantitative polymerase chain reaction
pH	"potentia hydrogenii"
P value	probability value
RAPD	random amplified polymorphic DNA
rcf	relative centrifugation force
RNA	ribonucleic acid
mRNA	messenger ribonucleic acid
RR	abbreviation for Roundup Ready resistant crops
RT	reverse transcriptase
s	second
ms	millisecond
SNP	single nucleotide polymorphism,
T_m	melting temperature ds-DNA [°C]
Tab.	table
Vol	volume
WAT	weeks after treatment

2 Introduction

The human population has more than doubled during the past 50 years - to around 6.75 billion people in 2008, passing 7 billion by the end of 2011 (FAO, 2011). During this period arable land has increased by only 9.6 %, and further increases will be limited (FAO, 2011). The area of available arable land per person has decreased by half over the last 50 years, from 1.45 ha per person in 1961 to approximately 0.72 ha 47 years later in 2008 (FAO, 2011). Despite this reduction, the global average yield of cereals, for example, has increased approximately 3-fold over this period, from 1.35 t ha^{-1} in 1961 to 3.57 t ha^{-1} in 2009, due to improvements in agricultural production and technology (FAO, 2011). However, the forecast for 2050 is for further human population growth to around 9.15 billion (FAO, 2011). To be able to produce enough food and feed, fibers and fuel, agriculture must find solutions to maintain and even increase yields to compensate for population growth.
The higher demand for food and feed is not only caused by the rapidly growing population, but also by the acceleration of economic development in several developing and third world countries (*i.e.,* China or Brazil). The growing incomes and growing settlements and infrastructure of these regions, world-wide demand for regenerable and CO_2 neutral energy *e.g.* biofuel, and the increasing demand for organic food is increasing the need for agricultural products while further decreasing the available arable land. The higher demand for agricultural products and the direct competition of renewable energy will lead to a higher volatility and prices in agricultural commodity markets, which might limit the available amount of food, particularly for the less developed countries (Owen, 2008; FAO, 2011). Between 2005 and 2007 about 13 % of the human population on earth was considered to be undernourished (FAO, 2011). Increasing food prices will also undermine political stability. For example, the Tortillia-crisis 2007 in Mexico was at least partly caused by rapidly rising costs for staple food corn (Rosset, 2009). Limits to increases in arable land and the required further increase in crop production will challenge agriculture to intensify and optimize all practices used.
One of the key areas of innovation necessary to maintain or even increase yields is protection of crops from abiotic and biotic stresses. Biotic stresses to crop can be caused by viruses, bacteria, fungi, insects or other plants, here called weeds. "Weeds are the most important pest complex impacting mankind" (Owen, 2011) and are, in general undesiderable, unattractive, or troublesome plants, especially those growing where unwanted (Harlan & deWet, 1965; Owen, 2011).

2.1 General Aspects in Weed Development and Management

As pioneer plants many weeds are well adapted to disturbed soils and have the ability to produce a high number of seeds to help increase the chances of survival and proliferation of the species. Weeds compete with crops for light, water, nutrients and space. They can

also interfere with cultivation, can harbor crop pests and diseases by offering conditions for their development and can weaken the health of crop plants by increasing their susceptibility to diseases, insects or abiotic stresses. Crops can also be considered as weeds. Oilseed rape[1], for example, is an important competitor of corn[2] or wheat[3] as a volunteer. Worldwide, potential yield losses of about 34 % are attributed to weeds (Basu et al., 2004). Oerke, (2006) reported losses of about US $ 20 billion each year in U.S. agriculture due to weeds, despite the use of herbicides. Furthermore, Owen (2011) predicted a decline in agricultural productivity by 20 % if herbicides would not be available.

The need to constrain yield losses and to practice sustainable weed control will require the farmer to adapt and combine weed control tools depending on crop, cropping system and weed population present in the fields. Although mechanical or chemical weed control tools have been availiable for a long time, not a single weed has been eradicated (Tuesca & Puricelli, 2007). Control options can therefore only provide a reduction in weed pressure and a shift in the species composition at a given site, depending on the level of selection pressure and the cropping system used. In fact, herbicides can both reduce or maintain weed diversity, depending on weed species and use pattern (Tuesca & Puricelli, 2007).

Several weed species have been spread worldwide through to commodities trade and cause problems in many different cropping systems, regions and countries. Weeds are often highly opportunistic plants and are able to survive and adapt to a wide range of ecological conditions. Thus weed populations vary between native and invasive species depending on region, climate, soil or cropping system and by the problems they cause (Neve et al., 2009). In 1995, for example, the Southern Weed Science Society, USA, published an annual survey of the most troublesome weeds, defined as those that cause the greatest monetary loss and are the most difficult to control. The four most troublesome weeds in Georgia cotton, in 1995 were *Senna obtusifolia*, *Senna occidentalis*, *Xanthium strumarium* and *Desmodium tortuosum*. Ten years later, in 2005, and 8 years after the introduction of glyphosate resistant cotton cultivars, the list changed completely. The previous weeds were replaced by weeds not effectively controlled by glyphosate like *Richardia scraba*, *Commelina communis*, *Commelina benghalensis* and *Amaranthus palmeri* (Webster & Sosnoskie, 2010).

Many weeds have been selected together with the crop or cropping system over centuries and are well adapted to agriculture through co-selection and co-evolution. The seeds or propagation organs of several weeds are able to withstand unfavorable conditions over extensive periods, either through high seed production, longevity in the soil seed bank or their ability to germinate over a very long time period, often throughout the entire growing season. In addition many weed seeds are easily distributed through wide distances *e.g.* by wind, water, animals or human activities and can infect even a whole continent within a

[1] *Brassica napus*
[2] *Zea mays*
[3] *Triticum aestivum*

few years (Christoffers, 1999). Nevertheless, detailed knowledge about the long distance seed- or trait-dispersal related to weeds still needs to be developed further. Especially long distance seed dispersal rates are difficult to measure and might be covered in a lot of models used by the high amount of seeds spread in a close distance around the mother plant (Cain et al., 2000). According to Cain et al. (2000) long distance seed dispersal rates are often underestimated and do not reflect true dispersal rates. Transport distances for example of more than 500 m are reported for the seeds of *Conyza canadensis*, dispersed by wind (Dauer et al., 2007). Some of these seeds have been collected at altitudes of 140 m in the Planetary Boundary Layer where seeds can easily be distributed over hundreds of kilometers (Shields et al., 2006). *Amaranthus palmeri* or *A. tuberculatus* seeds are able to germinate and propagate nearly throughout the entire growing season and quickly go to seed, giving it a competitive advantage (Keeley et al., 1987). In addition, *A. tuberculatus*, *A. retroflexus* and *Sorghum halepense* seeds have a high longevity in the soil seed bank and can germinate even after years. A germination rate of 14 % and 7 % 12 years after being buried 20 cm deep in fine sandy loam have been reported for *A. tuberculatus* and *A. retroflexus* seeds, respectively (Burnside et al., 1996). *A. palmeri* seeds are able to withstand the composting process in the surface of cotton gin trash piles for two years (Norsworthy et al., 2009). *A. retroflexus* seeds are able to withstand a silage process, rumen digestion or being buried in manure still showing a significant number of viable seeds (Blackshaw & Rode, 1991; Larney & Blackshaw, 2003). Egley & Chandler (1983) reported a germination rate for *S. halepense* seeds of 48 % after being buried in sandy loam for 5.5 years. Furthermore, *S. halepense* is another example of how weeds can regenerate after a long storage period in the soil. This weed is still able to regrow with high efficiency from rhizomes, even when heavily injured, making such weeds very competitive and in addition extremely difficult to control. Weeds have evolved mechanisms to ensure that each season enough plants bearing any favorable traits that help to overcome unfavorable conditions including herbicide treatments, are able to reproduce and multiply, and contribute to the soil seed bank (Mortimer, 1997; Tuesca & Puricelli, 2007). Several weeds are self-pollinating, limiting the abbility to distribute such traits but in many cases they are able to out cross to some extent (Barrett, 1982). A pollen-mediated spread of herbicide resistance over a distance of more than 21 km was reported for Creeping Bentgrass[4] (Watrud et al., 2004). This supports in addition the rapid and widespread distribution of favorable traits between weed populations.

The main task for farmers in controlling weeds is to delay weed-emergence and to slow or stop weed growth by mechanical or chemical weed control options, allowing the crop canopy to close in order to provide the crop a competitive advantage over the weeds. Farmers can choose between various mechanical, cultural and chemical weed control op-

[4] *Agrostis stolonifera*

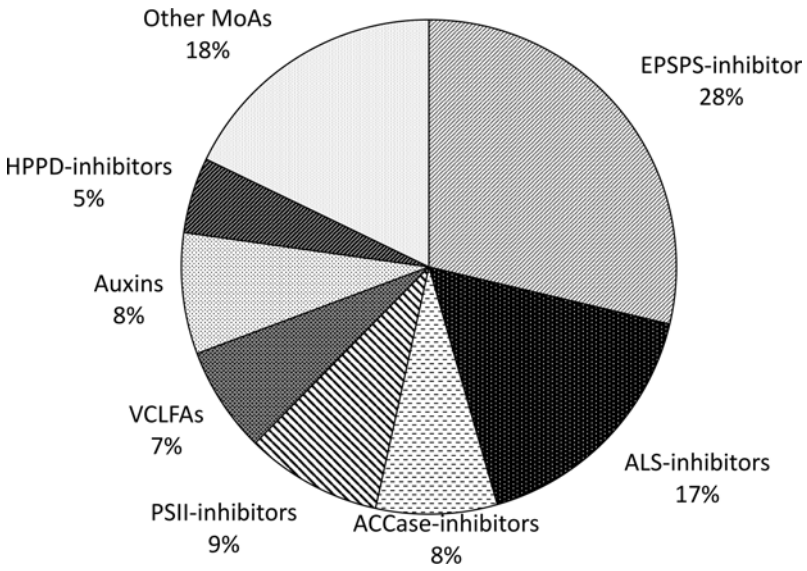

Fig. 1: World herbicide sales in 2011 according to herbicide mode of action (MoA) (Anonymous, 2012).

tions depending on several factors like the cultivated crop or the weed populations in field. The most common mechanical and cultural methods to control weeds are seed purification preventing settlement, plowing to bury seeds, or hoeing/harrowing to control weeds after germination. They are effective techniques but increase the risk of soil erosion. Several other mechanical methods, soil cultivation practices and applications methods have been adapted to different situations and cropping systems can also control weeds. One of these is crop rotation, which reduces weed establishment and allows the usage of different control methods during rotation period, but is used less and less.

Chemical weed control gives selective or non-selective weed control through the application of herbicides and is often more cost efficient than other weed control options. It will be described in detail in the following sections. Chemical and mechanical weed control or crop rotation can be used alone, but they are much more effective and sustainable in combination. To fulfill the requirements of a sustainable agricultural practice, an alternation of weed control options is necessary, especially to delay or circumvent the adaptation of weeds to a single approach or/and a single chemistry. Thus, it is of key importance in the battle of weed control, to understand how weeds can adapt by acquiring resistance to chemicals. This is necessary to help guide the development of new and alternative chemical solutions or/and to choose methods to help preserve the utility of existing products, such as using them in mixture or in series.

2.2 Chemical Weed Control

In the beginning of agriculture and for many thousands of years only mechanical and cultural methods were used to control weeds. Hand-weeding, plowing, hoeing, seed purification or/and crop rotation are common examples of those methods. They were used for ages, and are still important today, especially in Integrated Weed Management (IWM) approaches. A demand for easier and more efficient weed control was developed during the beginning of the industrialization period, which drew workers from the land and resulted in increasing agriculture labor expenses and led to an increase in mechanization. The first report of a broadscale use of chemicals was reported in 1840 with the application of lime to control *Equisetum* spp., followed in 1854 by a publication on the herbicidal uses of sodium chloride (Timmons, 1970). In 1855 the use of sulfuric acid and iron sulfate was proposed to control weeds (Timmons, 1970). This was in addition the first publication discussing selective chemical weed control in crops as reviewed by (Timmons, 1970). Inorganic chemicals were the first to be used for weed control, but the real beginning of the "Chemical era of agriculture (Timmons, 1970)" started during and after the second world war, with the discovery of the herbicidal properties of phenoxyacetic herbicide and growth regulator 2,4-D (Timmons, 1970). Since that time, inorganic chemicals have been superseded by organic chemicals. The number of chemicals with herbicidal activity has increased dramatically since then. Today commercial compounds representing 19 different modes of action (MoA) are known. For several MoAs, a large number of compounds were developed, sometime representing several chemical classes (HRAC, 2011). They provide today a selective or non-selective control of mono- or dicotyledonous, annual or perennial weeds, whether forbs or brushes, as seedlings or adult plants and applied differently, either in pre- or post-applications and under various environmental conditions.
Chemical weed control is divided into selective and non-selective weed control. While non-selective herbicides generally affect all green plants, the activity of selective herbicides is based on physiological differences between plant species that result in the control of well-defined weed species without affecting the crop (O'Sullivan et al., 2002). The first major goal in herbicide development was to find substances which keep the field clean and can non-selectively control all plants. Today these herbicides are used *e.g.* to maintain roadsides or industrial areas, or are used as pre-plant burndown herbicides in agriculture. The most important herbicides for non-selective weed control with decreasing worldwide market share is since 1974 the organophosphate glyphosate, which inhibits the shikimic acid pathway, in particular the 5-enolpyruvylshikimic acid-3-phosphate synthase (EPSPS; EC 2.5.1.19), followed since 1962 by Paraquat, which inhibits photosystem 1 (PSI) and glufosinate first reported in 1981, which inhibits the glutamine synthetase (EC 6.3.1.2.) (Fig. 1) (Tomlin, 2000; HRAC, 2011). According to the overall market share in 2011 are the selective ALS inhibitors, which inhibit the acetolactate synthase (ALS, EC 4.1.3.18) the second

most sold herbicidal class worldwide (Levitt, 1978; LaRossa & Schloss, 1984). HPPD inhibitors inhibiting the 4-hydroxyphenyl-pyruvat dioxygenase (4-HPPD, EC 1.13.11.27) are mostly selective, although one is sold in the non-selective market (sulfometuron methyl) (Tomlin, 2000; HRAC, 2011).

ACCase inhibitors, also called graminicides, are another class of herbicides providing a selective weed control and were introduced into the market in the late 1970's and early 1980's (Smeda et al., 1997). They provide monocotyledonous weed control but do not affect dicotyledonous plants. Several grass crops like *e.g.* *Triticum aestivum* L. are naturally tolerant to certain members of the three ACCase subfamilies, despite the sensitivity of most grassy weeds (Bradley & Hagood, 2001). The ACCase inhibitors are divided into three chemically distinct classes of aryloxyphenoxypropionates (APP/AOPP), phenylpyrazolines (PIZ) and cyclohexanediones (CHD) graminicides. All subclasses act on the Acetyl CoA Carboxylase (ACCase, EC 6.4.1.2), a key enzyme involved in the first step of the fatty acid biosynthesis pathway. It is nuclear encoded and localized in the chloroplasts of grass species (Burke et al., 2006; Manalil et al., 2011). The ACCase in most broad leaf plants is insensitive to ACCase inhibitors (Powles & Yu, 2010).

In addition to inherent selectivity, selective weed control can also be obtained by the use of the safener technology. Safener selectively activate crop detoxification processes to metabolize the herbicide, protecting crops against herbicidal injury. Safeners have been successfully used to safen cereal and maize crops to selected ACCase and ALS herbicides. No safeners have yet been found for broadleaf crops. This system is mainly used *e.g.* in the ACCase herbicide Puma® together with fenoxaprop-ρ-ethyl or in the Atlantis® formulation together with the ALS inhibitors iodo- and mesosulfuron protecting cereals (King, 2007).

2.2.1 The Herbicide Glyphosate

Glyphosate or N-phosphomethyl glycine, "the most successful pesticide ever" (Caseley & Copping, 2000) is today by far the most widely used herbicide worldwide. It inhibits the EPSPS enzyme (EC 2.5.1.19) the penultimate step in the synthesis of aromatic amino acids. The advantages of glyphosate are (1) its broad weed spectrum, (2) its good translocation throughout the plant, (3) its flexibility in application timing, because of good efficacy even on developed plants, (4) its favorable environmental profile, *e.g.*, low volatility, short half-life in soils, minimal movement to groundwater and (5) its classification as one of the least toxic pesticides to animals (Bromilow & Chamberlain, 2000; Duke & Powles, 2008; Webster & Sosnoskie, 2010). One of the biggest advantages of glyphosate is its favorable environmental and toxicity characteristics. The absence of the target enzyme and the entire shikimic acid pathway in mammals and insects is one reason for its low toxicity (Williams et al., 2000). Various investigations in birds, dogs, fish, mice, rats and other

animals have shown that toxicity can only be provoked at very high doses (Borggaard & Gimsing, 2008). In addition glyphosate was shown to be non-carcinogenic (Borggaard & Gimsing, 2008).

However, glyphosate is "a once in a century herbicide" and can be counted in the global food production as penicillin is for battling diseases (Powles, 2010). Glyphosate was introduced in the market in 1974 by Monsanto as a non-selective herbicide. When introduced, glyphosate could successfully control a wide spectrum of herbaceous, wooden, annual, perennial, forbs, brushes, monocotyledonous and dicotyledonous green plants with only a few exceptions. It is used in wide range of agricultural and industrial areas, along traffic infrastructure on roads or water channels and in urban areas. Glyphosate is taken up through all green plant tissues, but not through the bark (Powles, 2008). Due to the absence of any significant soil activity, seeds are not affected by glyphosate applications (Powles, 2008). Because of its lack of selectivity, glyphosate could not be used in cropping systems and therefore was used as burn-down herbicide just before sowing or in perennial crops like trees and brushes.

With the introduction of the Roundup Ready®(RR) system, containing a glyphosate resistant *Agrobacterium tumefaciens* EPSPS, it became a selective herbicide in key crops like corn[5], cotton[6], rapeseed[7], rice[8], soybean[9] and sugarbeet[10] (Powles, 2008; Tong et al., 2009). The first glyphosate resistant cotton cultivars were introduced in 1997. By 2008 glyphosate resistant cotton cultivars in the U.S. were planted on more than 95 % of total cotton grown area in Arkansas, Georgia, Louisiana, Mississippi, Missouri, North Carolina, South Carolina and Tennessee. The introduction of these GMO crop varieties changed cultivation practices dramatically (see also chapter 2.1). In the time prior to glyphosate resistant crops, several trips related to weed control through the field were required: (1) for soil cultivation with disk harrows or moldboard plows to eliminate weeds and to prepare the field, (2) for weed control with soil applied and postdirected herbicides and (3) additional trips for mechanical weed control (Webster & Sosnoskie, 2010). During that time in average 5 different herbicides throughout the growing season were used to maintain cotton (Webster & Sosnoskie, 2010). These cultivation practices were labor and cost intense and promoted soil erosion. With the introduction of glyphosate resistant cultivars, farmers had the opportunity to use only a single herbicide throughout the year. The advantage of this system is that the broad spectrum of controlled weeds reduced the need to scout weeds and the good activity on young and old weeds proveked nearly no limitations in application time. Farmers were able to introduce reduced or conservation tillage practices

[5] *Zea mays*
[6] *Gossypium hirsutum*
[7] *Brassica napus*
[8] *Oryza sativa*
[9] *Glycine max*
[10] *Beta vulgaris*

by replacing mechanical soil preparation or weeding with the herbicide, thus protecting the soil by decreasing dramatically the erosion. Pre-harvest labor and machinery related costs were reduced in that system by approximately 30 % (Webster & Sosnoskie, 2010). Glyphosate is highly charged in comparison to other herbicides with polar carboxyl-, amino- and phosphonate functional groups (Bromilow & Chamberlain, 2000). Through the high degree of polarity, glyphosate is quickly and strongly sorpted to soil minerals (Hance, 1976). The fast sorption reduces leaching and explains the almost complete absence of soil activity (de Jonge et al., 2000). Some exceptions are very sandy soils poor in oxides or macropore rich soils (de Jonge et al., 2000).

Glyphosate is quickly degraded mainly by microorganisms in soils (Hance, 1976). The DT_{50} has been described to be between 3 and 174 days, depending on soil microbiological activity (Grossbard & Atkinson, 1985; Hathway, 1989; Vereecken, 2005). The degradation of glyphosate leads either to glycine and sarcosine or to AMPA[11] which is less toxic than glyphosate itself (Borggaard & Gimsing, 2008). A rapid metabolisation has been described for several soil bacteria, from which a *Pseudosomonas* spp. strain spend the GOX-gene used to metabolise glyphosate in commercialized RR rapeseed varieties (Pline-Srnic, 2005).

While the degradation in soils is very rapid, there is little evidence of plant tolerance or resistance based on glyphosate metabolism. One RR-variety of the leguminose *Glycine max* shows a minor degree of glyphosate degradation, but is not enough to confer tolerance (Duke, 2011). A recent study reported degradation of glyphosate in *Digitaria insularis* of more than 90% in resistant species within 196 hours after treatment (De Carvalho et al., 2012).

Glyphosate is amphimobile and can be transported either symplastically or apoplastically (Dewey & Appleby, 1983). It is distributed similar to carbon assimilates, from source to sink issues, and accumulates mainly in the most active and youngest plant parts. Transport patterns are in addition depending on the physiological plant stage. The glyphosate concentration in meristematic tissues thus increases much faster than in vegetative tissues (Dewey & Appelby, 1983; Pline-Srnic, 2005).

Jaworski (1972) observed that aromatic amino acid biosynthesis in plant is inhibited by glyphosate application. Amrhein and coworkers subsequently identified the 5-enolpyruvoyl-shikimate-3-phosphate synthase (EPSPS) (EC 2.5.1.19) as the glyphosate target enzyme (Amrhein et al., 1980; Steinrucken & Amrhein, 1980). The EPSPS is nuclear-encoded but localized in the plastids (Amrhein et al., 1980; Steinrucken & Amrhein, 1980; Papanikou et al., 2004). It is the 6^{th} enzyme in the shikimic acid pathway, responsible for the formation of the aromatic amino acids tyrosine, phenylalanine and tryptophan present in all green plants, algae, bacteria and fungi but absent in mammalians and insects (Herrmann &

[11] aminomethylphosphonic acid

Weaver, 1999; Oliveira et al., 2003). The three amino acids are indispensable for protein biosynthesis and are the precursors for the production of many secondary compounds in plant, like auxins, pathogen defense compounds, phytoalexins, folic acid, lignins, flavonoids, plastoquinones and other phenolic and alkaloid plant metabolites (Alibhai & Stallings, 2001; Pline-Srnic, 2005). Approximately 35 % of the ultimate plant dry weight passes through this pathway.

The EPSPS catalyzes the conversion of shikimate-3-phosphate (S-3-P) to EPSP wherein the enolpyruvoyl group of PEP is transferred to the 5-hydroxyl of shikimate-3-phosphat to form 5-enolpyruvylshikimate-3-phosphate and inorganic phosphate (P_i). Glyphosate is a competitive inhibitor to phosphoenolpyruvat (PEP) and an uncompetitive inhibitor to S3P in the EPSPS-enzyme (Steinrucken & Amrhein, 1980; Schönbrunn et al., 2001). EPSPS, with an average relative molecular mass (M_r) of 46,000, folds into two similar domains which approach each other in a screw like movement with the active site in the interdomain cleft (Schönbrunn et al., 2001). Inhibition proceeds through the formation of a stable ternary EPSPS-S3P-glyphosate dead-end complex virtually identical with that of the EPSPS-S3P complex without perturbating the structure of the active-site cavity (Sammons et al., 1995; Schönbrunn et al., 2001). Schönbrunn et al. (2001) proposed that S3P triggers the enzyme's transition from the open to the closed state and not PEP or its competitive inhibitor glyphosate.

Due to a missing feedback inhibition of the EPSPS mediated reaction, the concentration of the precursor S-3-P rises dramatically upon glyphosate inhibition. S-3-P is an unstable molecule, because of the energy rich bond P_i. It is quickly degraded to the stable molecule shikimic acid. The shikimic acid content in the youngest and most active plant parts strongly increases after glyphosate treatment and can hence be used as an early plant injury indicator (Harring et al., 1998; Singh & Shaner, 1998; Koger et al., 2005). The high shikimic acid increase together with the starvation of amino acids, proteins and secondary plant metabolites, results in herbicidal injury or plant death (Pline-Srnic, 2005; Shaner et al., 2005). Aromatic amino acid biosynthesis inhibition affects also the synthesis of several plant signaling compounds such as salicylic acid. This inhibition could have also some detrimental effects in plants and might led to a higher susceptibility to plant pathogens, which promotes a faster plant death (Tamagnone et al., 1998; Pline-Srnic, 2005; Shaner et al., 2005; Johal & Huber, 2009; Lorentz et al., 2011). Immediate cytological modifications after glyphosate application such as decay of vascular tissues and pith as well as increased polyphenol and lignin concentrations in the intercellular space have previously been described by Franz et al. (1997) and Lorentz et al. (2011).

2.3 Herbicide Resistance

Darwin, Lamarck, Mendel and others introduced the idea of natural selection and the development of adaptations based on genetic diversity and mutations within species. Natural selection enables populations to survive successful changing environmental conditions. Some environmental conditions change slowly over centuries, while other changes occur in just minutes. Herbicides, as other pesticides and antibiotics, can be seen as such an event, which causes extreme stress and high selection pressure on targeted weeds, allowing the selection of the best adapted individuals. However, herbicide resistance in weeds is always an evolutionary process depending on various effects such as selection pressure, mutation rate and plant fitness influenced by resistance traits (Powles & Yu, 2010).
Herbicide resistance is usually caused by mutations within the plant genome. The spontaneous mutation rate in biological organisms is reported to be between 10^{-5} and 10^{-6} in gametes per generation and locus (Jasieniuk et al., 1996). In addition to natural variation the mutation rate under stressed conditions can be higher than under unstressed conditions (Mikula, 1995; Bridghes, 1997; Slack et al., 2006; Naito et al., 2009; Gressel, 2011). Nevertheless, a mutation, conferring resistance, is never caused by the herbicide application itself since each compound is tested for its mutagenic potential for approbation. Considering the number of seeds per surface unit and the natural mutation rate, an herbicide resistance trait might be already present in the population, evolved spontaneously or brought in by seed transfer. The advantages or disadvantages of each resistant trait might impact plant fitness and this will greatly influence its spread and its establishment in plant population (Christoffers, 1999).
Plants are sessile, but resistance traits are able to be transported over a long distance via pollen, seeds or other propagules and are therefore able to spread into new areas. A resistant trait to a given herbicide might be already present in a weed population or species, in most cases at a low frequency, even before the first application of the product. Thus, not each population needs to develop its own *de novo* resistance traits. However, the development and spread of such traits in weed populations still needs to be better understood.

In principle, the survival of a normally lethal herbicide dose rate by an individual plant, population or species is divided into herbicide tolerance or herbicide resistance. In the common use of both phrases are different definitions existing. In the present work and related to Fig. 2 we will call them the scientific definition and the commercial definition. They can be defined in two ways, respectively: (1) in an horizontal way based on the acquisition of the ability to survive an herbicide application, or (2) in a vertical way driven by the practical impact of this ability. Reasonable arguments can be found for both definitions. The **scientific definition** was given in 1998 by the Weed Science Society of America. Therefore **herbicide tolerance** is defined as *"the inherent ability of a species to survive and reproduce after herbicide treatment. This implies that there was no selec-*

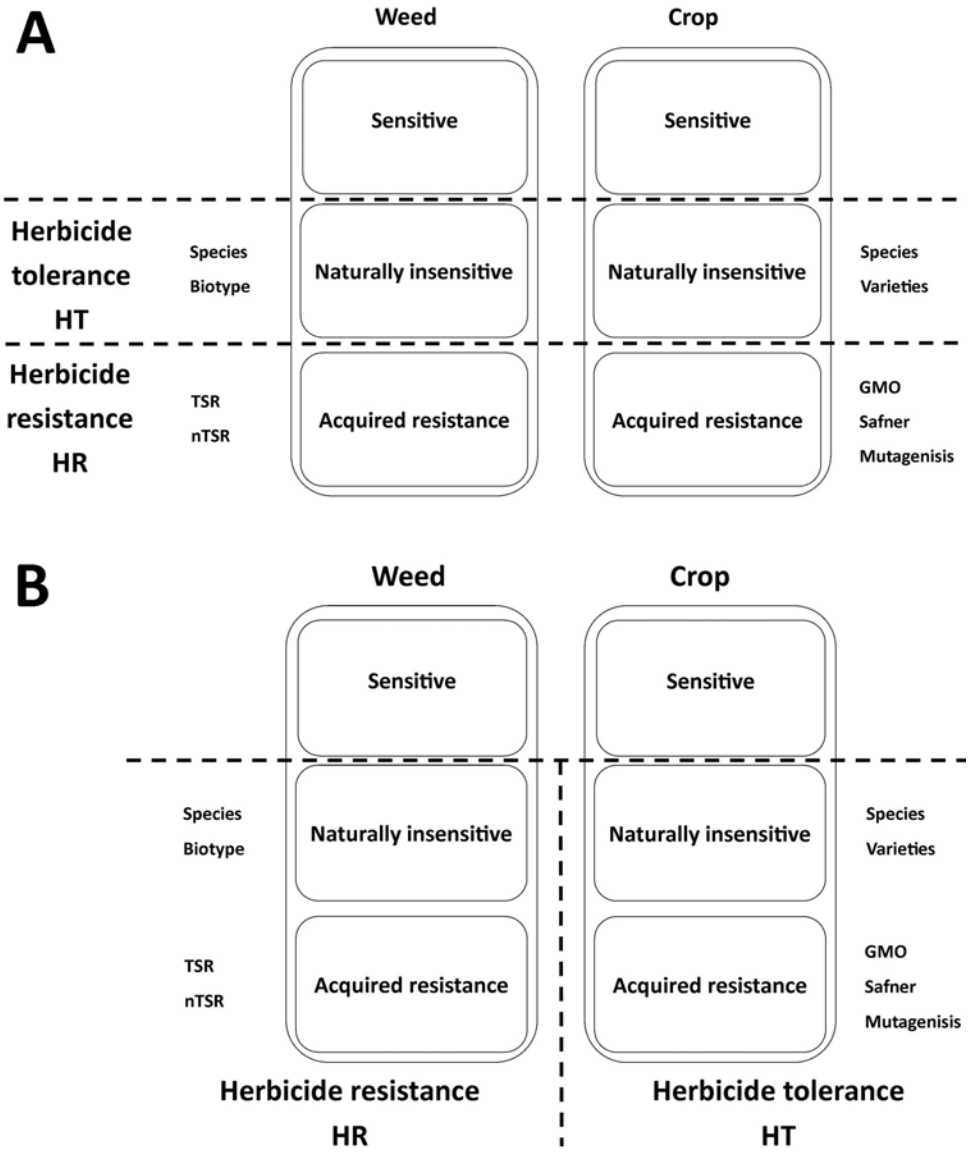

Fig. 2: Comparison of the two common approaches to define herbicide resistance and herbicide tolerance; **A** scientific definition according to Anonymous (1998) and **B** commercial definition.

tion or genetic manipulation to make the plant tolerant" (Anonymous, 1998), whereas the **herbicide resistance** is defined as *"inherited ability of a plant to survive and reproduce following exposure to a dose of herbicide normally lethal to the wild type. In a plant, resistance may be naturally occurring or induced by such techniques as genetic engineering or selection of variants produced by tissue culture or mutagenesis."* (Anonymous, 1998). The **commercial definition** for *"***herbicide tolerance** *is the ability of a crop to survive and to produce adequate yield after herbicide use at a normally lethal dose for plant, without any consideration concerning the method used to develop the tolerance in crop"*, whereas

herbicide resistance is defined as *"the naturally acquired ability of a weed to survive a normally lethal herbicide dose to plant, without any consideration if the ability was developed before or after the first application of herbicide."* As previously stated, reasonable pro or contra arguments can be found for both definitions. For example, in the scientific definition, Fig. 2 **A** it is questionable if the development of ACCase resistance in grassy weeds like *Alopecurus myosuroides* is herbicide resistance or herbicide tolerance. ACCase resistance has been shown to be present in several grass species within three to five years of repeated ACCase inhibitor treatments. Was ACCase resistance present in a population as a result of the natural genetic variation before the first use of the herbicide and then spread afterwards or was the resistance developed after the first use of ACCase inhibitors? As another example, volunteer glyphosate tolerant corn is one of the most important weeds in several cropping systems, but is it according to the commercial definition, Fig. 2 **B**, herbicide tolerant or herbicide resistant? Therefore both definitions have their weak points and doubtless the commercial definition is driven by the aim to prevent, in the general linguistic sense, the negative word "resistance" in relation to genetically modified and herbicide tolerant crops. In face of the difficult situation related to genetically modified crops in the public discussion, a neutral word instead of a prejudicial term might help to promote a knowledge-based discussion about this topic. Therefore the commercial definition will be used in the further text.

A weed is considered as herbicide resistant if its response to an herbicide application fulfills the current requirements of the following two definitions given by Heap (2011). The first, more scientific definition expects: *"a genetically inherited statistical difference in herbicide response between two weed populations of the same weed species"*, the second requires *"that the resistant population survive the recommended rate of herbicide under normal field conditions"*. Both definitions are required to avoid the proclamation of resistance cases without the requirements of it being a significant threat to the field (Stewart, 2009). Another, older definition but with comparable requirements was given by Shaner et al. (1997): herbicide *"resistance is the inherited ability of a weed population to withstand a herbicide at its use rate brought about by genetic change within the population due to selection by the herbicide"*(De Prado et al., 1997). However, a resistance trait in weeds confers the ability to overcome an herbicide treatment, independently, if the resistance trait depends on one or more genes.

Some weeds have not only evolved resistance to a single herbicide, but also resistance to several herbicides from the same or from different chemical classes. The resistance against two compounds from different chemical classes acting with the same Mode of Action (MoA) is called cross-resistance. The resistance of weeds to compounds acting with different MoAs is called multiple resistance (De Prado et al., 1997).

Each resistance mechanism developed in weeds to overcome selection pressure can be further divided into target site resistance (TSR) involving modifications of the gene en-

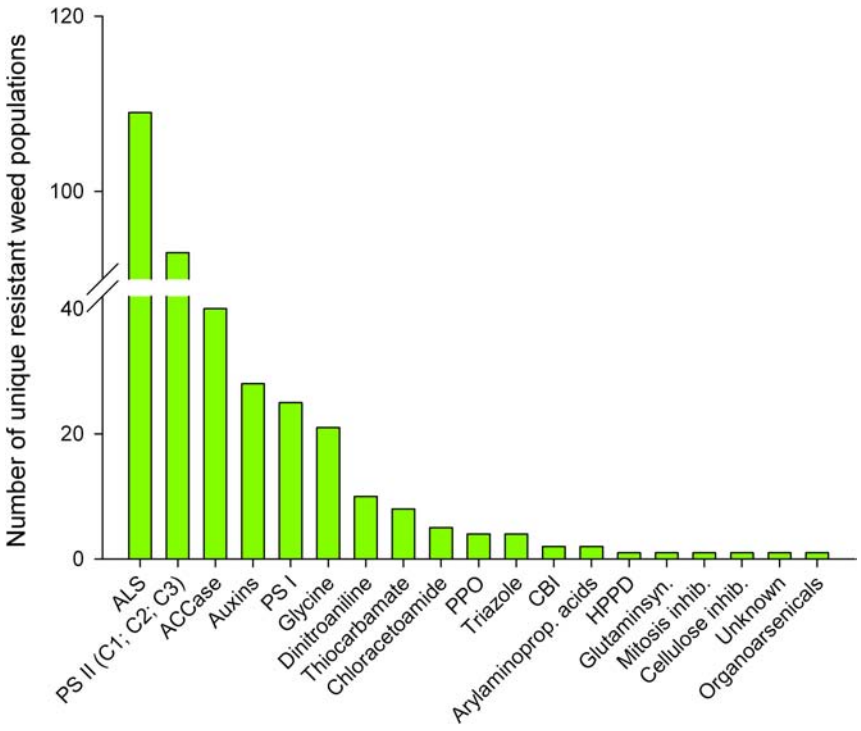

Fig. 3: Number of unique herbicide resistant weed populations sorted by herbicidal mode of action used in agriculture according to Heap (2011); resistance in populations independently confirmed.

coding the enzyme targeted by the herbicide and non-target site resistance mechanisms (NTSR) involving modification of herbicide uptake or transport, vacuolar sequestration, or enhanced metabolism (Powles & Yu, 2010). Today many herbicides with a significant market share show cases of weed resistance as displayed in Fig.3 (Heap, 2011).

Target site resistance can be conferred by a point mutation modifying one amino-acid in the binding pocket of the herbicide usually closely related with the catalytic site of the targeted plant enzyme. Powles & Yu (2010) have defined the target site resistance as a mechanism where the chemical properties still enable the molecule to reach the target site inside the plant cells. However, the target enzyme is no longer able to bind the herbicide but has kept its catalytic activity. A further mechanism related to target site resistance is gene amplification or over-expression. This was recently discovered by Gaines et al. (2010) in a single glyphosate resistant *A. palmeri* population showing partly a more than 100 fold *EPSPS* gene amplification in the genome of glyphosate resistant plants. A comparable mechanism but caused by *EPSPS* mRNA over-expression has been found before in a Malaysian *Eleusine indica* population (Baerson et al., 2002).

The second group of herbicide resistance mechanisms are non-target site based resistance mechanisms (NTSR). Non-target site resistance is caused by mechanisms that hinder the herbicide molecule from reaching the target enzyme in a concentrations adequate to inhibit

its activity (Powles & Yu, 2010). This resistance mechanism can be further divided into rapid xenobiotic metabolisation or differences in uptake, translocation or sequestration of the compound to circumvent plant herbicide injury. The development of non-target site resistance mechanisms is according to Manalil et al. (2011) directly linked to herbicide use below labeled rates (Busi & Powles, 2009). The metabolism of herbicidal compounds is becoming the focus of more and more investigations, although, it is more difficult to measure. Nevertheless, it seems that metabolic resistance is in its importance comparable to the target site based resistance mechanisms. However, herbicide resistance to a single herbicide can be caused by a single or a combination of several different mechanisms, even within the same plant. It is still questionable if such parallel mechanisms were developed in parallel in the same plant or if they are the result of inbreeding processes.

2.3.1 Glyphosate Resistance in Crops

The wide range of weeds controlled by glyphosate and the low environmental and mammalian toxicity helped to make glyphosate to an attractive herbicide to use for weed control in combination with crops bearing a resistant trait. This justfied the significant effort that was required to find a way to engineer glyphosate resistant cultivars. The approaches used included screens to find EPSPS with a lower glyphosate susceptibility, to find enzymes able to metabolize it or to select tolerant cells, callus cultures or whole plants under high glyphosate selection pressure. In principle the same mechanisms are used by nature to select resistant weeds. In the following the published EPSPS sequence of *Petunia hybrida* is used as reference to allow an easier comparison of mutation sites among plant species and/or microorganisms.

Several EPSPS enzymes from different organisms have been investigated in order to find glyphosate resistance including the EPSPS of *Salmonella typhimurium*, *Escherichia coli*, *Klebsiella pneumoniae* and *Petunia hybrida* (Comai et al., 1983; Sost et al., 1984; Kishore et al., 1986; Padgette et al., 1991; He et al., 2003). Several single mutation sites in the EPSPS sequence that are described to confer resistance have been found including T42, G68, G101, T102, P106, G144, P167, A183, R196, R416 or S428 (Bradshaw et al., 1997; Pline-Srnic, 2006; Yuan et al., 2006; Kahrizi et al., 2007). The commercialized corn variety GA21 contains a maize EPSPS transgene bearing the double mutation T102I and P106S in an usually highly conserved part of the EPSPS amino acid sequence, which should deliver a superior level of glyphosate resistance. However, the majority of commercialized glyphosate resistant crops like soybean, cotton, corn, sugarbeet and canola have been constructed with the CP4-EPSPS of the *Agrobacterium* spp. strain CP4, which is bearing a low homology to plant EPSPS. This highly tolerant CP4 EPSPS gene is fused to a petunia derived chloroplast transport sequence and is highly and constitutively expressed due to

the cauliflower mosaic virus 35S promotor (Pline-Srnic, 2006).
Two enzymes have been found that convert glyphosate into less toxic compounds. The first enzyme, a glyphosate oxireductase (GOX) was discovered in the bacterium *Ochronobactum anthropi*, isolated from a glyphosate waste stream treatment facility (Barry et al., 1992). It degrades glyphosate[12] through cleavage of the C-N bond to form glyoxylate and AMPA. It is used in the RR canola varieties together with the CP4-EPSPS. The second enzyme is a glyphosate acetylase (GAT) detoxifying glyphosate by adding an acetyl moiety (Castle et al., 2004).
Significant effort was also invested in finding natural mutants of plants resistant to glyphosate. Cell cultures were selected under continuous glyphosate selection pressure. In *Corydalis sempervirens*, *Euglena gracilis*, *Daucus carota* and other species a higher level of inherent glyphosate tolerance was found (Amrhein et al., 1983; Nafziger et al., 1984; Reinbothe et al., 1991). In all these species, resistance was based on higher enzyme activity mediated by a glyphosate sensitive EPSPS enzyme. Either overexpression, reduced enzyme turnover, higher enzyme stability or gene amplification were found to confer the resistance in cell cultures, but none of the cell lines gave stable regenerated and tolerant plants or products good enough to be commercialized (Pline-Srnic, 2006).

2.3.2 Glyphosate Resistance in Weeds

Since 1974, glyphosate has been used as a non-selective herbicide in orchards, vineyards or as a burndown herbicide, sometimes continuously for many years, often with several treatments during each growing season. Unsurprisingly, the intensive use of glyphosate has led to the selection of tolerant or resistant weed species. The first glyphosate resistant weed population was reported in 1996 in *Lolium rigidum* in a fallow cropping system in Australia, followed in 1997 by an *Eleusine indica* population collected in Malaysian orchards (Powles et al., 1998; Baerson et al., 2002). With the introduction of the glyphosate tolerant crop varieties and the intensive use of glyphosate, resistant weed populations have also developed in row crops like corn, cotton or soybean. The adoption of glyphosate tolerant crops was a revolution in agriculture that became the measuring stick for all current weed management programs. Costs for weed management and soil cultivation plummeted by an average of \$45, \$24, and \$37 ha^{-1} in U.S. soybean, corn and canola in 2005, respectively (Gianessi, 2008). The advent of glyphosate resistant weeds in these crops caused a dramatic increase in cultivation costs, a turnaround from the decrease in cultivation, which the adoption of glyphosate tolerant crops had enabled. Cultivation cost increases of up to 58 % in cotton were reported due to the advent of glyphosate resistant *A. palmeri* (Webster & Sosnoskie, 2010). The cost increases are mostly due to additional soil cul-

[12] N-(phosphonomethyl)glycine

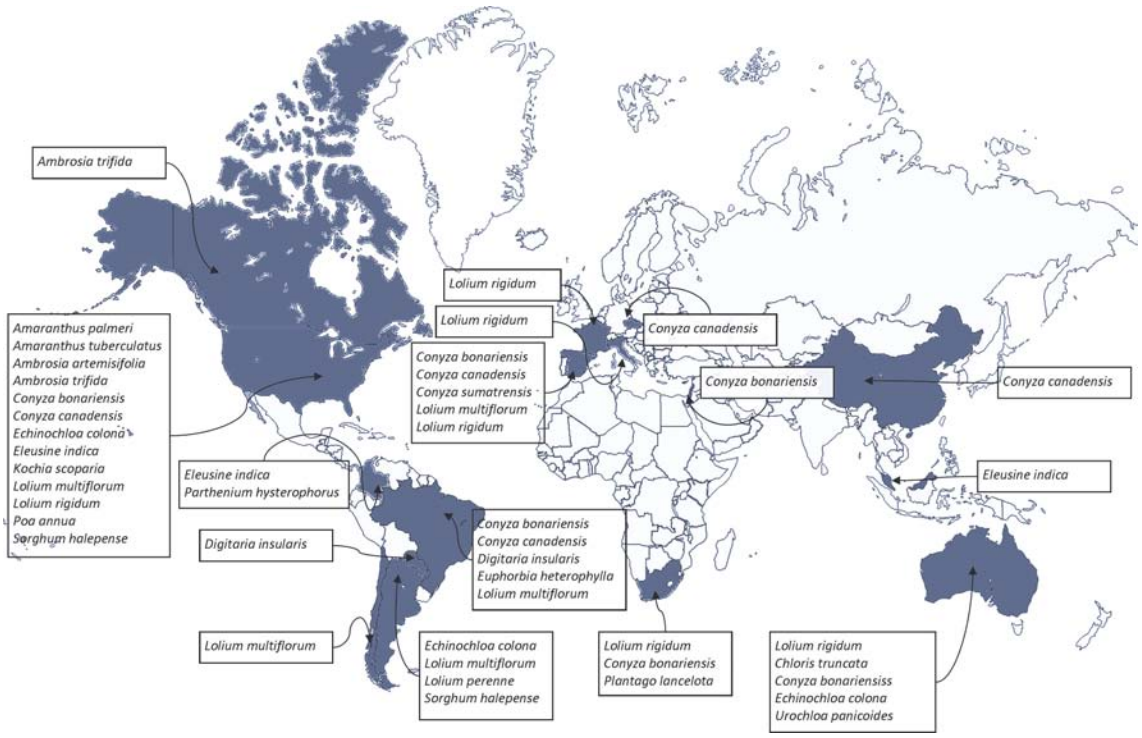

Fig. 4: Global distribution of glyphosate resistant weed species independently confirmed; distribution provided on country level according to Heap (2011); dark color indicates the presence of at least a single glyphosate resistant weed population of different weed species within a country.

tivation measures and conspicuous weed management programs (Webster & Sosnoskie, 2010). Today glyphosate resistant weed populations are present on each continent and found in 21 different weed species (Fig: 4). Species and infested areas are thoroughly documented on the website found under URL: http://www.weedscience.org by I. Heap (Heap, 2011). However, as different the locations and the resistant species are, so are the mechanisms causing glyphosate resistance also as diverse.

Although many different resistance mechanisms in these glyphosate resistant weeds have been discovered, new one keep being found. Both target- and non-target site based resistance mechanisms are responsible for glyphosate resistance (Powles & Yu, 2010). Alterations in the *EPSPS* gene sequence have been reported in a minority of resistance cases, *i.e.* the P106S/T/A target site mutations in *Eleusine indica* and *Lolium rigidum* (Baerson et al., 2002; Wakelin & Preston, 2006). Differences in glyphosate translocation have been found in other populations of *Lolium rigidum* and in *Lolium multiflorum*, *Sorghum halepense* and *Conyza canadensis* (Lorraine-Colwill et al., 2002; Feng et al., 2004; Perez-Jones et al., 2007; Riar et al., 2011). Glyphosate translocation in these populations is mostly restricted within the treated leaves and it is translocated significantly less throughout the whole plant. This mechanism is inherited as a single dominant

Tab. 1: Glyphosate resistance mechanisms found in important global weeds species; within a plant mechanisms can occur either alone or in combination.

Resistance mechanism	Weed species	Reference
Target site	*E. indica, L. rigidum, L. multiflorum* (P106S/A/T)	Baerson et al., 2002; Wakelin & Preston et al., 2006; Perez-Jones et al., 2007
Dif. uptake	*L. multiflorum*	Michitte et al., 2007;
Dif. translocation	*C. bonariensis, C. canadensis, L. multiflorum, L. rigidum, S. halepense*	Riar et al., 2011, Dinelli et al., 2006; Wakelin et al., 2004;
Sequestration	*C. canadensis*	Ge et al., 2010
EPSPS overexpression	*L. rigidum*, suggested for *C. canadensis*	
EPSPS gene amplification	*A. palmeri, A. tuberculatus*	Gaines et al., 2010, Tranel et al., 2010
Metabolism	*Digitaria insularis*	de Carvalho et al., 2012
CP4-EPSPS gene transfer	*Brassica rapa*	Hall et al., 2000; Warwick et al., 2008

or semi-dominant trait (Preston & Wakelin, 2008). In *Conyza canadensis* the reduced translocation is based on a rapid glyphosate sequestration into vacuoles (Ge et al., 2010). In a population of *Lolium multiflorum* found in Chile, the resistance is based on differences in the spray solution retention and foliar absorption on abaxial leaf surfaces (Michitte et al., 2007).

A new herbicide resistance mechanism was found by Gaines et al. (2010) based on *EPSPS* gene amplification in a *A. palmeri* population from Georgia. This population was collected in 2005 in a cotton field that had been managed without tillage and using glyphosate as the only weed management tool (Culpepper et al., 2006). In these plants the *EPSPS* gene was amplified more than 100 fold, comparably what was previously observed in glyphosate resistant cell cultures (chapter 2.3.1). This gene amplification is polygenetic transmitted to the progeny and no mutations in the *EPSPS* gene sequences were found. This resistance is therefore based on the overproduction of the enzyme targeted by the herbicide (Gaines et al., 2010). To summarize, glyphosate resistance mechanisms are highly diverse and can vary even within a species from population to population (Tab. 1).

2.3.3 ACCase Resistance in Weeds

The first case of ACCase resistance was found 1982 in blackgrass (*Alopecurus myosuroides* Huds.) in the United Kingdom and is now found in at least 39 weed species widely spread over Europe, Australia and the Americas. The resistance to ACCase inhibitors is caused

either by target site mutations in the Acetyl CoA Carboxylase (EC 6.4.1.2) gene or by enhanced plant herbicide metabolism (Vila-Aiub et al., 2005). Several single nucleotide mutations conferring resistance to at least one of the subclasses of ACCase inhibitors in the amino acid positions I1781, W1999, W2027, I2041, D2078, C2088 and G2096 have been described (Yu et al., 2007; Powles & Yu, 2010). The mutation site W2027 confers resistance to aryloxyphenoxypropionates (APP) herbicides like fluziafop or quizalofop and a weak resistance to cyclohexanediones (CHP) ACCase herbicides like clethodim (Powles & Yu, 2010). Kaundun (2010) described in addition the influence of the zygosity of an ACCase mutation in *Lolium multiflorum* and obtained a higher resistance value to APP, CHD and phenylpyrazoline (PPZ) ACCase inhibitors for homozygous than for heterozygous or sensitive plants with the mutation D2078G.

2.4 Biology of the Weed Species Studied

2.4.1 Biology of the Genus: *Amaranthus*

The origin of the name *Amaranthus* is derived from the Greek word *amarantos* meaning immortal, and originated from the persistence of the inflorescence beyond the death of the plant (Häfliger & Brun-Hool, 1969; Steinau et al., 2003). Thus, with the view of the present and with the development of each new herbicide resistance case the name is a fitting description of the nature of this genus of weeds.

The genus *Amaranthus* belongs to the family of the dicotyledonous *Amaranthaceae* (Pigweeds) and was originally distributed in the northern hemisphere. They settle mainly dry arable land, dry grassland, and waste areas. The species of this genus are C4 plants and thus well adapted to dry conditions. They occur only rarely in wet or poorly drained soils. Of the approximately 100 members of this genus only two species, *Amaranthus lividus* L. and *A. graecizans* are native to Europe. However, through global trade of agricultural commodities several of *Amaranthus* species are now distributed worldwide and have become endemic and often troublesome weeds (Häfliger & Brun-Hool, 1969). The usually black, glossy, smooth and strongly compressed seeds of *Amaranthus* species are edible and were used as an important grain crop in early American agriculture, which continues until today (Häfliger & Brun-Hool, 1969). Other species of this genus are, due to their attractive inflorescences and colored leaves, used as ornamentals.

The close genetic relationships between species within the genus are highlighted by the occurrence of fertile interspecies hybrids in several species combinations, e.g. between *A. palmeri* and *A. tuberculatus* plants. The genetic diversity is also supported by several species being dioecious (Steinau et al., 2003). Within the *Amaranthus* genus, *A. palmeri* is the fastest growing and tallest weed species (Sellers et al., 2003). Horak & Loughin (2000) reported growth rates of 0.18 - 0.21 cm per growing degree day, while *A. tuberculatus*

had a growth rate of 0.11 - 0.16 cm per growing degree day. The close relationships between *A. palmeri* and *A. tuberculatus* are also apparent when comparing the *EPSPS* gene coding sequences. The homology between the *A. palmeri EPSPS* cDNA sequences published at NCBI[13] FJ861242 (1599 bp) and FJ861243 (1603 bp) is 96.6 %. Whereas the homology between the published *A. tuberculatus EPSPS* cDNA sequences FJ869880 (1967 bp) and FJ869881 (1958 bp) is 98.1 %. The identity among the 4 reported *EPSPS* cDNA sequences of both species is around 96.1 %.

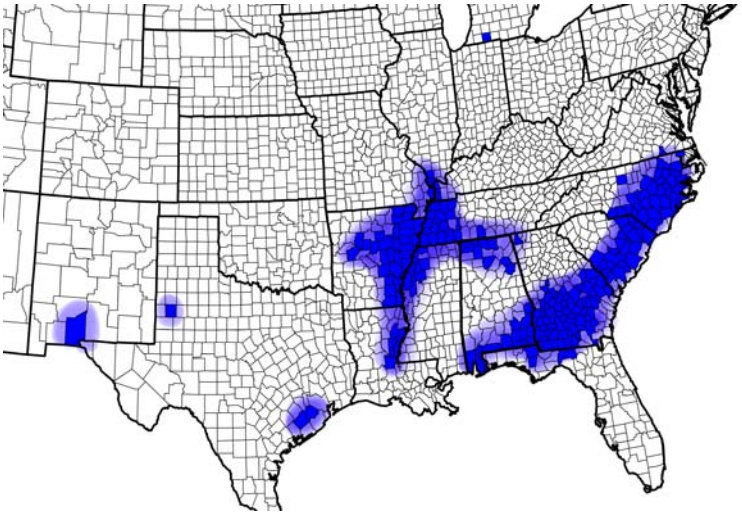

Fig. 5: US counties with confirmed glyphosate resistant *Amaranthus palmeri* populations (intensive color) and the probable further spread of resistant populations (light color), data according to Strek et al. (2012).

***Amaranthus palmeri* S. Wats.**, also called Palmer Pigweed is a dioecious and wind polinated C4 plant with female and male flowers on different plants. It is a branched herbaceous and summer annual plant, native to the southern Great Plains, where it can cause severe interference and yield reductions in corn, cotton and soybean (Klingaman & Oliver, 1994). It is an erect plant, growing up to 2.30 m tall with stiff bracts at the female inflorescence. Within a 7 month growing period it can reach an average plant dry weight of 5 kg (Horak & Loughin, 2000; Bond & Oliver, 2006). *A. palmeri* can emerge in California from the beginning of March until October and will grow until the first frost. Under optimal conditions female plants are able to produce up to 0.5 m long seedheads containing between 200000 and 600000 seeds (Sellers et al., 2003; Bond & Oliver, 2006). In direct contradiction to current weed control strategies, where "weed competition is greatly diminished after canopy closure (Wright et al., 1999)", *A. palmeri* is able to emerge and compete with soybean plants even after the canopy closes (Wright et al., 1999; Jha et al., 2008). The problems caused by *A. palmeri* are not only due to growth competition, they

[13]National Center for Biotechnology Information, U.S.A.

can also hinder the mechanical harvest of cotton due to thick and strong stems interfering with, and even in some severely infested fields, breaking metal parts.

A. palmeri shows the ability to rapidly adapt to new herbicides. Cases of resistance in the US have been reported to Dinitroaniline, Photosystem II inhibitors, ALS-inhibitors and glyphosate (Heap, 2011). This species was not among the five most troublesome weeds in the most southeastern states (Webster, 2005) until glyphosate resistance occurred. The first glyphosate resistant *A. palmeri* population was found in 2005. Today, glyphosate resistance is widely present in *A. palmeri* populations across the whole southeastern US with resistant populations found in Georgia, North Carolina, Arkansas, Tennessee, New Mexico, Mississippi, Alabama, Missouri and Louisiana (Fig. 5). Wright et al. (1999) predicted that *A. palmeri* will cause significant problems in soybean fields of the southeast for the foreseeable future given the current weed control strategies being used. Therefore, *A. palmeri* has grown to become one of the most troublesome weeds in the southeastern US.

Amaranthus tuberculatus and ***Amaranthus rudis*** were originally divided into sep-

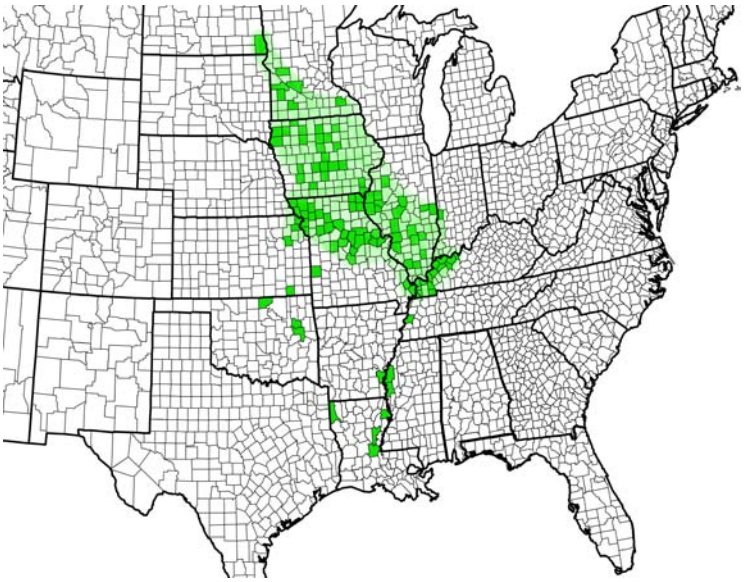

Fig. 6: US counties with confirmed glyphosate resistant *Amaranthus tuberculatus* populations (intensive color) in Dec. 2011 and the probable further spread of resistant populations (light color), data according to Strek et al. (2012).

arate species primarily according to differences in tepals of the pistillate flowers and indehiscent fruits (Sauer, 1967; Sauer, 1972). However, a proposal to classify them into a single species *A. tuberculatus* divided into two varieties *A. tuberculatus.* var. *rudis* and *A. tuberculatus.* var. *tuberculatus* was given by Pratt & Clark (2001) based on isoenzyme comparisons and morphology inhomogenity. In the work presented herein, the studied plants belonged to the species *A. tuberculatus* var. *rudis* and will be referred to solely as

A. tuberculatus. Pratt & Clark (2001) proposed also that both groups, native to North America, were originally geographically divided, but differences were erased through agricultural and human impact.

Amaranthus tuberculatus (Moq.) Sauer var. *rudis* (Sauer) Costea & Tardif (common waterhemp) is a dioecious erect weedy C4-plant which can grow easily up 3 m in height with roots reaching a depth of 70 cm. It can occupy a radial area of 2 m with a growing rate of 0.11 - 0.16 cm per growing degree day (Horak & Loughin, 2000; Costea & Demason, 2001; Tranel et al., 2010). The reported growth rate of *A. tuberculatus* is less than that for *A. palmeri*, but still higher than the values reported for other Amaranthus species (Sellers et al., 2003). The female plant can produce between 35 000 to 1 200 000 seeds during the growing season (Costea et al., 2005).

Its ecological preferences are summarized by Costea et al. (2005), where the species is described as thermophytic, hygrophytic to mesophytic, heliophytic and nitrophilous. It prefers well drained and nutrient rich soils, *e.g.* alluvial sands also well suited for agricultural production. Therefore, *A. tuberculatus* is an important weed, especially under drought stress, and can vigorously interfere with corn and soybean for water and nitrogen (Steckel & Sprague, 2004; Costea et al., 2005). *A. tuberculatus* has also developed resistance to several previously highly effective herbicidal classes like PSII, ALS, PPO, EPSPS (Fig. 6) and HPPD inhibitors (Sprague et al., 1997; Foes et al., 1998; Legleiter & Bradley, 2008; Heap, 2011).

2.4.2 Biology of *Sorghum halepense*

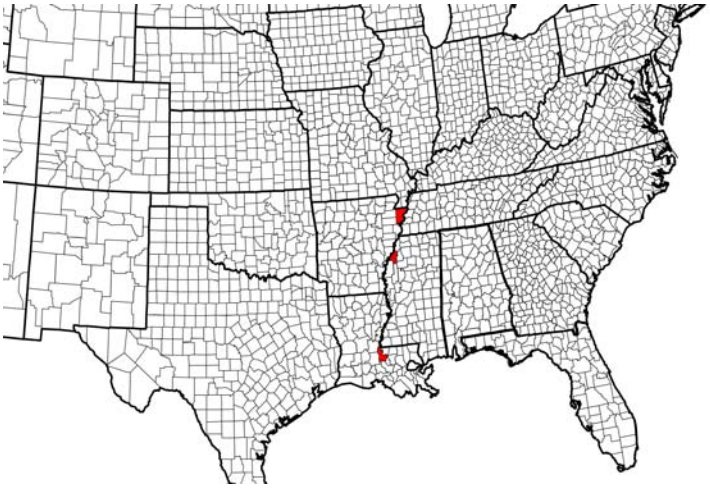

Fig. 7: US Counties with confirmed glyphosate resistant *Sorghum halepense* populations in Dec. 2011, data according to Strek et al. (2012).

Sorghum halepense (L.) Pers. or Johnsongrass is a tetra-allopolyploid (n = 10, 2n = 40)

invasive grassy weed species found worldwide and is a problematic weed mainly in warm climates (Celarier, 1958; Monaghan, 1979; Price et al., 2005; Vila-Aiub et al., 2005). It is also a weed in corn fields in southern Germany. It was introduced into the U.S. from the Mediterranean region around 1800 as a grain or forage crop. The origin of *Sorghum halepense* is believed to be the Near East including Iraq, Iran, Turkey and Israel where it was used in early agriculture (Monaghan, 1979). Its distribution north is mainly limited due to the lack of cold hardiness most likely caused by the storage of starch instead of fructosans in rhizomes like in other temperate grasses (Hull, 1970). It is an aggressive C4 perennial weed species, which propagates by seeds or rhizomes and possesses a high reproductive ability (Anderson et al., 1960; Kaloumenos & Eleftherohorinos, 2009). A single plant can produce up to 28 000 seeds and between 60 and 90 m of rhizomes per growing season (Horowitz, 1972; Warwick & Black, 1983). *S. halepense* affects crop yield not only by competition for nutrients and water, but also by producing xenobiotic componds in root exudates that have an herbicidal activity (Bertin et al., 2003). It can grow up to 3 m in height and is difficult to control with herbicides because of its vigorous regrowth from rhizomes (Horowitz, 1972). Yield reductions up to 50 % in soybeans are reported for full season competition (Williams & Hayes, 1984).

S. halepense was listed as one of the ten world's worst weeds, being difficult to control in crops (Holm et al., 1991). The introduction of selective ACCase inhibitors and later the glyphosate tolerant crops along with the use of glyphosate permitted excellent control of Johnsongrass until resistance evolved. Five US *S. halepense* populations have been reported to be resistant to ACCase inhibitors with the first reported case being reported in 1991 (Smeda et al., 1997). Different populations from the same geographical region have been found to be resistant to either a single or to different chemical classes of ACCase herbicides (Burke et al., 2006). The resistance mechanisms conferring resistance to ACCase inhibitors were proposed to be either based on a higher expression of the *ACCase* gene or due to an insensitive ACCase enzyme (Bradley et al., 2001). Differences in the absorption or translocation of ACCase inhibitors, as well as the metabolism of the herbicide as reported in other ACCase resistant weed species have so far not been found (Bradley et al., 2001; Vila-Aiub et al. 2005).

One of the biggest advantages of glyphosate for controlling *S. halepense* plants is its good translocation pattern throughout the whole plant, including the difficult to kill roots and rhizomes (Kivlin & Doll, 1988; Warwick & Black, 1983; Hamill & Zhang, 1995). In 2005 the first confirmed case of glyphosate resistant *S. halepense* was reported in Argentina followed by a report in Arkansas (Heap, 2011). Both were found in RR soybean fields. Several locations throughout the mid United States were noted in 2011 for a lack of control of Johnson grass with glyphosate (Fig: 7) (Anonymous, 2011). Most recently Riar et al. (2011) found that glyphosate resistance of the Arkansas *S. halepense* population were based on an altered glyphosate translocation in plant. Resistance cases to both ALS and

VLCFAs[14] herbicides are also mentioned for some populations (Burke et al., 2006; Heap, 2011).

2.5 Scope of this study

Weed control by herbicides is necessary in most of the cropping systems worldwide. The continuous evolution of regulatory requests increases, the time and costs to develop novel herbicides. In addition the discovery of an herbicid with a novel mode of action and a good biological efficacy is a rare event. Therefore it is important to delay or circumvent herbicide resistance development in order to preserve existing solutions and MoA as long as possible. This is similar to the concept of preserving antibiotics important for human health (Shaner et al., 1997; Allen et al., 2010). In this respect, the scope of the present work is to increase and improve our knowledge about (1) the occurrence and importance of herbicide resistance mechanisms towards inhibitors targeting two major modes of action, *i.e.* glyphosate and ACCase inhibitors, and (2) the evolution of herbicide resistance mechanisms at the level of weed populations both in terms of trait longevity and in terms of resistance development or trait spreading. Key weeds from the U.S. resistant to glyphosate were studied, in particular *A. palmeri*, *A. tuberculatus* and *S. halepense*. This work was extended to the study of other herbicidal classes and resistance mechanisms in *S. halepense* because of the necessity to control the strong vegetative reproduction of that weed in case of glyphosate failure. Populations collected from the field, described as being either sensitive or resistant to glyphosate or/and other herbicides, were first characterized in the greenhouse after treatments with the chosen herbicides. Visual ratings or/and fresh weight assessments were performed along with a biochemical characterization of the shikimic acid content to characterize the plant response to glyphosate. In a second step, biochemical characterization of the target enzyme activity and molecular characterization of the corresponding genes and transcripts were performed. Finally, in a third step, representative populations were selected to study the genetic relationships among these populations.
In the recent past, significant effort has been invested to develop a weed control strategy that was simple and easy to use, relying on a single herbicide to protect a wide range of agricultural crops and cropping systems. Therefore it is of key interest to evaluate this popular and low cost weed control strategy in terms of herbicide resistance development, especially since agricultural production systems are strongly dependent on national and international trade and transportation.
The results will be discussed in term of the mechanisms evolved by the weeds to overcome herbicide treatments, both at the individual and the population levels. Finally, according to the results obtained, Integrated Weed Management strategies will be reviewed.

[14] Very Long Chain Fatty Acid synthase

3 Material & Methods

3.1 Origin of Plant Populations & Cultivation

The term "population" in the present thesis is in general used for plant seeds collected at a one location or are the result of one breeding processes between plants. A population indicated as glyphosate resistant can contain therefore glyphosate sensitive and resistant individuals depending on the heterogeneity of each single population. The classification into glyphosate resistant and sensitive populations was depending on the glyphosate control efficacy in field or greenhouse. A pre-selection for glyphosate resistant individuals was not performed in any of the studies and all values obtained, reflect therefore the average results of the individuals of a given population.
Glyphosate can be used in Germany with a concentration of up to 1800 g ae ha^{-1} glyphosate in the beginning of the work the lowest recommended field rate for the control of broad-leaf weeds was at 720 g ae ha^{-1} (Tomlin et al., 2011; Anonymous, 2012a). To keep consistency this rate is in the present work used as the recommended field rate. In the U.S. the lowest recommended field rate is at 560 g ae ha^{-1} glyphosate, but the recommended dose rate to control *A. tuberculatus* plants is today at 1120 g ae ha^{-1} glyphosate. Specifically *Amaranthus palmeri* is not anymore mentioned on the U.S. Roundup Ultra®label (Anonymous, 2012a).

3.1.1 Origin & Cultivation of *Amaranthus palmeri* & *Amaranthus tuberculatus*

The *Amaranthus palmeri* and *Amaranthus tuberculatus* seeds were collected between 2005 and 2009 at different fields throughout the southeast and mid U.S. as indicated for *A. palmeri* in Tab. 2 and for *A. tuberculatus* in Tab. 3. They were kindly provided by T. Gaines, P. Westra, S. Culpepper, L. Steckel, B Young, A Hager, A. York and K. Bradley for further work. Some of the populations, GA_R & GA_S, have been partly evaluated (Culpepper et al., 2006; Gaines et al., 2010). Steckel et al., (2008) reported first data related to the population TN_{LR} and Legleiter & Bradley (2008) to the populations Mo_{13}, Mo_{15} & Mo_{16} (Tab. 2 & 3). Their results and also the glyphosate resistance status of the remaining populations in field is described in Tab. 2 & 3.

The glyphosate resistance status of *A. palmeri* and *A. tuberculatus* populations in their response to glyphosate was conducted in spring 2009 at the Weed Research Laboratory, Colorado State University, Ft. Collins, U.S. in one experiment using the same conditions

Tab. 2: ***Amaranthus palmeri***; name, origin and year of collection; field or greenhouse response to glyphosate mentioned as reported and indicated by the subscripts: R - resistant, S - sensitive, LR - low level resistant; further information given as reported by Culpepper et al. (2006), Steckel et al. (2008) and Gaines et al. (2010); TN_{LR} was kindly provided by L. Steckel, Associate Professor, University of Tennessee; $NC_{R,R1,LR\,\&\,S}$ were kindly provided by A. York, William Neal Reynolds Professor Emeritus, North Carolina State University, U.S.; $GA_{S\,\&\,R}$ were kindly provided by S. Culpepper, Associate Professor and Extension Agronomist University of Georgia.

Name	Glyphosate resistance status	Year of sampling, origin & comments	
TN_{LR}	Resistant	2005	Crockett county, Tennesse (Steckel et al., 2008) glyphosate RF 1.5 - 5 x shikimate accumulation in sensitive and resistant
TN_R	Resistant	2009	Millington, Tennesse
NC_S	Sensitive	2007	North Carolina, collected close to NC_{R1}
NC_{LR}	Resistant	2007	Collected at a site between NC_S and NC_{R1}; weak and heterogeneous glyphosate resistance in field.
NC_{LRS}	Sensitive	2010	Inbred line using the individuals of NC_{LR} bearing a low amount of EPSPS gene copies
NC_{LRR}	Resistant	2010	Inbred line using the individuals of NC_{LR} bearing a high amount of EPSPS gene copies
NC_{R1}	Resistant	2007	North Carolina, Cotton field resistant population with unknown resistance mechanisms
NC_R	Resistant	2007	Cross between two highly resistant plants of NC_{R1}
GA_S	Sensitive	2005	Tift county, Georgia, (Culpepper et al., 2006; Gaines et al., 2010)
GA_R	Resistant	2005	Macon county, Georgia, (Culpepper et al., 2006; Gaines et al., 2010) $RF_{glyphosate}$ to GA_S 6 - 8 x lower shikimate accumulation than GA_S no resistance related changes in EPSPS gene sequence no changes in ploidy no changes in glyphosate absorption or translocation higher *EPSPS* gene copy number in genomic DNA (60 - 160 times more)
AR_R	Resistant	2009	Arkansas, West Memphis resistant field population with unknown resistance mechanisms
Herbiseed	Sensitive		commercial available seeds, purchased: Herbiseed New Farm, Mire Lane, West End Twyford, England, RG10 0NJ

for all populations of a species. To increase and promote the consistency of germination the different populations were sown in petri dishes containing 0.7 % agar type A (plant cell culture tested; A4550 Sigma Aldrich). The sown plates were stored for two days at 4 °C in the dark, before they were placed in the greenhouse to initiate germination at a photoperiod of 16 h light, 28 °C / 8 h dark, 15 °C. About 300 seedlings per population at the cotyledon developmental stage were initially transplanted into a 2 cm diameter pot system with a single plant per pot and grown for about three weeks, before 200 - 250 young and uniform plants for each population were subsequently transplanted into 8 cm diameter plastic pots containing a peat-vermiculite mixture (Fafard 2B Mix). The seeds of *A. palmeri* and *A. tuberculatus* used for all further steps in the Bayer CropScience, Weed Control Biology facilities in Frankfurt (Germany) were similarly cultivated but the seedlings were transplanted at the seedling first leaf stage into 4.5 cm Fertilpots containing peat/loam 1:1 soil mixture and were cultivated in the greenhouse at a photoperiod of 16 h light, 22 °C / 8 h dark, 14 °C with a light intensity of at least 220 μE m^{-2} s^{-1} (Phillips Son-T AGRO). The growing conditions between the greenhouses at the CSU Weedlab in Ft. Collins and at BayerCrop Science AG Weed Research Center in Frankfurt were initially standardized to allow the comparison of results.

The *A. palmeri* strains NC_{LRR} and NC_{LRS} were produced out of NC_{LR} plants bearing either a high or a low amount of *EPSPS* gene copies. Therefore, both groups of individuals were separated in two greenhouses, where 5 female and 3 male plants for each cross were grown together. To obtain a good fertilization plants were shake daily. Seeds were harvested when the majority were ripe, indicated by their black color. Watering and fertilization with 0.4 % Wuxal Super solution was in all cases done when needed.

3.1.2 Origin and Cultivation of *Sorghum halepense*

The glyphosate resistant *S. halepense* rhizomes and seeds, named GLY_R, were kindly provided by J. Norsworthy, University of Arkansas and collected by A. Hopkins, Sr. Field Development Specialist, in a site near West Memphis, AR in autumn 2009. The *S. halepense* rhizomes were sectioned into short rhizome pieces with 2 - 3 nodes, planted in 15 diameter plastic pots containing a peat/loam 1:1 soil mixture and placed in the greenhouse at a photoperiod of 16 h light, 22 °C / 8 h dark, 14 °C at a light intensity of at least 220 μE m^{-2} s^{-1} (Phillips Son-T AGRO) light intensity.

Seeds collected in the U.S. and commercially available at Herbiseed, named GLY_S, were used as sensitive control and grown from seeds. They were soaked for 12 hours in water at 22 °C, and then planted in peat/loam 1:1 soil mixture and stored for about 2 weeks at 4 °C in the dark, before they were allowed to germinate in the greenhouse at a photoperiod of 16 h light, 22 °C / 8 h dark, 14 °C with a light intensity of at least 220 μE m^{-2} s^{-1}

Tab. 3: ***Amaranthus tuberculatus***; name, origin, year of collection; field or greenhouse response to glyphosate mentioned as reported; further information given as reported by Legleiter & Bradley (2008); Il_1 was kindly provided by A. Hager Associate Professor University of Illinois; B. Young, Professor for Weed Science, Southern Illinois University, Carbondale U.S.; Mo_{13-18} were kindly provided by K. Bradley, Associate Professor, State Extension Weed Scientist, University of Missouri, U.S.; Co_1 was kindly provided by P. Westra, Professor and Extension Specialist, Weed Science, Colorado State University, U.S..

Name	Glyphosate resistance status	Year of sampling, origin & comments	
Il_1	Tolerant	2006	Illinois
Mo_{13}	Tolerant	2008	Missouri, Plate county, harvested from a single plant; resistance to glyphosate RF 19; PPO resistant, harvested from different plants probably heterogeneous (population MO1 Legleiter & Bradley 2008); resistance to PPO- & ALS-inhibitors, weak shifting for lactofen.
Mo_{14}	Tolerant	2008	Missouri, Pettis county
Mo_{15}	Tolerant	2008	Missouri, Plate county, harvested from several plants; glyphosate: RF 19; PPO resistant, (population MO1 Legleiter & Bradley 2008); resistance to PPO- & ALS-inhibitors , weak shifting for lactofen.
Mo_{16}	Tolerant	2008	Missouri, Holt county, (population MO2 Legleiter & Bradley 2008); glyphosate RF 9,
Mo_{17}	Tolerant	2008	Missouri, Callaway county
Mo_{18}	Sensitive	2006	Missouri, Howard county
Co_1	Sensitive		Colorado, *Amaranthus blitum*

Tab. 4: ***Sorghum halepense***; name, origin, year of collection; field or greenhouse response to glyphosate mentioned as reported; further information given as reported by Riar et al. (2011); GLY_R was kindly provided by B. Scott, Professor Extension Weed Science, University of Arkansas.

Name	Glyphosate resistance status	Year of sampling, origin & comments	
GLY_R	Tolerant	2009	Arkansas, West Memphis; Glyphosate resistance RF: 11; glyphosate resistance probably based on an altered glyphosate translocation (Riar et al., 2011)
GLY_S	Sensitive	2009	commercially available at Herbiseed, formally collected in U.S..

(Phillips Son-T AGRO).
Germinated plants (BBCH 11) were further cultivated in the greenhouse at a photoperiod of 16 h light, 28 °C / 8 h dark, 14 °C with a light intensity of at least 220 μE m^{-2} s^{-1} (Phillips Son-T AGRO) untill they were pruned approximately 1 cm above soil surface when they reached BBCH 50 (Hess et al., 1997). Afterwards they were allowed to regrow to obtain plants with well-developed and strong rhizomes. The further propagation was in all cases done by rhizome plantings using the conditions previously described. Plants propagated from the same rhizome can be considered as genetically identical clones.
Watering and fertilization with 0.4 % Wuxal Super solution was in all cases done when needed.

3.2 Bioassay

3.2.1 *A. palmeri* & *A. tuberculatus* Glyphosate Resistance Assessment

A dose response study was established to verify the previous reports of glyphosate resistance in *A. palmeri* and *A. tuberculatus* populations in the conditions used and to compare the different populations under the same conditions. The dose response relationship was established according to previous reports (Seefeldt et al., 1995; Michel et al., 1999; Ritz & Streibig, 2005; Knezevic et al., 2007; Onofri et al., 2009).
In the dose response study 13 decreasing doses of glyphosate were applied to compare the populations in their glyphosate resistance using a stationary sprayer outfitted with Teejet 8001 nozzles at 280 kpa and a spray volume of 300 L ha^{-1} (11520; 5760; 2880; 1440; 1080; 720; 540; 360; 180; 90; 45; 22.5; 0 g ae ha^{-1} glyphosate formulated as Roundup®Weathermax, 660 g acid equivalent (ae) L^{-1} glyphosate). The different dose rates were chosen in order to cover the whole range from 0 % until 100 % plant damage. Plants were treated when they reached in average BBCH 16 (Hess et al., 1997). Sensitive populations were used as control. In each population uniform plants in size and appearance were selected and 9 plants per dose of glyphosate were used to assess each population. All populations of one species were applied together and in equal conditions to allow unbiased comparisons within a given species. To assess the response to glyphosate of the *A. palmeri* populations NC_{LRS} and NC_{LRR} a dose response study was performed using the glyphosate dose rates of 2880; 1440; 720; 360; 180; 90; 45 and 0 g ae ha^{-1} glyphosate formulated as Roundup®UltraMax. The dose response study was applied to seven plants at BBCH stage 14 in greenhouse at the previous described conditions.
The highest dose rate represented 16-fold and the lowest 1/128-fold the labeled field rate of 720 g ae ha^{-1} glyphosate. An additional nonionic surfactant, 0.5 % NIS, was applied in all treatments and controls. The fresh weight was determined of the entire above ground plant material of each single plant, when the first plants start to regrow after the initial

glyphosate damage. In *A. palmeri* the fresh weight was assessed 16 days after treatment (DAT) and in *A. tuberculatus* 14 DAT.
The fresh weight data were statistically evaluated as described on page 32 (Knezevic et al.; 2007; R Development Core Team, 2010).

3.2.2 Glyphosate Resistance Assessment of *S. halepense*

To assess the glyphosate resistance of *S. halepense* populations a dose response study was established. The glyphosate resistance factor (RF) was assessed in mature plants, grown for 6 month in 13 cm diameter pots before they were finally pruned. Two weeks later, after regrow, eight doses of glyphosate (Roundup®Ultramax, 450 g ae L^{-1} glyphosate) were applied and compared to untreated control. The pots of the sensitive and resistant populations contained one plant each with 5 - 9 tillers at BBCH 42 (Hess et al., 1997). They were treated with 0; 90; 180; 360; 720; 1080; 1440; 2160; 2880 g ae ha^{-1} glyphosate using a stationary sprayer outfitted with Teejet 8001 nozzles at 280 kpa and a spray volume of 300 L ha^{-1}. To determine the glyphosate efficacy the plants were harvested 14 DAT, when the first regrow after initial glyphosate damage was visible. The entire above ground plant material was harvested and the fresh weight was expressed in mean weight per tiller. The fresh weight data were statistically evaluated as described on page 32 (Knezevic et al.; 2007; R Development Core Team, 2010).

3.2.3 ACCase Inhibitor Resistance Assessment of *S. halepense*

To assess the sensitivity to APP and CHD ACCase inhibitors, plants were grown for about nine month in 5 L pots under the previous described conditions. Two weeks before treatment they were finally pruned to allow regrow. At the time of treatment each pot contained a single plant with in average 24 tillers at BBCH 42 (Hess et al., 1997). Plants were considered as target site resistant when bearing at least a single mutated W2027C ACCase allele. Plants were separated into W2027 target site resistant and sensitive plants. The quantitative assessment of W2027C resistant *ACCase* alleles was performed by pyrosequencing analysis for each single plant as described on page 36. A selection for homogeneous plants in size and shape was done before application. The W2027C resistant plants were treated with 7 doses of fluziafop-ρ-butyl (Fusilade®max, Syngenta Agro GmbH) at 50; 250; 500; 750; 1000; 1250; 1500 g active ingredient (a.i.) ha^{-1}, 8 doses of quizalofop (Targa®Super, Nufarm limited) at 10; 30; 60; 80; 100; 200; 300; 500 g a.i. ha^{-1} and 7 doses of clethodim (Select 240 EC®, Stähler Deutschland GmbH & Co. KG) at 5; 15; 30; 60; 90; 120; 240 g a.i. ha^{-1}. The sensitive, wild type plants were used as susceptible standard and doses at and below field rate were applied, in particular 50; 250 g a.i. ha^{-1} fluazifop-ρ-butyl, 5;

15; 30; 60 g a.i. ha^{-1} quizalofop and 5; 15; 30; 60; 90 g a.i. ha^{-1} clethodim. Spraying was accomplished using a stationary sprayer at 280 kpa outfitted with Teejet 8001 nozzle at a spray volume of 200 L ha^{-1}. The fresh weight of entire above ground plant material was determined 21 DAT. Targa®Super and Select 240 EC®were applied together with 2 L ha^{-1} Para Sommer as adjuvants.
In a further experiment 100 g a.i. ha^{-1} fluziafop-ρ-butyl (Fusilade®max) were applied to determine the influence of the number of W2027C resistant alleles in *S. halepense* at BBCH 42 (Hess et al., 1997). At all 31 mature plants bearing none, a single or two W2027C resistant ACCase alleles were compared to six untreated control plants.
The plant injury in all cases was determined by fresh weight assessment and expressed as mean weight in g / tiller. Statistic significant differences between wild type and mutant plants were determined for each doses rate separately by using t-test with a probability value of P = 0.05 (SigmaPlot 11.0).

3.2.4 Sensitivity of *S. halepense* to Different Herbicidal Mode of Action

Since ACCase inhibitors and glyphosate failed to control *S. halepense*, herbicides with other modes of action were evaluated, in particular. Herbicides providing selective control by themselves or with the use of GMO tolerant crops like glutamine-synthetase-, ALS- or HPPD inhibitors, were applied to test their efficacy on *S. halepense* plants. Glufosinate as glutamine synthetase inhibitor was applied, formulated as Ignite®(Bayer CropScience AG), at 35; 71; 140; 285; 570; 1140 and 2280 g a.i. ha^{-1}, the combination nicosulfuron and prosulfuron (Milagro®Forte Peak®Pack, Syngenta Agro GmbH) as representative for the class of ALS inhibitors was applied at 11/21; 22.5/42; 45/ 84 and 90/168 g a.i. ha^{-1} and tembotrione was tested for its efficacy as representative of the class of HPPD-inhibitors, formulated as Laudis®(Bayer CropScience AG) at 20; 40; 76; 152; 218; 284 and 410 g a.i. ha^{-1}. The herbicides were applied as mentioned before, to three glyphosate resistant and sensitive mature plants with 5 - 9 tillers at BBCH 42 (Hess et al., 1997). The efficacy was visually rated 14 DAT.

3.3 Biochemical and Physiological Studies

3.3.1 Roundup Ready® Trait Test

To exclude a glyphosate resistance trait transfer between Roundup Ready®crops and weeds causing glyphosate resistance, 5 random plants of each population were first checked for the presence of the Roundup Ready®gene construct. The SDI TraitChek®crop and grain test kit (SDI; Strategic Diagnostics Inc.; 111 Pencader Drive; Newark, DE 19702 USA) was therefore used. Fresh leaf material was tested with a CP4-EPSPS specific antibody

according to manufacturer instructions.

3.3.2 Determination of Plant Shikimic Acid Accumulation

The plant shikimic acid concentration has been shown to increase after the EPSP-Synthase inhibition by glyphosate and is directly correlated with the degree of herbicide injury (Harring et al., 1998; Koger et al., 2005). The methods described by Cromartie & Polge (2000) and Koger et al. (2005) were used to determine the shikimic acid accumulation in plant 4 days after glyphosate application.
To identify the right leaf developmental stage to collect leaf samples for a shikimic acid assessment in *A. palmeri* and *A. tuberculatus* populations, nine plants of the glyphosate sensitive population Herbi (BBCH 18) were treated with the field dose of 720 g ae ha^{-1} glyphosate (Roundup®UltraMax, 450 g ae L^{-1} glyphosate) and in their shikimic acid content compared to three untreated control plants.
The plants were harvested 4 DAT and divided into single leaves, shoot tips, stem segments and roots. The stems were divided into the upper four nodes, the following three nodes and the remaining nodes. The material was weighed, and frozen at -20 °C. Then the tissues were incubated in the three fold amount (*w/v*) of 0.25 M HCl (Sigma-Aldrich®) at 60 °C for approximatively 1 h until complete digestion occurred. Digested plant material was disrupted with 5 mm stainless steel beads in a ball mill (Retsch®300MM) and centrifuged at 6000 g (Sigma®, 4K15) for 20 min afterwards, 10 μl of the supernatant was oxidized at 37 °C for 1 h in 100 μl periodate solution (0.3 % periodic acid, 0.3 % sodium-meta-periodate, Sigma®). The solution was quenched directly before measurement by adding 100 μl colour reagent (0.6 M NaOH, 0.22 M Na_2SO_3, Sigma®). The colorimetric determination of shikimic acid was conducted at a wavelength of 390 nm using a Fluostar plate reader. The shikimic acid values, expressed in μg / mg fresh weight, were calculated by using a standard curve. Statistic significant differences among the shikimic acid concentrations in tissues were evaluated by using a t-test at a probability value of $P = 0.05$ (SigmaPlot 11.0).

Shikimic Acid Accumulation in Glyphosate Sensitive and Resistant *A. palmeri* and *A. tuberculatus* Populations

To compare the rise of shikimic acid and the glyphosate mediated plant growth reduction, the shikimic acid content was measured in the glyphosate treated plants of the previous dose response experiment (see pp. 28). Four 0.4 cm diameter leaf discs of the youngest fully expanded leaf of each plant were harvested, with exception of plants of the highest dose rate tested. At this dose rate of 11520 g ae ha^{-1} glyphosate the young leaves were

that strongly stunted that an adequat sampling was impossible. The dose rate of 5760 g ae ha^{-1} glyphosate or 8-fold the labeled field rate represented therefore the highest glyphosate concentration tested in the shikimic acid assay. To determine the shikimic acid concentration in the leaf discs, the plant material was frozen at -20 °C before it was incubated in 250 μl 0.3 M HCl (Sigma-Aldrich®) at 60 °C until the material was completely digested, indicated by turning from green into a brownish color. A 25 μl aliquot was mixed with 100 μl periodate solution (0.3 % periodic acid, 0.3 % sodium-meta-periodate) and incubated for 1 h at room temperature. The colour reaction was obtained and stabilized by addition of 100 μl colour reagent (0.6 M NaOH, 0.22 M Na_2SO_3) and read at 380 nm on a 96-well plate reader (Biotek®Synergy microplate reader).

3.3.3 Dose Response Relation - Data Processing

The dose response relation data obtained by fresh weight and shikimic acid assessment were fitted by using the GNU-licensed statistical program "R" and the supplemented package "drc" to calculate the 4-parameters sigmoidal log-logistic dose-response model (1)(Knezevic et al., 2007; R Development Core Team, 2010). The parameters are "c" and "d" as the lower and upper limits and "b" as the slope in the turning point "e" of the log-logarithmic curve. The turning point "e" is also representing the effective dose rate causing 50 % (ED_{50}) growth reduction or to increase the shikimic acid concentration to 50 % of the maximum value measured in each population. The fresh weight and shikimic acid data were fitted to the following formula when the data are in a lack of fit test not significant (P = 0.05) (Knezevic et al., 2007).

$$y = c + \frac{d - c}{1 + e^{(b(ln(x) - ln(\mathrm{ED}_{50})))}} \tag{1}$$

Upper and lower limits were in the second step used to normalize the raw data allowing unbiased comparisons of dose response curves fitted to the model. ED_{50} and ED_{90} values were calculated with the raw data by using the 4 parameter model. ED_{90} values are only provided for *A. tuberculatus* populations. The dose-response curves were considered to be statistically significant different if the p-values in the ANOVA were $P \leq 0.05$. The resistance factor (RF) of population is calculated based on the quotient between the ED_{50} value of the population in question and most sensitive population of each species.

3.3.4 EPSPS Enzyme Activity

To measure EPSP-synthase activity, the enzymes were extracted, fractionated, concentrated and desalted to remove unwanted ions like inorganic phosphate (P_i) and other enzymes like phosphatases which might inhibit or disturb the EPSPS activity test.
The enzyme extraction itself was performed according to Arnaud et al. (1998) at 4 °C with minor modifications. The youngest leaves and shoot tip of young and actively growing plants were harvested (BBCH 18) and ground with mortal and pestle in liquid nitrogen. The powdered plant material was mixed with buffer solution containing 50 mM Tris-HCl pH 7.5, 5 mM EDTA, 5 mM $MgCl_2$ and 50 mM mercaptoethanol until a homogenous solution was reached and then filtered through 5 layers of cheesecloth. The extract was centrifuged for 30 min at 10000 g to remove cell debris. Unwanted proteins in solution were removed by a fractionated ammonium sulfate precipitation. The fraction between 40 - 70 % (w/v) ammonium sulfate was dissolved in a buffer, containing 20 mM Tris-HCl, pH 7.5, 5 mM $MgCl_2$ and 1 mM mercaptoethanol and desalted using NAP^{TM} 5 columns (GE-Healthcare). The enzyme extraction was mixed with 20 % glycerol (v/v) and stored at -20 °C. The protein concentration in solution was determined in a Bradford assay (Bio-Rad Protein assay, Cat.No. 500-0006) adjusted with a BSA calibration curve (Bradford, 1976). Results are given as μg protein in BSA equivalent protein.
The EPSPS activity was measured by monitoring the P_i release of the crude protein extract incubated together with the two EPSPS substrates S3P and PEP according to Gaines et al. (2010). The P_i release was determined with EnzCheck®Phosphat assay kit (E6646; Invitrogen) by a continuous color reaction due to the enzymatic conversion of 2-amino-6-mercapto-7-methylpurine riboside (MESG) to ribose 1-phosphate and 2-amino-6-mercapto-7-methylpurine by purine nucleoside phosphorylase (PNP). The crude enzyme extract was therefore incubated in a total volume of 250 μl containing 50 mM MOPS, 10 mM $MgCL_2$, 1 mM $NaMoO_4$, 100 mM KF, 280 μM MESG, 0.5 mM PEP, 0.5 mM S3P and 2 U μl^{-1} PNP. The color reaction was continuously measured at 360 nm in a Fluostar Optima 96 well plate reader to determine EPSPS enzyme parameters. The amount of catalytic active EPSPS enzyme (K_{cat}) in the *A. tuberculatus* populations Il_1, Mo_{13}, Mo_{15}, Mo_{16} and Mo_{18} was compared by using a Biotek Synergy microplate reader.
To determine the enzymatic parameters of the EPSPS the S3P concentration was held constant at 0.5 mM while the PEP concentration varies (0.5, 0.25, 0.125, 0.0625, 0.031, 0.016 and 0 mM PEP). The experiments were conducted with 2 technical and at least 2 biological replicates of each population (NC_S, NC_R, Il_1 & Mo_{18}). The optical density was measured continuously and transformed by a calibration curve into μM of P_i release. The data were fitted to the linear Lineweaver-Burk model to obtain the K_m, V_{max} and K_{cat} values of the EPSPS by using Sigmaplot 11.0 (Lineweaver & Burk, 1934). A probability value of $P \leq 0.05$ was chosen to determine significant differences in a t-test between pop-

ulations (Sigmaplot 11.0).

3.3.5 Glyphosate Absorption and Translocation

Alterations in glyphosate uptake and translocation are, according to SHANER (2009) one of the major resistance mechanisms in glyphosate resistant weeds and were therefore tested in the *A. palmeri* populations NC_S and NC_R and in the *A. tuberculatus* populations Mo_{18}, Mo_{13} and IL_1. To allow the development of a leaf cuticle as close as possible to the one which occurs in natural conditions, all plants were propagated for one week under natural open air conditions during summer 2009 before glyphosate application. The application solution was adjusted to a final rate of 720 g ae ha^{-1} glyphosate using Roundup®Ultramax (450 g ae L^{-1} glyphosate) and radiolabeled glyphosate (phosphonomethyl-[^{14}C]) solution with a specific activity of 50 mCi $mmol^{-1}$ (ARC, American Radiolabeled Chemicals). Twelve 0.5 μl droplets containing 1000 Bq each were spotted onto the youngest fully expanded leaf. The treated plants were incubated until harvest in a growth chamber with a photoperiod of 16 h light and a light intensity of 120 μE m^{-2} s^{-1} (IWASAKI EYE MT150D) at a temperatur of 25 °C/18 °C day/night and a constant relative humidity of 70 %. To determine the glyphosate leaf uptake, treated leaves were cut from the plant at harvest time and rinsed during twenty seconds in 3 ml 0.1 % Triton X100 and 4 % methanol. An aliquot of rinseate was mixed with 20 ml scintillation liquid (Roth; Eco Plus) and measured in a scintillation counter (Packard 2000CA TriCarb Liquid Scintilation counter). The radioactive leaf uptake in *A. palmeri* populations was calculated as % of total applied radioactivity, assuming it as ^{14}C-labled glyphosate. In *A. tuberculatus* populations the leaf uptake and translocation is expressed as the absolute values of radioactivity, calculated in Bq per leaf.

To determine plant glyphosate translocation quantitative and qualitative measurements were performed. The translocation was determined quantitatively by combustion of different plant sections and CO_2 trapping. The harvest time points were set for the *A. palmeri* populations NC_S and NC_R at 4, 8, 24, 32, 48, 120 HAT, respectively, whereas the *A. tuberculatus* populations Mo_{18}, Mo_{13} and IL_1 were tested at 8, 24, 48, 72 HAT, respectively. At the harvest time points the soil was washed off the roots and plants were sectioned into root, above treated leaf, the remaining shoot material and the treated and rinsed leaf. The sectioned plant material was dried at 60 °C in an oven and combusted afterwards in a biological oxidizer (OX-300, R.J. Harvey Instrument Corp.). The released CO_2 was collected in 10 ml trapping and scintillation solution (Oxysolve C400, Zinsser Analytics) after 2 min oxidation period (1.5 min oxygen, 0.5 min nitrogen) at 900 °C. The radioactivity was measured in a liquid scintillation counter (Packard 2000CA TriCarb Liquid Scintillation counter). Values for translocation into roots, shoot, shoot tips or remaining radioactivity

in the treated leaf were calculated for *A. palmeri* in % of total absorbed ^{14}C-glyphosate. For *A. tuberculatus* the results represent the absolute values per combusted plant part in Bq. Significant differences were calculated by ANOVA at a probability value of $P \leq 0.05$ (SigmaPlot 11.0).

To visualize the time course of glyphosate translocation and to determine qualitative differences in the glyphosate transport, autoradio-grams were performed with treated plants. The timepoints were set at 4; 8; 16; 24; 74 and 96 HAT for the *A. palmeri* populations NC_S and NC_R, while no sample was harvested at 16 HAT for the *A. palmeri* populations HERBI. For the *A. tuberculatus* populations Il_1, Mo_{13} and Mo_{18} the time points were set at 8; 18; 48; and 96 HAT. Five treated plants of each population and at each time point including the washed leaf and rinsed root were fixed on a paper sheet (20 x 40 cm) and pressed until complete dryness for 2 days at 50 °C in an oven. The dry plants were exposed to a phosphoimaging film (Fujifilm BAS-MS-2040) for 48 h before reading the plates in a BAS-reader 1000 (Fujix). Background reduction and evaluation was done with AIDA Image Analyzer-Software 4.19.

3.4 Molecular Biology Studies

3.4.1 DNA and RNA Extraction and Purification

DNA or RNA extractions were performed mainly using the Qiagen®DNeasy plant mini kit and the Qiagen®RNeasy mini kit according to the manufacturer instructions, respectively. To determine the genomic *EPSPS* gene copy number in the *A. palmeri* populations NC_S, GA_S, TN_{LR}, NC_{LR}, NC_R, NC_{R1}, GA_R, AR_R & TN_R and in the *A. tuberculatus* populations Il_1, Mo_{13}, Mo_{14}, Mo_{15}, Mo_{16}, Mo_{17} & Mo_{18}, the DNA was extracted with the "Promega Wizard Magnetic 96 DNA Plant system kit" according to manufacturer instructions. The plant material for all DNA extractions was homogenized with 5 mm stainless steel beads (Qiagen®) in a ball mill (Retsch 300MM) at 30 Hz for 3 - 5 min at room temperature. For RNA extraction, using the RNeasy mini kits the samples were frozen in liquid nitrogen and homogenized for 30 sec, before the plant material was cooled down again in liquid nitrogen. This step was repeated until well homogenized samples.

To perform the Southern blot analysis higher pure and less shared DNA was extracted according to the modified CTAB methode of Porebski et al. (1997). All centrifugation steps were carried out at 4 °C and 6000 g (Beckmann Avanti®J-26 XP) until complete clarity of supernatant.

The extracted DNA concentration and quality was measured in all cases using a nanodrop device (Thermo Scientific®Nanodrop 1000), before the samples were stored at -20°C in ddH_2O.

Tab. 5: Oligonucleotide sequences for molecular biology studies in *A. palmeri* and *A. tuberculatus*; diluted to a concentration of 100 pmol μl^{-1}; forward primer indicated by subsequent "f"; reverse primer indicated by subsequent "r"; subsequent "s" indicates pyrosequencing primer; the amino acid (AA) position gives the target site to analyze in the *EPSPS* gene sequence; "*" indicates primer published by Gaines et al. (2010); oligonucleotides purchased by Eurofins MWG Operon, Ebersberg, Germany.

Name	AA position	Sequence		Tm[°C]
gly.a.1f		5' GCCTTCATCTGTCCCAGAAATTG 3'		60.6
gly.a.2f		5' AGAAATTGTGTTACAACCCA 3'		50.2
gly.a.3f		5' ATGTTGGACGCTCTCAGAACTCTTGGT 3'	*	65.0
gly.a.4r		5' TGAATTTCCTCCAGCAACGGCAA 3'	*	65.1
gly.a.5f		5' CGGAGTACCAAGAATGAGGGAGC 3'		64.2
gly.a.6r		5' CCTTTAGCATTGACCCGAACAG 3'		60.3
gly.a.7f		5' GCTCTCTGGATCGGTTAGTA 3'	*	57.3
gly.a.8f		5' CTCCTGGAAAGGCATATGTTGAGG 3'		67.7
gly.a.9r		5' CCTCAACATATGCCTTTCCAGGAG 3'		62.7
gly.a.10r		5' GCAAGAGTCATAGCAACATCTGGC 3'	*	62.7
gly.a.11f		5' CAGGGAATCATCTGGAAGGAAACATTTG 3'	*	63.7
gly.a.12r		5' CTTCGGCAAATTTTACATCACC 3'		56.5
gly.a.13r		5' GCTTTCTCAGTTCTGTGCAAATGG 3'		61.0
gly.a.14f		5' GGCACAACTGTGGTCGACAACTTG 3'		64.4
gly.pyro.a1.1s	G101, T102	5' GGAAATGC(AT)GGAACAGCGATGCG 3'		64.2
gly.pyro.a1.2s	P106	5' CAACTTTT(CT)CTTGGAAATGC 3'		52.2
als.a.1f		5' GCTGCTGAAGGCTACGCT 3'	*	58.2
als.a.2r		5' GCGGGACTGAGTCAAGAAGTG 3'	*	61.8
act.a.1f		5' GACTCTGGTGATGGTGTGAGTC 3'		62.1
act.a.2r		5' GAGCTGCTCTTGGCAGTCTC 3'		61.4

3.4.2 *ACCase* and *EPSPS* Target Site Detection

Pyrosequencing™ was used to detect target site mutations in the *A. palmeri* and *A. tuberculatus EPSPS* gene sequence and in the *S. halepense EPSPS* and *ACCase* gene sequences. The sequencing reaction was performed as described by Peterson et al. (2010). The DNA purification was done with the Qiagen®DNeasy Plant mini Kit as previously described. For the sequencing reactions the DNA fragment containing the suggested target site mutations was amplified in a polymerase chain reaction (PCR), using the oligonucleotides mentioned in Tab. 5 & 6. To sequence the *A. palmeri* and *A. tuberculatus EPSPS* target sites G101, T102 and P106 a 195 bp long fragment was amplified. The forward primer gly.a.3f and the 5'-biotinylated reverse primer gly.a.4r were therefore used (Tab. 5). The PCR conditions were 15 min preincubation at 95 °C, followed by 45 cycles at 94 °C de-

Tab. 6: Oligonucleotide sequences for molecular biology studies in *S. halepense*; initial "acc" indicates oligonucleotides to sequence *ACCase* target site mutations; initial "gly" oligonucleotides to sequence target site mutations in *EPSPS* gene sequence; oligonucleotides diluted to a concentration of 100 pmol μl^{-1}; forward primer indicated by the subsequent "f"; reverse primer indicated by subsequent "r"; "s" indicates pyrosequencing primer; the amino acid (AA) position gives the target site to analyze in the *ACCase* and *EPSPS* gene sequence; oligonucleotides were purchased by Eurofins MWG Operon, Ebersberg, Germany.

Name	AA position	Sequence	Tm[°C]
acc.s.1f		5'TTGTCCCTGCTGATCCAGGT3'	59.4
acc.s.2r		BT-5'CCCTTGAGGCTCGAGAACAT3'	59.4
acc.pyro.s.3	W2027	5'CCTCTGTTTATCCTGGCTAAC3'	57.9
acc.pyro.s.4	I2041	5'ACAGAGAGATCTCTTTGAAGGA3'	56.5
acc.pyro.s.5	D2078	5'GTGGAGGAGCTTGGGTTGTGGTC3'	66.0
acc.pyro.s.6	C2096	5'GTGGAGGAGCTTGGGTTGTGGTC3'	61.0
acc.s.7f		5'ACTATGGCCGTATTAGCTCT3'	55.3
acc.s.8r		BT-5'AAACACCTTCAAGGTCATCT3'	53.2
acc.pyro.s.9	I1781	5'ATGGACTAGGTGTGGAGAAC3'	57.3
gly.s.1f		5'CCCTCGGRCTCTCTGTGGAAGC3'	66.7
gly.s.2r		BT-5'TAGGTCGCTCCCTCATTCTT3'	57.3
gly.pyro.s.3	G101, T102, P106	5'AGCTCTTCTTGGGGAATGC3'	59.4

naturating step (30 sec.), 55 °C annealing temperature (40 sec), 70 °C elongation time (40 sec.) and a final extension step for 10 min at 70 °C in an Eppendorf Thermal cycler (Eppendorf Mastercycler® ep Thermal Cyclers). The primer gly.pyro.a1.2 was used to sequence the coding sites G101 and T102, while the primer gly.pyro.a1.1 was used to sequence the coding site P106 in a Pyromark PSQ 96 device.

To determine the *ACCase* and *EPSPS* target site mutations in *S. halepense*, approximately 1 cm^2 leaf of the tested plant was disrupted with 5 mm stainless steel beads in 400 μl 100 mM Tris(HCl) and 1 M KCl, pH 9.5 in a ball mill (Retsch 300MM) at 30 Hz for 3 min at room temperature. After homogenization, the plant material was centrifuged at 3000g and the supernatant was used as template in the PCR amplification in a 25 μl total reaction volume, where 0.1 μl of the supernatant was mixed 1:1 with Hotstart Taq (Qiagen®): ddH_2O and 0.1 pM of each primer to perform the PCR. A 380 bp long biotinylated (BT) *ACCase* fragment containing the W2027 mutation site was amplified using the forward and reverse primers as mentioned in Tab. 6, acc.s.1, and acc.s.2, respectively. The PCR conditions were 95 °C preincubation for 15 min followed by 45 cycles at 94 °C denaturating step (30 sec.), 54 °C annealing temperature (40 sec.), 70 °C elongation time (40 sec.) in 45 cycles and a final extension step for 10 min at 70 °C. The pyrosequencing reaction was performed using the pyrosequencing primer acc.s.3 in a Pyromark PSQ 96 device. The

reactions to sequence the mutation sites coding for the aminoacid (AA) positions I2041, D2078 and G2096 in the *ACCase* sequence were performed using the same fragment but with the sequencing primer acc.s.4, acc.s.5 and acc.s.6, respectively. To sequence the mutation site I1781 of the *ACCase* gene sequence a 405 bp long fragment was amplified by using the forward primer acc.s.7 and acc.s.8 as the reverse primer, acc.s.9 was used as the sequencing primer.
To sequence the G101, T102 and P106 on the *EPSPS* sequence, the forward primer gly.s.1 and the reverse primer gly.s.2 were used to amplify the 389 bp long fragment before the pyrosequencing analysis using gly.s.3. Evaluation was in all cases done with the by the manufacturer supplemented software (Qiagen®).

3.4.3 Determination of the Relative Genomic *EPSPS* Gene Copy Number

The *EPSPS* gene copy number in the *A. palmeri* and *A. tuberculatus* genomic DNA was determined by quantitative PCR (qPCR) in a LightCycler 480 (Roche) in relation to the *ALS* gene as reference gene according to Gaines et al. (2010) with minor modifications. Each plant was tested in three technical replicates for the *EPSPS* and *ALS* gene sequence. A negative control to test the absence of DNA contaminations, the supplied Mastermix and primers was done in addition on each plate. The DNA was purified using the "Promega Wizard Magnetic 96 DNA Plant system kit" according to manufacturer instructions and 0.001 μg DNA of these extracts was used as qPCR template. The mastermix was mixed according to the manufacturer protocol and contained 20 μl reaction solution per well (10 μl Roche LightCycler 480 H.R. Melting Master, 2.7 μl ddH_2O and 2.3 μl 25 mM MgCl solution). To amplify the *EPSPS* fragment, 0.12 pmol of each of the forward primer gly.a.3f and of the reverse primer gly.a.4r were used per reaction whereas, to amplify the *ALS* gene fragment 0.12 pmol of each of the forward primer als.a.1f and the reverse primer als.a.2r were used per well. The gene fragments were amplified in a Roche LightCycler 480 device after a 15 min preincubation time period at 95 °C followed by 35 cycles with a 95 °C denaturating step and a annealing and elongation step for 1 min at 60 °C. The florescence of Cyber green agent was determined after each annealing and elongation step. Cybergreen is only inserted into the double stranded DNA and the occurring florescence reflects therefore the amount of amplified DNA. To determine the relative genomic *EPSPS* gene copy number in relation to the *ALS* gene, the increase of the double stranded DNA for both gene fragments was measured. The comparison was done at a level of 12 times the standard deviation of the background by using the model of crossing points with the supplemented software (Roche LightCycler 480 1.5). The comparison was done within a single individual by using the average of the three replicates of the *EPSPS* and *ALS* gene fragments. The difference between the *ALS* gene and the *EPSPS* gene was either expressed as difference

in PCR cycles between crossing points ($\Delta\Delta$ cp) or as relative *EPSPS* gene amount in relation to the reference gene (Livak & Schmittgen, 2001; Larionov et al., 2005; Gaines et al., 2010).
A calibration curve using increasing DNA concentrations to determine the amplification efficacy of each primer combination used was done before. The relative *EPSPS* gene copy number *in planta* was calculated in relation to the *ALS* gene by using the formula (2).

$$r = x^n \tag{2}$$

Whereas "r" is the ratio between *EPSPS* and the ALS gene, "x" is the amplification efficacy of the fragment at the used conditions while "n" is the calculated difference in PCR-cycles between the crossing points of double stranded DNA concentration present in the probe during each PCR-cycle.

3.4.4 *EPSPS* Expression Characteristics

Plants at four-leaf stage, BBCH 14, were sprayed with 720 g ae ha^{-1} glyphosate (Roundup® Ultramax, 450 g ae L^{-1} glyphosate) using a stationary sprayer outfitted with Teejet 8001 nozzle at 2.8 bar and a spray volume of 200 L ha^{-1}. Plant samples from shoot tip were taken directly before treatment, and at 4; 8; 24 and 48 HAT from both treated and untreated plants maintained under identical conditions (Zhu et al. 2008). After being harvested, the samples were immediately frozen in liquid nitrogen and stored at -80 °C until RNA extraction. RNA was purified with RNeasy mini kit (Qiagen®) according to manufacturer instruction, followed by an DNase I (Invitrogen™) treatment. The cDNA synthesis was performed using the Superscript kit (Invitrogen™) in an Eppendorf thermalcycler (Eppendorf Mastercycler®ep Thermal Cyclers).
To determine the *EPSPS* mRNA expression the previous described method to measure gene amplification in genomic DNA was used. In addition, a 242 bp long fragment from the *Actin* gene was used as reference to assess the *EPSPS* expression, known to be constitutively expressed at a constant level. The *Actin* gene fragment was amplified with the primer pair act.a.1f and act.a.2r (Tab. 5). The primer sequences were derived from an alignment of different *Actin* genes together with the known *Actin* sequence from *Amaranthus tricolor* to find conserved areas within the gene sequence (NCBI Accession number: EF452618). Evaluation was done as previously described with the supplemented software (Roche LightCycler 480 1.5). The obtained crossing points of the *Actin* fragment were tested for statistic differences in all timepoints with a t-test at a probability value of P = 0.05 throughout the populations NC_S, NC_R, Mo_{18}, Mo_{13} and Il_1 and for all time points and used as stable expressed reference gene. The values obtained for the *Actin* gene were tested for their homogenity by ANOVA (t-test). The relative expression values of the *EPSPS* and *ALS* gene were given by the ratio of their respective values with those obtained

for the *Actin* gene. Significant differences between the relative *ALS* and *EPSPS* gene expression values at different time points were tested by ANOVA at a probability value of P = 0.05 (Sigmaplot 11.0).
A DNA dilution series was performed before, to determine the *EPSPS*, *ALS* and *Actin* amplification efficacy in each PCR amplification step during qPCR. The dilution series showed that the gene fragments of sensitive and resistant plants were doubled in each PCR cycle. According to these results, the amplification factor was "2", and the relation (r) between the *EPSPS* and the reference gene could be determined by the equation $r = 2^n$, whereas n = difference of crossing points in PCR cycles.

3.4.5 Internal Structure of the *A. palmeri EPSPS*

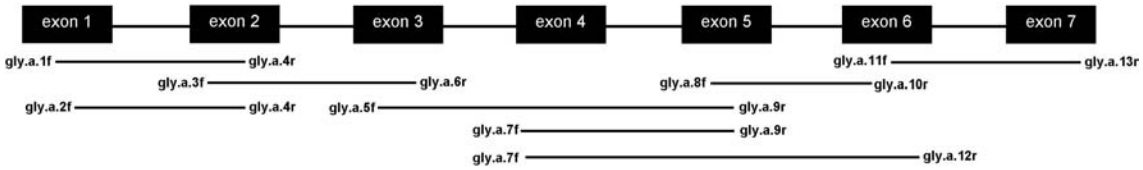

Fig. 8: Primer localisation in suggested *A. palmeri EPSPS* exon-intron structure; schematic graphic derived from published *Conyza canadensis* and *Eleusine indica EPSPS* gene structure; description of oligonucleotide sequences according to Tab. 5.

The *EPSPS* cDNA sequence of *A. palmeri* is known but the genomic sequence and the structural organisation (intron-exon) of the gene is still unknown. To perform the Southernblot it was important to gain more knowledge on this genomic *EPSPS* gene structure. The published *Eleusine indica* and *Conyza canadensis EPSPS* sequences showed 7 Exons, equal in size whereas the intron length varies. The structure of both sequences was therefore used to determine the probable *A. palmeri EPSPS* structure. Primers were designed located in adjacent exons overlapping the suggested introns, based on the published cDNA sequences of *A. palmeri*. The fragments were amplified using purified DNA either from NC_S and from NC_R as template and HotStar Taq DNA polymerase (Qiagen®). The primer to amplify the fragments are described in Tab. 5 and were used at a concentration of 0.4 μM each as indicated in Fig. 8 in a total volume of 50 μl. The PCR conditions were 15 min preincubation at 95 °C followed by 45 cycles at 94 °C denaturating step (30 sec.), 50 °C annealing temperature (30 sec), 70 °C elongation time (2 min) and a final extension step (10 min) at 70 °C in an Eppendorf Thermal cycler. The whole gene was amplified using LongRange PCR Kit (Qiagen®) at 3 min initial activation step 93 °C followed by 35 cycles of 15 sec denaturation at 93 °C, 30 sec at 55 °C annealing step and an elongation step of 11 min at 68 °C. The forward and reverse primer gly.a.1f and gly.a.13r were used to

amplify the gene at 0.4 μM each in a total volume of 50 μl. The fragments were evaluated in a 0.8 % agarose gel stained with ethidium bromide. Length determination was done according to the 100 bp Invitrogen™TrackIt™DNA plus ladder.

3.4.6 Southern blot

The Southern blot was performed to validate the PCR data related to the *A. palmeri EPSPS* gene amplification and to detect quantitative differences between the amplified *EPSPS* gene sequences from the glyphosate resistant and sensitive plants, respectively (Southern, 1975). In a Southern blot differences in the DNA sequence between *EPSPS* copies within or among individuals or populations can be detected by comparing the fragment length, whereas alterations in the gene amount will be visualized according to the signal intensity.
The genomic DNA was digested by restriction enzymes and separated in an agarose gel, before the transfer to the membrane. The labeling of the DNA probe can be obtained by radioactive or digoxigentin labeling, assembly of fluorophores or other labeling systems. Here the digoxigentin (DIG) labeling system in combination with a chemiluminescent detection, based on CDP-star alkaline phosphatase substrate was used (Roche). The preparation of the Southern blot is following the "DIG application Manual for filter hybridization" supplied by Roche.
To perform the Southern blot, 10 μg of genomic DNA, were restricted by the DNA restriction enzymes Hind III, Xba II, Not I and Btg I (New England Biolabs®) at 37 °C for 7 h in 25 μl of the supplemented restriction buffers. To get a complete DNA digestion the restriction enzymes were 5 fold higher dosed than in the recommended protocol. The same amount of enzyme was added 5 h after the start of the digestion for an additional incubation period of 2 h. The digested DNA solution was heated up for 5 min to 95 °C prior to mixing with the loading buffer (Blue juice; Invitrogen™) and loaded on a 0.8 % agarose gel, stained with ethidium bromid. The DNA was slowly separated in a distance of about 10 cm over a time period of 5 h together with 100 bp TrackIt™DNA ladder (Invitrogen™) and DIG labeled 0.08 - 8.57 kbp DNA molecular weight marker VII (Roche). The DNA was depurinated by soaking the gel for 7 min in 0.25 M HCl before the DNA was denaturated by soaking the gel for 30 min in 0.4 M NaOH. The capilary transfer to a positively charged nylon membrane (Roche) was done with 0.4 M NaOH buffer over-night. Afterwards, the membrane was neutralized by soacking it in 2 x SSC buffer (0.3 M NaCl, 0.03 M sodium citrate, pH 7.0) for 3 min. The DNA was crosslinked to membran by 1.30 min exposure to UV-radiation. The membrane was stored dry and at roomtemperature until probe hybridization.
The probe was PCR labeled using the PCR DIG Probe synthesis kit (Roche) according to

manufacturer instructions to incorporate DIG-11-dUTP into the amplified DNA-strand, using the forward primer gly.a.14f (Tab. 5) and the reverse primer gly.a.4r to obtain a 195 bp long PCR fragment. The probe covers the second exon of the *A. palmeri EPSPS* gene. The probe hybridization to membrane was done in rolling bottles in an oven at the required temperatures and using the DIG Easy Hyb buffer (Roche). The hybridization temperature (T_{hyp}) was determined according to the following formulas.

$$T_m = 49.82 + 0.41(\%\ G + C) - 600/l \tag{3}$$

Whereas the % G+C = 47.2 % in the l = 195 bp long probe and results in T_m = 66.1 °C. The hybridization temperature for this probe was calculated by the following formula (4)

$$T_{hyp} = T_m - (20°C\ to\ 25°C) \tag{4}$$

therefore is T_{hyp} = 41.1 °C. The membrane was equilibrated prior hybridization in prewarmed, 41.1 °C hybridization buffer for 1.30 h. During the membrane equilibration the probe was denaturated in a boiling water bath for 5 min and quickly chilled on ice. The chilled probe was mixed with prewarmed hybridization buffer (DIG Easy Hyp, Roche) and immediately added to the equilibrated blot. Hybridization was completed for 14 h over night at 41.1 °C. The following washing steps were performed as described in the manual. The high stringency washing was accomplished at 68 °C with 0.1 x SSC-buffer and 0.1 % SDS for 5 min. The visualization of chemiluminescent labeled probe on the Southern blot was performed as described in the manual by using the DIG Wash and Block buffer set (Roche), the Anti-Digoxigentin-alkaline phosphatase antibody (Roche) and the CDP-Star solution (Roche). The chemiluminescence detection was done by 10 h exposure to a X-ray film (Kodak BioMax). The X-ray film was developed under weak red light by shaking the film for several seconds in developing solution (Kodak GBX developer/replenisher). When the picture starts to be visible, the film was directly washed for 5 min in fixing solution (Kodak GBX fixer/replenisher). Finally, the X-ray film was washed in dH_2O, dried and evaluated.

3.5 Population Analysis by RAPD Markers

The analysis of the relationship analysis between populations was performed by using Rapid Amplified Polymorphic DNA (RAPD) marker according to Huff et al. (1993) and Rowe et al. (1997), first described by Williams et al. (1990). A single 10 bp oligonucleotide is used to detect inverted repeats in the genomic DNA by PCR amplification and evaluation in an agarose gel. Each primer can produce, depending on the sequence, non, a single or a nearly endless amount of marker amplified out of the whole genome. Each

Tab. 7: Oligonucleotide primers for RAPD analysis in *A. palmeri* (AP) and in *A, tuberculatus* (AT) populations; oligonucleotides diluted to a concentration of 100 pmol μl^{-1}; oligonucleotides purchased by Eurofins MWG Operon, Ebersberg, Germany.

Primer	Sequence	AP	AT	Primer	Sequence	AP	AT
OPW 1	5' CTCAGTGTCC 3'		X	OPW 18	5' TTCAGGGCAC 3'	X	
OPW 2	5' ACCCCGCCAA 3'	X	X	OPW 20	5' TGTGGCAGCA 3'	X	
OPW 4	5' CAGAAGCGGA 3'	X	X	OPN 2	5' ACCAGGGGCA 3'	X	
OPW 5	5' GGCGGATAAG 3'	X	X	OPN 4	5' GACCGACCCA 3'	X	
OPW 6	5' AGGCCCGATG 3'	X		OPN 6	5' GAGACGCACA 3'	X	
OPW 7	5' CTGGACGTCA 3'	X	X	OPN 11	5' TCGCCGCAAA 3'	X	
OPW 9	5' GTGACCGAGT 3'		X	OPN 12	5' CACAGACACC 3'	X	
OPW 11	5' CTGATGCGTG 3'		X	OPN 15	5' CAGCGACTGT 3'	X	
OPW 15	5' ACACCGGAAC 3'		X	OPN 16	5' AAGCGACCTG 3'	X	
OPW 16	5' CAGCCTACCA 3'	X	X	OPN 18	5' GGTGAGGTCA 3'	X	

Tab. 8: Amount of *A. palmeri* plants tested in relationship analysis per population and per set of individuals; plants randomly chosen within each population.

Population	NC_S	NC_R	NC_{LRR}	TN_R	AR_R	GA_S	GA_R	$\sum$
Ind. / set	4 5 5	4 5 5	- - 6	- 7 8	4 5 -	4 5 5	4 5 5	20 32 34
Individuals	14	14	7	14	9	14	14	86

amplified fragment will be counted as a dominant marker assuming them as evenly distributed in the genome. A comparison of the marker pattern will give the relation between the investigated individuals.

The RAPD oligonucleotide sets OPW and OPN (MWG Eurofins Munich), containing 20 primer each, were evaluated for suitable oligonucleotides according to Huff et al. (1993). Leaf material of 4 randomly chosen individuals of the *A. palmeri* populations NC_S and NC_R and 4 individuals of the *A. tuberculatus* populations Il_1 and Mo_{18} were therefore harvested. Prior to DNA extraction samples were divided by two and the resulting 16 samples were extracted as previously described by using DNeasy mini kits (Qiagen®). In a pre-screen assay the 40 primers were tested in a single individual per species and in two replicates per PCR reaction at a final primer concentration of 0.4 μM in an 25 μl PCR

Tab. 9: Amount of *A. tuberculatus* plants tested in relationship analysis per population and per set of individuals; plants randomly chosen within each population.

Population	Mo_{13}	Mo_{18}	IL_1	$\sum$
Individuals	5	5	5	15

reaction volume. The used conditions were 15 min initiation step at 95 °C, 40 cycles of 30 sec annealing at 40 °C, 2.5 min elongation time at 70 °C and a final extension step at 70 °C for 10 min in an Eppendorf thermal cycler using Qiagen®Hotstart Taq. The marker evaluation was done in 1 % agarose gels stained with ethidium bromide. Oligonucleotieds with an appropriate marker pattern in the first evaluation were further tested with the remaining 14 samples in two replicates each. Oligonucleotides producing a reproducible marker pattern in replicates were used for the further studies and are described in Tab. 7. Several different *A. palmeri* and *A. tuberculatus* populations as described in Tab. 8 & 9 were evaluated to determine their relation. To decrease the error an estimation of marker length was not accomplished. A comparison between individuals was only conducted within the same gel. Therefore, the amount of individuals within a comparison was limited. In the beginning only 20 individuals were compared at the same time. In later experiments up to 34 individuals were tested and evaluated within the same gel.
All markers were treated as dominant alleles and counted by absence (0) or presence (1). The Hardy-Weinberg equilibrium was also assumed for each locus. The calculation of relationship and phylogenetic trees was either done with genedata, Fig. 26, or with Phylip 3.69 (Felsenstein, 1985; Felsenstein, 1989). The cladograms were either calculated by a consensus of the most parsimonyous tree according to Kluge & Farris (1969) using the packages "PARS" and "CONSENSE" of Phylip 3.69, or based on genetic divergence according to Nei & Li (1979) and the "Neighbor Joining Method" of Saitou & Nei (1987) using the packages "RESTDIST" and "NEIGHBOR" of Phylip 3.69. The mean values and the standard deviation of the plant genetic distance per population are also given. The heterozygosity (H_S) of each population was computed by using Popgene 32 according to Nei et al. (1973)(Yeh & Boyle, 1997). The same program was used to display polymorphic marker (P_L) within each population. The analysis of molecular variance (AMOVA), was calculated in "R" using the package "ade4" to calculate the influence of geographical distance and the response to glyphosate on the genetic variation among tested individuals (Gower & Legendre, 1986; Excoffier et al., 1992; Garland et al., 1993). The variance components of each hierarchical level are extracted by equating the mean squares (MSDs) to their expectations of the F-statistics (Excoffier et al., 1992; Sachs, 2002). Whereas the MSD were obtained by dividing the sum of squared deviations (SSD) by the appropriate degrees of freedom (Excoffier et al., 1992). The supplemented Φ-statistics yield according to Excoffier et al. (1992) the correlation between different tested groups depending on σ^2. Therefore Φ_{ST} is the correlation of random individuals grouped by population to the total variation of all populations tested. It will provide also the genetic differentiation among populations (G_{ST}; Nybom, 2004). Φ_{SR} provides the correlation of random individuals grouped by population to the variation of random individuals grouped by origin. Φ_{RT} provides the correlation of random individuals grouped by origin to the variation of random individuals of the total variation of all populations tested. Φ_{SG} gives

the correlation of random individuals grouped by population to the variation of individuals grouped by glyphosate appearance. Φ_{GT} is the correlation of individuals grouped by glyphosate appearance to the total variation of all tested populations. The AMOVA was calculated based on the Euclidean genetic distance matrix for all pairwise comparisons, which was shown to yield almost identical results as the calculation based on Nei & Li (1979) genetic distance matrix (Huff et al., 1993; Nybom & Bartish, 2000). A statistical evaluation of RAPD-marker was only performed in the second and third set among *A. palmeri* populations containing 32 and 34 individuals, respectively (Tab. 8).

4 Results

Herbicide - and especially glyphosate - resistant weeds are causing significant problems in cultivation and harvest of key crops around the world. The glyphosate-resistant weeds *Amaranthus palmeri*, *Amaranthus tuberculatus* and *Sorghum halepense* are important weed species in Roundup Ready®(RR) corn, cotton and soybean cropping systems in the south-eastern U.S. The first populations of glyphosate-resistant *A. palmeri*, *A. tuberculatus* and *S. halepense* were reported in 2005, 2006 and 2007 seasons, respectively (Culpepper et al., 2006; Legleiter & Bradley, 2008; Riar et al., 2011).
An increased *EPSPS* gene copy number, the target enzyme of glyphosate, has recently been reported to be the glyphosate resistance mechanism in an *A. palmeri* population collected in Georgia (Gaines et al., 2010). The identification of the importance of *EPSPS* gene amplification for glyphosate resistance of other *A. palmeri* populations and to identify the glyphosate resistance mechanism in *A. tuberculatus* was of main interest in the present work. The plant populations were therefore first characterized in their response to glyphosate treatments. Different glyphosate resistance mechanisms, reported in other weed species, were then tested in selected populations of both species. A relationship analysis based on RAPD markers was used to better characterize the evolution and spread of glyphosate resistance among *A. palmeri* populations. The glyphosate resistance of the *S. halepense* population is caused by alterations in glyphosate translocation (Riar et al., 2011). Therefore the *S. halepense* population was tested for the response to glyphosate and potential alternative herbicides. ACCase resistance was confirmed and characterized in this tetraploid weed species. Finally, it was shown that the biological efficacy of ACCase inhibitors was correlated with the number of ACCase alleles mutated encoding for a ACCase resistant protein.

4.1 Investigations into the *Amaranthus palmeri* Glyphosate Resistance

The aim was to examine the importance of an increased *EPSPS* gene copy number to confer the resistance to glyphosate and whether gene amplification is the only resistance mechanism present in *A. palmeri* (Gaines et al., 2010). Therefore, seven different *A. palmeri* populations were compared related to their response to glyphosate and their *EPSPS* gene copy number in the greenhouse. Field data showed that five of these populations were difficult to control with glyphosate, while 2 were still sensitive (Tab. 2). In the following experiments the populations NC_R and NC_S were evaluated concerning their glyphosate uptake and translocation, possible *EPSPS* target site mutations and the expression of *EPSPS* after glyphosate treatment. Modifications in the EPSPS protein structure and the overall amount of active EPSPS in the protein pool of cell was tested by assessing the K_{cat}, K_m and V_{max} values of the native EPSPS protein. Moreover, a single glyphosate sensitive

population with a high variation in the *EPSPS* gene amplification was selected and the plants were propagated according to their *EPSPS* gene copy number into two daughter populations, one bearing a high amount and the other a low *EPSPS* gene copy number. Both populations were evaluated in the greenhouse for their response to glyphosate in order to compare the *EPSPS* gene copy number with the biological behavior towards glyphosate. Finally, in order to better understand the evolution of glyphosate resistance across the U.S. the relationship of several *A. palmeri* populations were tested by using RAPD-markers.

4.1.1 Response of the *A. palmeri* Populations to Glyphosate

To assess and to characterize the response to glyphosate of the seven sensitive and resistant *A. palmeri* populations dose response curves were established by shikimic acid and fresh-weight assessments 4 and 16 days after treatment (DAT), respectively.
Approximately 250 plants of each of the populations NC_S, GA_S, TN_{LR}, NC_{LR} NC_{R1}, NC_R and GA_R were grown in the greenhouse and 117 plants of each of the 7 populations were treated with increasing glyphosate dose rates. During the propagation period and until untreated plants were 3 month old, no significant differences in plant phenotype or growth were observed among these populations.
In treated plants, two days after glyphosate application the first injury symptoms appeared as chlorotic, yellowish, and in a later stage, stunted young leaves (Fig. 45). These symptoms were observed in both, in sensitive and resistant populations, but expressed at different dose rates. In addition to the described symptoms necrotic lesions on the leaf surface of resistant plants were visible approximately 6 - 7 DAT at glyphosate dose rates higher than two 1440 g ae ha^{-1}, probably caused by the high surfactant content at these dose rates. The first injury symptoms appeared in the sensitive populations at less than 50 g ae ha^{-1} glyphosate and at a dose rate of 540 g ae ha^{-1} glyphosate no survivor was observed. The resistant populations showed the same response with a yellowing of young leaves starting at above 300 g ae ha^{-1} glyphosate in only a few plants. Even with the highest dose rate tested - 16 fold the field dose and 11520 g ae ha^{-1} glyphosate - not all resistant *A. palmeri* plants of the populations NC_R, NC_{R1} and GA_R were killed. However, the injury symptoms in some resistant plants at 5760 g ae ha^{-1} glyphosate were higher than at 11520 g ae ha^{-1} glyphosate which is probably caused by the self limiting effect of glyphosate action (Geiger & Bestman, 1990; Geiger et al., 1999; Shaner, 2009).
The fresh weight of the glyphosate treated plants in the dose response study was assessed 16 DAT, when a visible regrowth of treated plants started. According to this data the populations were grouped into three statistically significant groups of sensitivity to glyphosate ($P = 0.05$). The sensitive group contained, in opposite to the shikimic acid assessment, both sensitive and the low level resistant population NC_S, GA_S and NC_{LR} with a mean

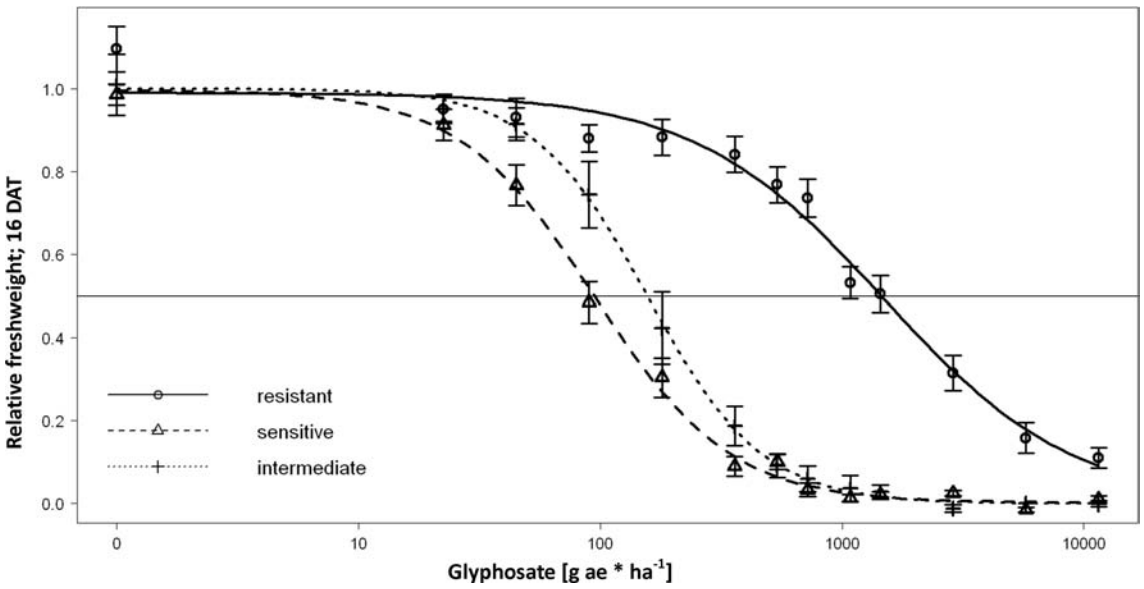

Fig. 9: Effects of glyphosate on the entire above ground plant fresh-weight of six sensitive and resistant *A. palmeri* populations based on relative plant growth 16 DAT; expressed as relative biomass reduction in comparison to nontreated control; the sensitive group contains NC_S and GA_S; intermediate group contains TN_{LR}; resistant group contains NC_{R1}, NC_R and GA_R; ED_{50} values, confidence intervals and R/S ratios are given in Tab. 10.

ED_{50} value of 93.5 g ae ha^{-1} glyphosate. The population NC_{LR} is also not significant different to the low level resistant population TN_{LR} with an ED_{50} value of 156.4 g ae ha^{-1} glyphosate and a resistance factor of 1.8. The third group contains the resistant populations NC_R, NC_{R1} and GA_R with ED_{50} values between 924 and 2038 g ae ha^{-1} glyphosate and a mean resistance factor of 22.7 (Tab. 10).

Since the inhibition of the EPSPS activity in plant tissues leads to the accumulation of the enzyme substrate, shikimate-3-phospate (S3P), which will be rapidly degraded in plant to shikimate (Shaner et al., 2005). In treated plants the rise of shikimate after glyphosate treatment can be used as an early marker of plant injury and shows the highest increase in the youngest and most active leaf tissues 4 DAT (Shaner et al., 2005). The leave stage best suited to differentiate between sensitive and resistant *A. palmeri* plants in the dose response relation the shikimate concentration was first assessed in different *A. palmeri* plant tissues.

Glyphosate sensitive plants of the commercial available population Herbiseed were treated with 720 g ae ha^{-1} glyphosate and the shikimic acid concentration in different plant sections was determined 4 days after treatment (DAT). In the untreated control plants, shikimic acid was present at a low level with the same content based on the tissue fresh weight in all plant tissues excepted in roots where a lower content was found (Fig. 10).

The shikimic acid concentration in treated plants had the highest rise of about 17 times

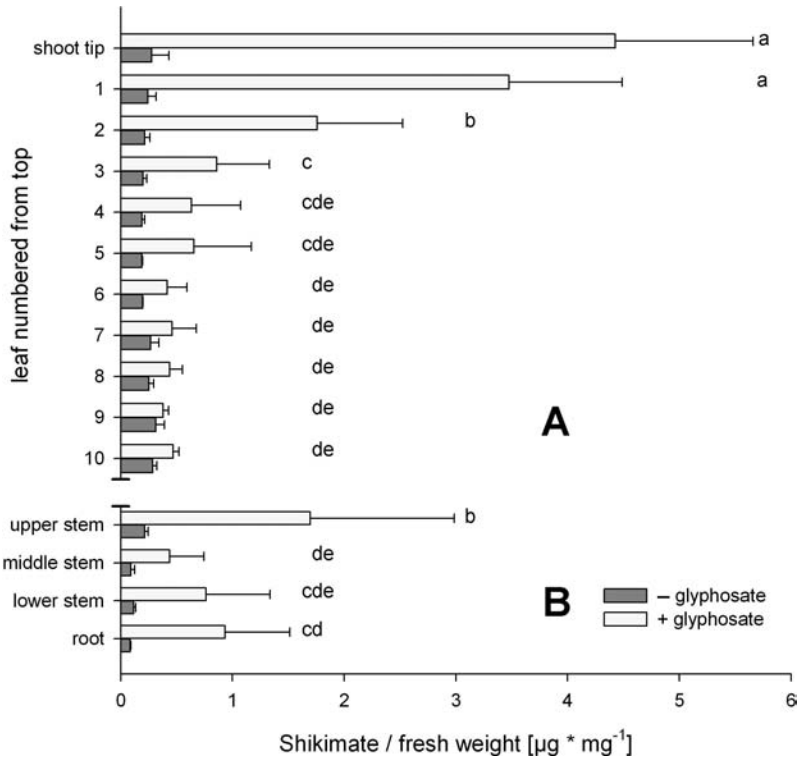

Fig. 10: Comparison of shikimate accumulation in *A. palmeri* 4 days after treatment with 720 g a.e. ha^{-1} glyphosate in comparison to nontreated plants; (A) shikimic acid distribution in the leaves and in the shoot tip; (B) shows the shikimate accumulation in the stem and the root; "a", "b", "c", "d" and "e" represent statistical differences ($P < 0.05$) in treated plants only.

in the shoot tip, followed by an increase of 14 times in the youngest nearly full expanded leaf in comparison to untreated plants. The content in the whole stem increased in average by more than 6 times whereas the youngest 4 nodes alone had an about 9 times higher content compared to untreated plants. The root was not divided into younger and older parts and showed an shikimic acid increase by 11 times despite the absence of any chloroplasts. In general, the overall shikimic acid accumulation decreased the older the tissue is. The highest shikimic acid increase was measured in the youngest, nearly fully expanded leaf of *A. palmeri*, which is also well suited to harvest leaf discs.

The changes of the shikimic acid content in plants of the dose response study were assessed 4 DAT for all glyphosate doses, excepted for the highest dose rate (11520 g ae ha^{-1} glyphosate) for which an equal sampling of leaf tissue was due to strongly stunted leaves not possible. Effective doses (ED_{50}) provoking either 50 % growth reduction or 50% of the mean maximal observed shikimic acid increase after glyphosate treatment were used to compare the populations in their glyphosate response. According to the shikimic acid measurement 4 DAT, the seven *A. palmeri* populations can be divided into three different groups showing a low, intermediate and high shikimic acid accumulation after glyphosate

treatment. The sensitive population NC_S and the by STECKEL et al. (2008) as low level glyphosate resistant classified population TN_{LR} cluster together and show an ED_{50} value of around 84 g ae ha^{-1} glyphosate (Tab. 10). The second sensitive population GA_S and the low level resistant population NC_{LR} are significantly different from the previous sensitive group and show an ED_{50} value of 143.5 g ae ha^{-1} glyphosate with a resistance factor of RF = 1.8 towards the most sensitive. The resistant populations NC_R, NC_{R1} and GA_R have a resistance factor of RF = 16.3 towards the most sensitive and in average an ED_{50} value of 1313 g ae ha^{-1} glyphosate. The highest resistance factor was measured in GA_R with RF = 21.7.

The *A. palmeri* populations NC_S and GA_S are therefore sensitive to glyphosate and are

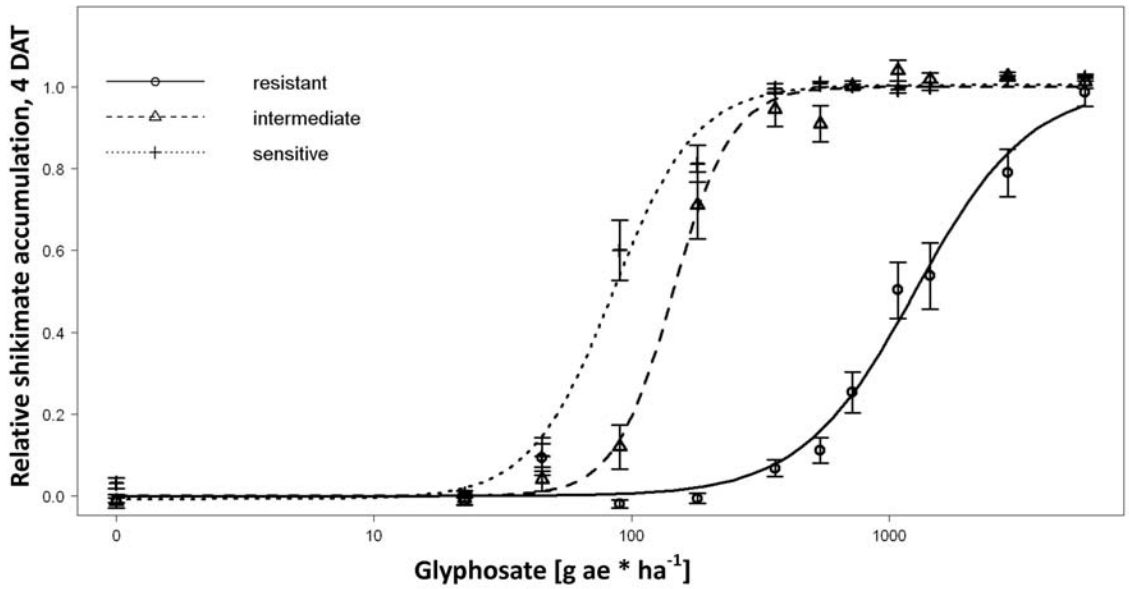

Fig. 11: Relative shikimic acid accumulation 4 DAT in the youngest fully expanded leaf of different *A. palmeri* populations, treated with increasing glyphosate dose rates; the sensitive group was formed by plants of the populations NC_S and GA_S; the intermediate group was formed by plants of the population TN_{LR} and the resistant group by plants of the populations NC_R, NC_{R1} and GA_R; error bars represent the standard error of the mean; ED_{50} values, confidence intervals and R/S ratios are given in Tab. 10.

easy to control with the recommended field rate of 720 g ae ha^{-1} glyphosate (pp. 24). The populations NC_R, NC_{R1} and GA_R are highly glyphosate resistant and some plants even survived 16 fold the recommended field rate (11 520 g ae ha^{-1} glyphosate).

Tab. 10: Effects of glyphosate on the shikimic acid content and the fresh weight of different *A. palmeri* populations; shikimic acid measurement 4 DAT; fresh weight assessment 16 DAT (entire above ground plant); ED_{50} values provide the effective dose rate provoking 50 % shikimic acid accumulation or growth of the mean maximal observed effect, resistance ratio (R/S) given in comparison to the most sensitive: results of NC_{LR} on 6 plants per dose and 8 different dose rates in comparison to the other populations tested.

Pop.	Shikimate acc. (4 DAT) ED_{50} [g ae ha^{-1}]	95 % confidence interval		R/S ratio	Fresh weight (16 DAT) ED_{50} [g ae ha^{-1}]	95 % confidence interval		R/S ratio
TN_{LR}	**80.6**	67.7 - 93.6	a	1	**156.7**	119.8 - 195.6	C	1.8
NC_S	**87.5**	67.7 - 107.4	a	1.1	**91.7**	66.0 - 117.6	A	1
NC_{LR}	**146.0**	113.6 - 178.5	c	1.8	**101.8**	73.3 - 130.4	AC	1.1
NC_R	**1131.8**	817.9 - 1445.6	b	14.0	**1416.1**	836.6 - 1995.6	B	15.8
NC_{R1}	**1059.5**	928.0 - 1191.0	b	13.1	**924.0**	272.9 - 1575.5	B	10.3
GA_S	**141.2**	119.9 - 162.5	c	1.8	**88.0**	58.5 - 116.4	A	1
GA_R	**1747.0**	589.0 - 2904.9	b	21.7	**2038.0**	930.6 - 3146.5	B	22.7

4.1.2 Glyphosate Absorption and Translocation in *A. palmeri*

Alterations in glyphosate absorption or translocation have been important resistance mechanisms in weeds, but have not been found in each case (Shaner, 2009). Culpepper and cooperators (2006) found no difference in glyphosate uptake and translocation between the resistant and sensitive *A. palmeri* populations GA_R and GA_S 48 hours after treatment (HAT). For a better characterization of glyphosate resistance *A. palmeri* the glyphosate uptake and translocation was assessed quantitatively and qualitatively in a second glyphosate resistant population NC_R with a different origin as those described by Culpepper et al. (2006) in comparison to the sensitive population NC_S. For both experiments plants were treated with radio-labeled glyphosate on the youngest fully expanded leaf and tissues were harvested at 4, 8, 24, 32, 48 and 120 HAT.

The ^{14}C-glyphosate translocation and the velocity of transport into the different plant parts was determined by combustion of the sectioned and dried plant material. The $^{14}CO_2$ was trapped and then measured in a scintillation counter. A total recovery of about 90 % of radio-labeled compound in the washing and the combusted plant material was reached during the experiment. The losses of about 10 % of radioactivity can be explained since the plants were grown in a peat:loam mixture to provoke more natural growing conditions, indeed the extraction of the roots is more difficult than for *e.g.* sand grown plants. Therefore the losses are most probably caused by remaining root tips in the soil or due to secreted radioactivity into the soil.

The glyphosate uptake of NC_R and NC_S during the first 48 HAT was approximately 40 %

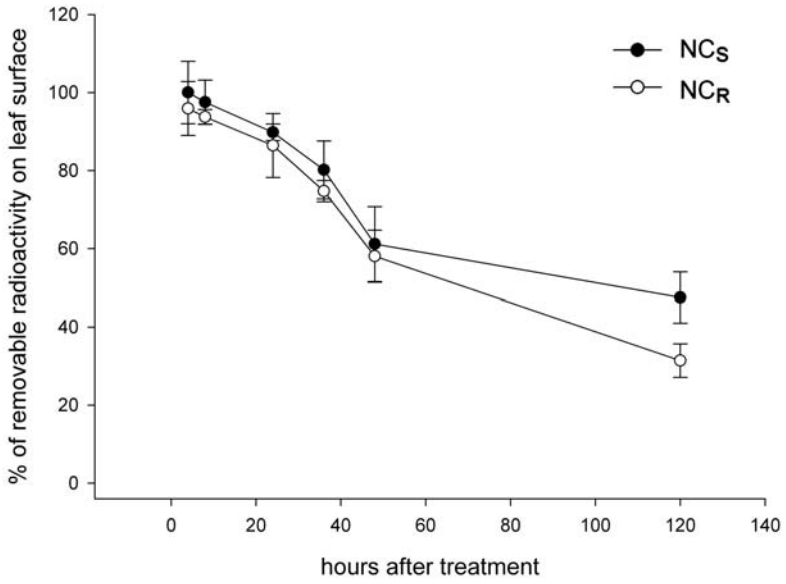

Fig. 12: Glyphosate uptake by the surface of the youngest fully expanded leaf of plants of the *A. palmeri* populations NC_R and NC_S at 4; 8; 24; 32; 48 and 120 HAT using ^{14}C-glyphosate; standard error is indicated by error bars.

of applied radioactivity with the highest velocity between 24 and 48 HAT (Fig. 12). At 48 HAT also the first herbicide injury symptoms in the sensitive population were visible, probably causing the significant lower uptake at 120 HAT. At that time point the sensitive plants have absorbed 50 % of the applied radioactivity, while the resistant plants showed a further uptake until 65 % of applied radioactivity without herbicidal injury symptoms. However, already 4 HAT radioactivity in shoot, shoot tip and root was detectable by combustion. A strong increase in translocation was found between 24 and 36 HAT without differences between populations (Fig. 13 & 14). At 24 HAT still 86 % of the total absorbed radioactivity remained in the treated leaf with the highest export rates between 24 and 48 HAT. At 48 HAT 12 % of the absorbed radioactivity was detectable in the shoot tip, between 10 and 15 % in shoot and in average 8.5 % was translocated into the roots. After 120 HAT in the resistant plants 36 % of absorbed radioactivity remained in the applied leaf while the sensitive plants kept 50 %. Also in shoot tip - 32 % to 19 %, in shoot - 20 % to 14 % and in root - 17 % to 10 % the velocity of translocation was in the resistant plants higher than in the sensitive at later timepoints.

The translocation of radioactivity into the shoot tip was in resistant plants nearly constant from 24 until 120 HAT whereas the sensitive plants showed a reduced translocation into the shoot tip 48 HAT, indicating an overall herbicidal damage of plant. Statistically significant differences ($P = 0.05$) were measurable at 48 HAT in root and shoot. At 120 HAT the translocated amount of radioactivity in all captured plant parts was significant different between NC_R and NC_S. The first herbicidal injury symptoms of the applied plants were

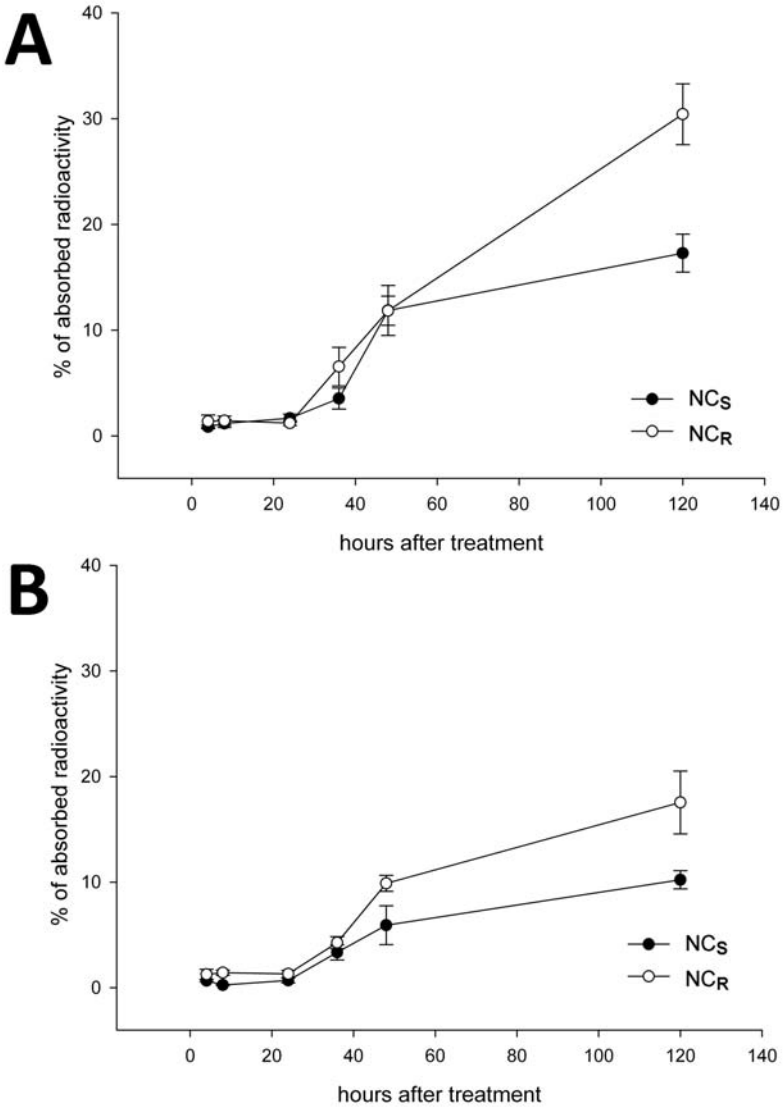

Fig. 13: Glyphosate translocation throughout plants of the *A. palmeri* populations NC_S and NC_R at 4; 8; 24; 32; 48 and 120 HAT using ^{14}C-glyphosate; (A) shows the translocation into the shoot tip; (B) the translocation into the root; standard error is indicated by error bars.

observed 48 HAT in plants of the sensitive population NC_S with droppy leaves. Thus, significant differences between both populations that could explain the glyphosate resistance of NC_R were not found.

Resistance based on altered glyphosate translocation is not necessarily caused by differences in the transport rates out of the treated leaf as found *e.g.* in *S. halepense* (Riar et al., 2011). Also a higher translocation to the borders of the applied leaf of resistant plants can be found as *e.g.* in *C. canadensis* where this phenomenon is most probably caused by a glyphosate sequestration into the vacuole (Ge et al., 2010). To evaluate alterations in the

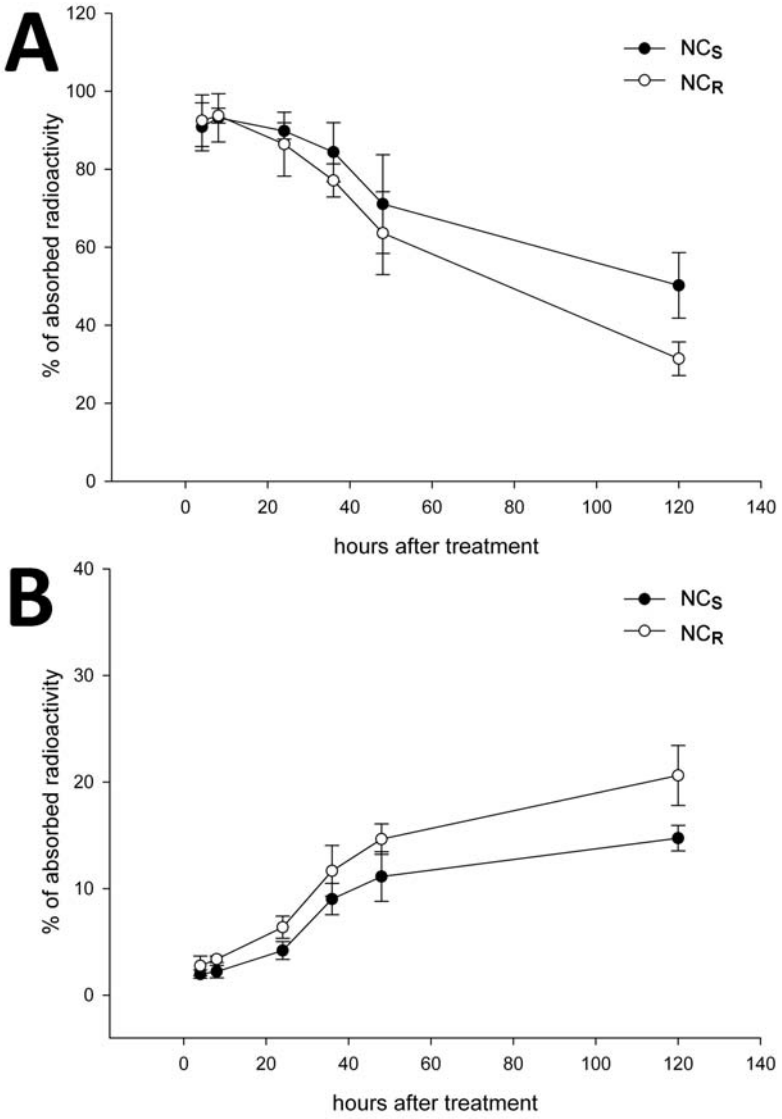

Fig. 14: Glyphosate translocation throughout plants of the *A. palmeri* populations NC_S and NC_R at 4; 8; 24; 32; 48 and 120 HAT using ^{14}C-glyphosate; (A) shows the translocation out of the applied leaf; (B) the translocation into the remaining shoot; standard error is indicated by error bars.

glyphosate translocation within a single plant part the translocation was quantitatively visualized at different time points in 5 plants each of the *A. palmeri* populations NC_R, NC_S and HERBI treated with ^{14}C-glyphosate (Fig: 15).

The first radioactivity in the root and the shoot tips was already visible 4 HAT. After 96 HAT radioactivity was completely distributed in the whole plant, even in the oldest and physiologically less active tissues. The highest amount of radioactivity was found in the root and shoot tip and also in sprouting auxiliary buds, whereas the detectable amount was decreasing with age and physiological activity of plant organ. Differences between

sensitive and resistant plants either in the distribution of radioactivity in the whole plant or within a single organ were not detected. The distribution and movement of radioactivity in a single plant organ or within the whole plant was comparable between sensitive and resistant plants until the latest time point of 96 HAT.
Thus, the glyphosate resistance in the tested *A. palmeri* populations NC_R is unlikely to be due to differences in the glyphosate uptake or translocation as indicated in Fig. 12, 13 & 14 or in the auto-radiograms, Fig. 15. Therefore the populations NC_R and NC_S showed the same behavior than the population GA_R described by Culpepper et al., (2006).

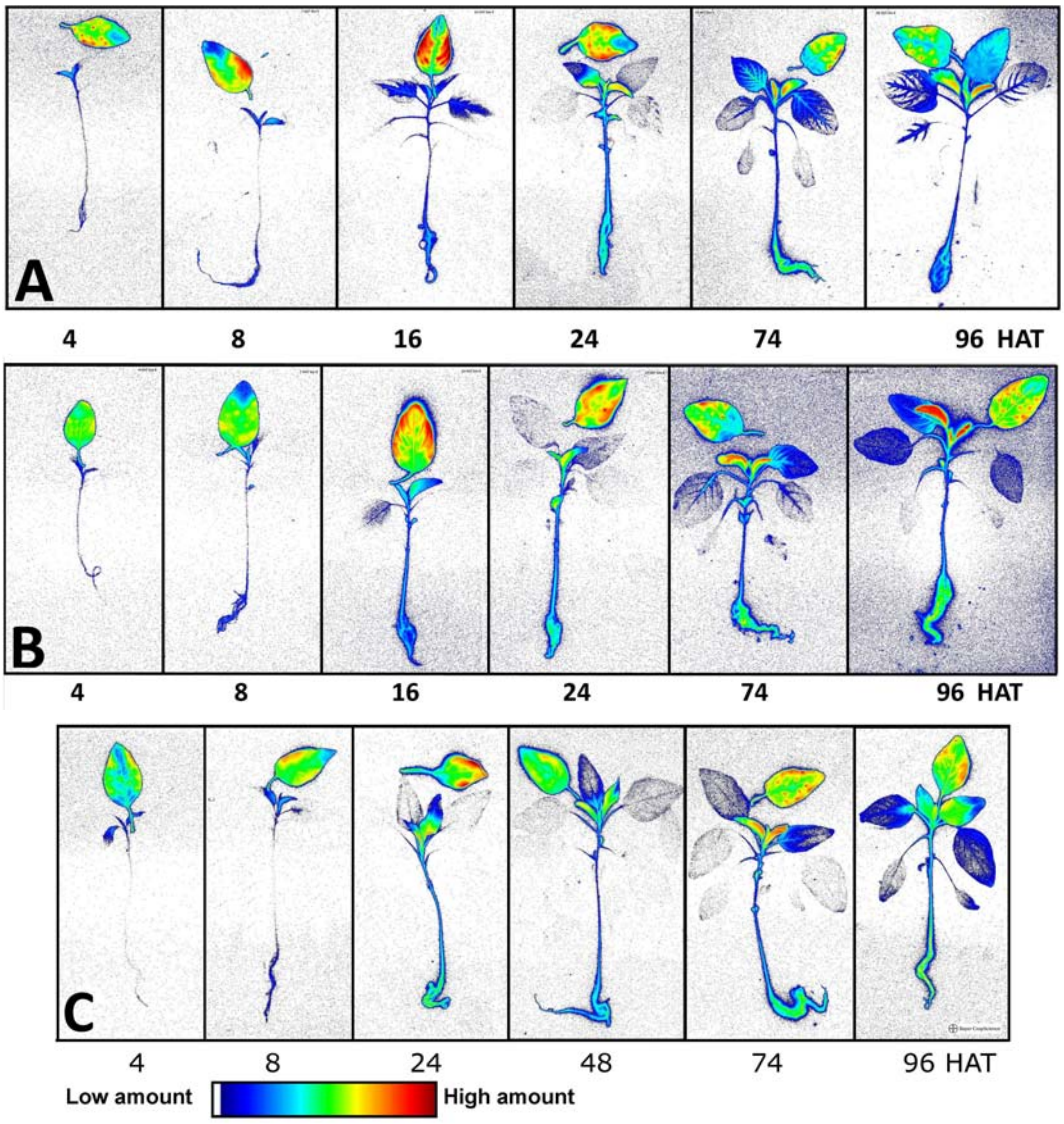

Fig. 15: Auto-radiograms of ^{14}C-glyphosate translocation in *A. palmeri* populations NC_S shown in **A**; NC_R shown in **B** both tested at 4, 8, 16, 24, 74 and 96 HAT; the population from Herbiseed shown in **C** was observed at 4; 8; 24; 48; 74 and 96 HAT; red and blue colors indicates high and low amounts of radioactivity in plants, respectivly; Each picture represent one representative of 5 plants per time point and population.

4.1.3 Alterations in the *A. palmeri EPSPS* gene sequence

Several weed species evolved glyphosate resistance due to mutations in the genes encoding the target enzymes *e.g., Eleusine indica* and *Lolium rigidum* where mutations at position P106S/T/A were described (Baerson et al., 2002; Wakelin & Preston, 2006; Powles & Yu, 2010). In contrast, Gaines et al. (2010) found no target site mutation in the *EPSPS* coding sequence of the *A. palmeri* population GA_R. To exclude target site based glyphosate resistance in the *A. palmeri* populations studied, the *EPSPS* gene sequence was analyzed at the amino acid positions G101, T102 and P106 using Pyrosequencing™ technology, well suited to detect single nucleotide polymorphisms (SNP) present in a low frequency in the samples (Pielberg et al., 2003).
No mutation was detected at the positions analyzed, supporting that an *EPSPS* target site mutation in the amino acid positions G101, T102 or P106 is not responsible for glyphosate resistance in *A. palmeri* populations NC_R, NC_{R1}, NC_{LR}, NC_S and TN_{LR}. Therefore the observed resistance in the investigated populations is not due to mutations in the *EPSPS* gene.
The weed *Brassica rapa* evolved herbicide resistance by the pollen-mediated transfer of CP4-EPSPS gene from glyphosate resistant *Brassica napus* cultivars (Warwick et al., 2008). To determine if pollen-mediated or any type of horizontal gene transfer has occurred between Roundup Ready®crops and the *A. palmeri* populations studied herein, all populations were assessed for the presence of the Roundup Ready®trait. The test revealed the absence of the RR-gene-construct in all glyphosate resistant *A. palmeri*. Therefore, the glyphosate resistance in *A. palmeri* is not due to horizontal gene transfer between Roundup Ready®crops and weeds. Neither the target site mutations G101, T102 or P106 nor an horizontal gene transfer between crop cultivars and weeds is responsible for the observed glyphosate resistance in *A. palmeri*.

4.1.4 *EPSPS* Gene Amplification in *A. palmeri*

None of the previous evaluated mechanisms could explain the resistance to glyphosate of the tested *A. palmeri* populations. Gaines and his collaborators (2010) found in GA_R 60 to 120 times more genomic *EPSPS* gene copies in comparison to the sensitive *A. palmeri* population GA_S. They described the *EPSPS* gene amplification as a further resistance mechanism to class G9 herbicides (glyphosate). The *ALS* gene was used as reference gene to determine the amount of *EPSPS* in the genomic DNA. In the present study the same set-up was used to evaluate the *EPSPS* gene copy number in the resistant and sensitive *A. palmeri* populations NC_S, GA_S, TN_{LR}, NC_{LR}, NC_R, NC_{R1}, GA_R, AR_R and TN_R (Tab. 2).
As found by Gaines et al. (2010) the *EPSPS* gene copy number in the sensitive popula-

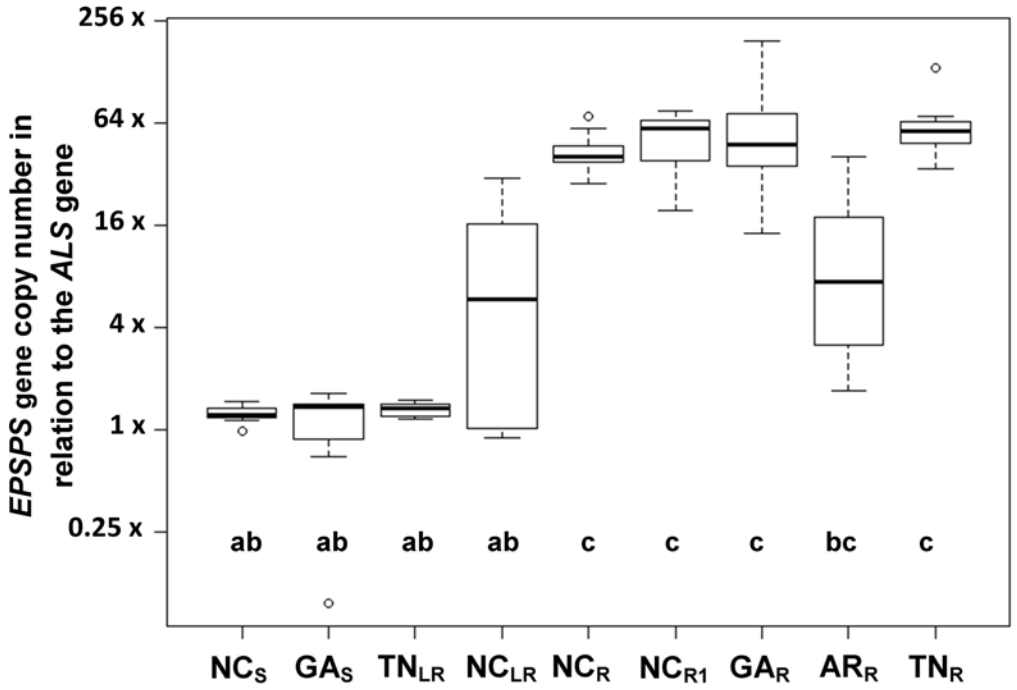

Fig. 16: Comparison of genomic *EPSPS* gene copy number relatively to the *ALS* gene of different *A. palmeri* populations; the mean relative *EPSPS* gene copy number and the standard deviation is in $NC_S = 1.2 \pm 0.13$ relative *EPSPS* genes, in $GA_S = 1.2 \pm 0.5$, $TN_{LR} = 1.3 \pm 0.12$, $NC_{LR} = 9 \pm 9.5$, $NC_R = 45 \pm 11$, $NC_{R1} = 51 \pm 18.3$, $GA_R = 65 \pm 49$, AR_R 11.5 ± 12.1, $TN_R = 65.6 \pm 32.7$; different letters inside the graph provide significant differences among populations (P=0.05).

tion GA_S correspond to the number of *ALS* genes. Therefore GA_S had 1.2 ± 0.5 relative *EPSPS* gene copies, whereas the sensitive population NC_S had with 1.2 ± 0.13 relative *EPSPS* gene copies a tighter *ALS* - *EPSPS* relation. In average, both populations had the same amount of relative *EPSPS* genes. The low level resistant population TN_{LR} had with 1.3 ± 0.12 relative *EPSPS* gene copies a comparable amount of *EPSPS* than the sensitive populations analyzed and therefore no gene amplification, which explain the low level of glyphosate resistance.

NC_{LR} and AR_R were both classified as medium glyphosate resistant in the field and had in average 9 ± 9.5 and 11.5 ± 12.1 relative *EPSPS* gene copies, respectively.

The glyphosate resistant population NC_R had 44.5 ± 11.0 and NC_{R1} 51 ± 18.3 relative *EPSPS* gene copies. Both are significantly different from the sensitive populations. Though, the population NC_R, a cross between two highly resistant plants of NC_{R1}, had a less wide distribution of *EPSPS* -gene copies but was more glyphosate resistant than the population NC_{R1}. Nevertheless the amount of *EPSPS* genes was not significantly different to the amount found in GA_R with 65 ± 45 times the *ALS* gene or to TN_R with 65.6 ± 32.7 relative *EPSPS* gene copies.

As described above the *EPSPS* gene is present in a high number in the genomic DNA of glyphosate resistant *A. palmeri* plants (section 3.4.3). Therefore, a Southern blot was performed either to visualize the differences in the amount of gene copies and to assess length and sequence differences of the *EPSPS* gene sequence between resistant and sensitive plants of the populations NC_R and NC_S.

In *A. palmeri* only the *EPSPS* mRNA coding sequence of GA_S and GA_R is known and published at NCBI (Accession numbers: FJ861242.1; FJ861243.1). The whole *EPSPS* fragment is approximately 9 000 bp long when amplified out of genomic DNA and estimated in a 1 % agarose gel. It was therefore important to evaluate the occurence and length of internal intron sequences in the *A. palmeri* EPSPS sequence. As previously described the genomic *EPSPS* DNA sequence of *Eleusine indica* (NCBI Accession number: AY157642.1; AY157643.1) and *Conyza candensis* (NCBI Accession number: AY545666.1; AY545667.1) inclusive the intron-exon structure is known and published. The *EPSPS* gene

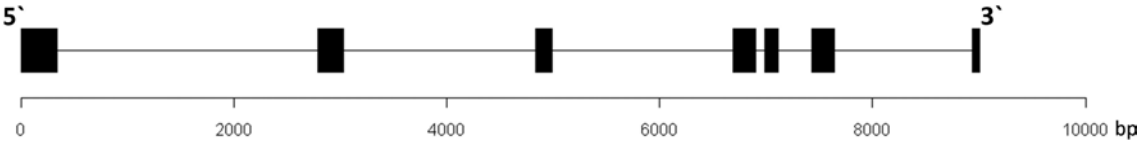

Fig. 17: Putative scheme of the *A. palmeri EPSPS* intron-exon structure; boxes indicate exons; lines are introns; localisation of ATG-start codon and UTR-stop-codon was not determined.

sequence in the genomic DNA of both species contains 7 coding exon sequences of equal length, while the overall gene length in the genomic DNA including introns varies in *E. indica* between 3079 and 3115 bp, and in *C. canadensis* between 6988 and 7954 bp. The mRNA sequence identity between *E. indica* (NCBI Accession number: AY395699.1) and *C.canadensis* (NCBI Accession number: AY545668.1) is 74 % (84 % in the amino acid sequence) while the homology between *C. canadensis* and *A. palmeri* (NCBI Accession number: FJ861242.1) is 77 % (84 % in amino acid-sequence). Therefore, length differences are caused by differences in the intron length. The intron-exon structure of *E. indica* and *C. candensis* was used to predict the probable *EPSPS* gene structure of *A. palmeri*. Primer combinations located in the known exon sequence, overlapping the introns, were used to make a rough estimation of the intron size in a 1 % agarose gel (Fig.17). Therefore the length of the *EPSPS* gene from the first until the seventh exon in *A. palmeri* is approximatly 9000 bp and gave the same result as indicated by amplification of the whole gene. Differences in the size of the fragments obtained either from NC_S or NC_R plants were not obvious.

The high coding sequence identity of 99 % does not exclude higher differences in the intron sequences (NCBI Accession number GA_S: FJ861242.1 GA_R: FJ861243.1 $\rightarrow$ 1596 bp / 1599 bp = 99 % identity). To avoid a mismatch and an unclear band pattern in the

Southern blot only a single exon sequence was used as labeled probe. The second exon with a size of 244 bp and with a highly conserved sequence between *E. indica*, *C. candensis* and *A. palmeri* was chosen to generate the probe. The non radioactive DIG-technology was here used to detect the hybridization fragments.

The Southern blot was performed to clarify if size differences among the inserted *EPSPS* gene sequences exist which might be generated during the amplification process and as a visual control of *EPSPS* gene amplification in *A. palmeri*.

The DNA of the sensitive and resistant populations NC_S and NC_R was compared in an equal concentration and cut by the restriction enzymes Hind III, Xba II, Not I and Btg I (Fig. 18). The Hind III enzyme is cutting inside the 1^{st} exon and the 3^{rd} intron and produced an approximately 1700 bp long fragment with a sharp single band in resistant individuals of NC_R and a single thin band in the sensitive population NC_S. Btg I also yields a single band in both populations with a length of approximately 4000 bp. Both enzymes yield a single strong band in resistant and a thin band at the same hight in the sensitive population (Fig. 18).

A problem of Southern blot was the *EPSPS* gene structure with long intron sequences, which offer only the possibility to design short probes located in the exons with a length of less than 300 bp. The short probes might be one reason for the weak signal of *EPSPS* in sensitive individuals, which we were not able to intensify despite several trails of optimization. In addition to the signal intensity, the gene size of approximately 9 Kbp prevented to find restriction sites outside the gene which gave meaningful results in both populations.

The Southern blot results suggest that the *EPSPS* gene structure was, despite gene amplification not changed between sensitive and resistant plants. These results confirmed also the data obtained using PCR analyses, *i.e.* an amplification of the *EPSPS* gene in the population NC_R as they showed a stronger *EPSPS* signal intensity in glyphosate resistant than in sensitive plants.

In summary, the sensitive populations NC_S and GA_S had a comparable amount of *EPSPS* and *ALS* genes. The resistant populations NC_R, NC_{R1} and GA_R had with in average 47 times more *EPSPS* genes a strong increase in the number of genomic *EPSPS* genes, ranging from 19 until 196 relative *EPSPS* gene copies. This supports that the most cases of glyphosate resistance in *A. palmeri*, so far analyzed, are caused by *EPSPS* gene amplification. Nevertheless, since this mechanism was not found in the population TN_{LR}, most likely *A. palmeri* has developed other glyphosate resistance mechanisms in addition.

4.1.5 Effect of glyphosate on the *EPSPS* gene expression

The high *EPSPS* gene amplification in glyphosate resistant *A. palmeri* plants raised the question for the transcriptional regulation of these genes. Gaines et al. (2010) found GA_R

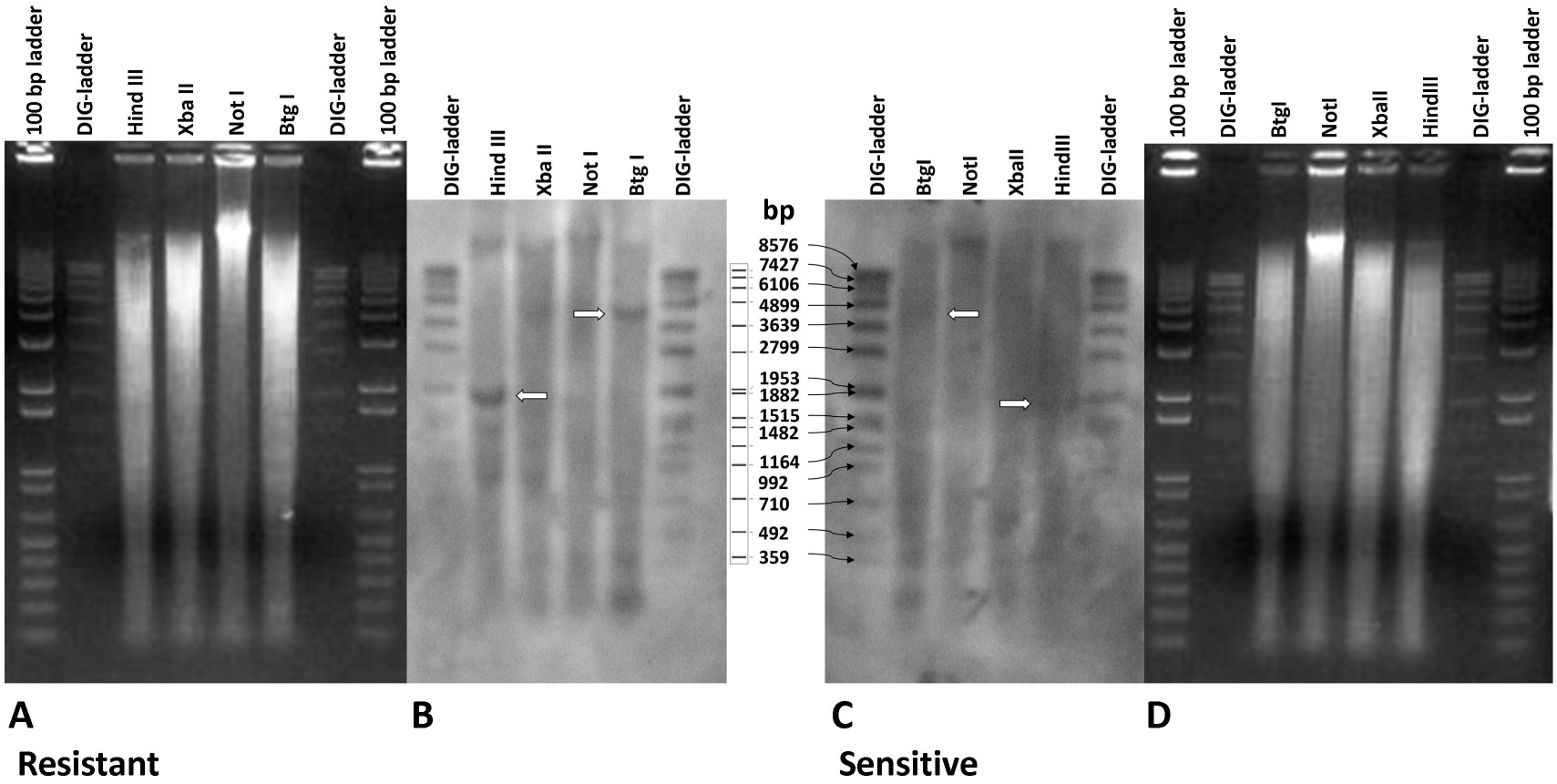

Fig. 18: Southern blot of the NC_S (C) and NC_R (B) *EPSPS*, 10 μg DNA each, cut with the restriction enzymes Btg I, Not I, Xba II and Hind III; white arrows indicate the DIG labeled restriction fragments; Length determination by Roche®DIG ladder VII; the 100 bp ladder refers to TrackIt™ 1 kb DNA ladder (Invitrogen™); A & D shows the agarose gel prior to Southernblot.

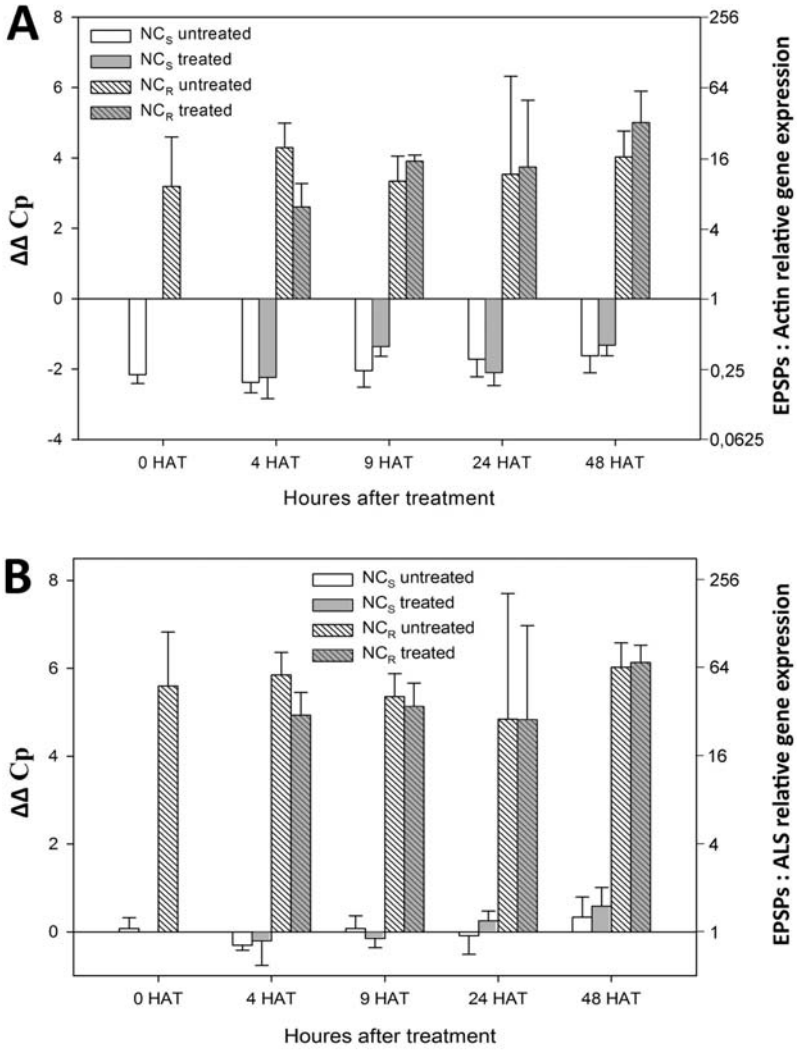

Fig. 19: Relative *EPSPS* mRNA expression of plants of the *A. palmeri* populations NC_S and NC_R relatively to the *Actin* (A) and *ALS* (B) gene expression of untreated and treated plants at 0; 4; 9; 24 and 48 h after treatment with 720 g ae ha^{-1} glyphosate; the columns represent the differences in expression in ΔΔ Cp and in relative *EPSPS* expression in comparison to the *Actin* and *ALS* gene expression; the standard deviation is given by the error bars.

plants a strong correlation between the number of *EPSPS* gene copies and the mRNA expressed. The *EPSPS* expression in NC_R and NC_S was analyzed at different time points after glyphosate treatment to confirm this finding in a second glyphosate resistant population and especially to evaluate the effect of glyphosate treatment on the expression of the *EPSPS* gene. The *Actin* gene was used as an additional housekeeping gene, known to be stable expressed at a constant level in a wide range of environmental conditions.

Indeed, significant differences in the *Actin* gene expression between time points and treatments were not observed (data not shown). *Actin* is therefore suitable as reference gene dur-

ing the glyphosate treatment in the *A. palmeri* populations NC_R and NC_S. The *ALS* gene was also expressed at a constant level throughout the treatment period, but with a higher variation between plants, time points and treatments.
Therefore, the *EPSPS* gene expression of NC_R and NC_S in Fig. 19 A was normalized to the expression of the *Actin* gene and in Fig. 19 B to the *ALS* gene. According to these results no significant differences between treated and untreated plants were found. Therefore the expression of the *EPSPS* gene was not modified by a glyphosate treatment in the sensitive and resistant plants of NC_S and NC_R.

4.1.6 A Glyphosate Sensitive Population Bearing *EPSPS* Gene Amplification

The population NC_{LR} has been found to be heterogeneously in its glyphosate tolerance. Nevertheless, the dose response relation showed that it can be considered as glyphosate sensitive with an ED_{50} value, measured by shikimic acid concentration, of 146 g ae ha^{-1} glyphosate and an ED_{50} value, based on fresh weight, of 101.1 g ae ha^{-1} glyphosate (Tab. 10). In comparison to the other populations these results were based on less doses rates and less individuals per dose rate, caused by the low number of plants available. Despite the same apparent seed quality this population had a low germination rate, high susceptibility towards fungal infections and a poor growth. However, an average of 9 ± 9.5 relative *EPSPS* gene copies were detected in this population, ranging from 0.9 (*i.e.* 1) - 30 relative *EPSPS* gene copies. This populations contains therefore individuals bearing the same *EPSPS* gene copy number as found in glyphosate sensitive and glyphosate resistant *A. palmeri* individuals (Fig. 16).
To get a better understanding of the NC_{LR} glyphosate sensitivity, despite some of this indi-

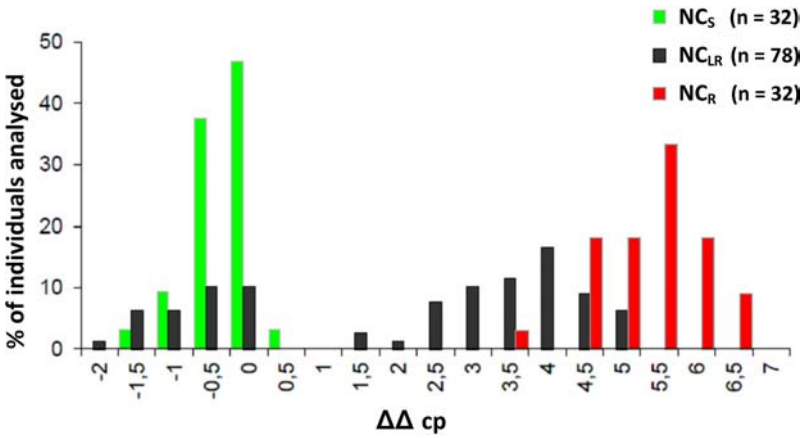

Fig. 20: Comparison of *EPSPS* gene copy number in *A. palmeri* populations NC_{LR}, NC_S and NC_R indicated as ΔΔ Cp in relation to the ALS gene in percent of individuals analyzed; determination of *EPSPS* gene copies based on 3 technical replicates of the *EPSPS* and *ALS* gene fragment by qPCR in genomic DNA.

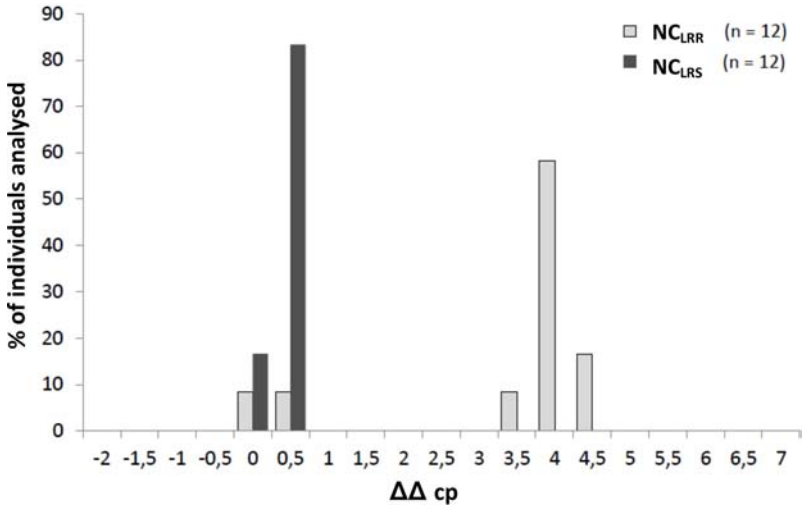

Fig. 21: Distribution of *EPSPS* gene copy number in *A. palmeri* F_1 generations NC_{LRS} and NC_{LRR}; each a cross of NC_{LR} individuals divided according to their EPSPS gene copy number; indicated as ΔΔ Cp in relation to the *ALS* gene in percent of individuals analyzed; the determination of *EPSPS* gene copies based on 3 technical replicates of the *EPSPS* and *ALS* gene fragment by qPCR in genomic DNA.

viduals bearing *EPSPS* gene amplification, 78 individuals were analyzed for their genomic *EPSPS* gene copy number. The *EPSPS* gene number in this population was therefore bisferious distributed. Some individuals contained up to a quarter less *EPSPS* gene copies than *ALS* copies but there were also individuals bearing up to 32 times more *EPSPS* than *ALS* genes (Fig. 20). The left peak of the distribution is comparable to the *EPSPS* copy number distribution of the sensitive population NC_S, while the right peak of the bisferious distribution is left shifted in comparison to the glyphosate resistant individuals of NC_R. These results suggest that NC_{LR} is most probably the result of a cross between glyphosate resistant and sensitive individuals.

According to these results the population was splitted into two different groups of plants in order to be reproduced. Plants of the first group had an equal *ALS* and *EPSPS* gene copy number, while the second group contained individuals bearing the highest amount of *EPSPS* gene copies found in the investigated plants. The two groups of plants were placed in separate greenhouses where male and female plants were grown together and shake daily to obtain a good fertilization.

The copy number in the daughter accessions was checked by random sampling of 12 individuals of each daughter population. No individual of the first group, named NC_{LRS}, was bearing a high number of genomic *EPSPS* copies and had an *EPSPS* gene copy number comparable to that of NC_S. In the second group, named NC_{LRR}, the *EPSPS* gene copy number was in 83 % of the individuals at the expected high number of *EPSPS* gene copies, but 16.6 % of the individuals had a lower number of *EPSPS* in the genome and were in

their *EPSPS* gene copy number comparable to NC_{LRS} (Fig. 21).

Both daughter accessions NC_{LRS} and NC_{LRR} were tested in their response to glyphosate.

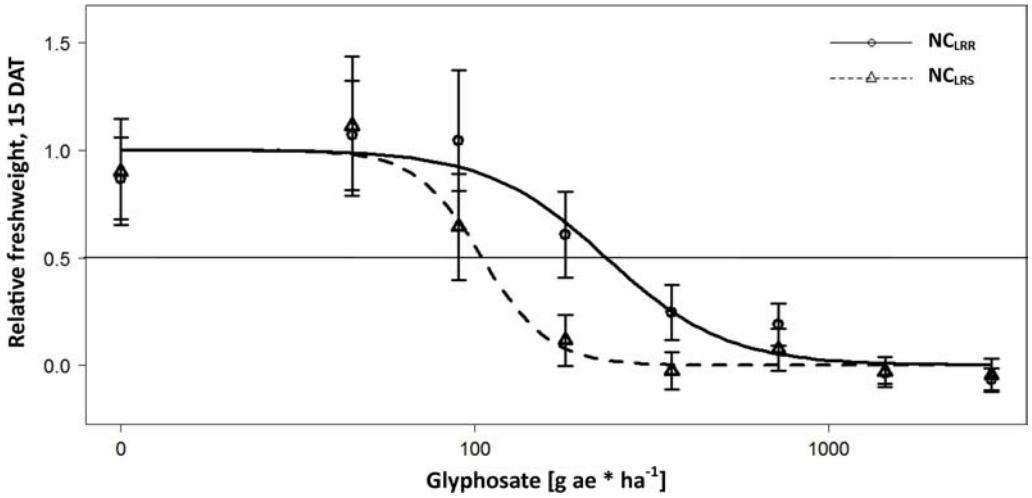

Fig. 22: Glyphosate tolerance of the NC_{LR} daughter lines NC_{LRS} and NC_{LRR} based on an entire above ground fresh weight assessment 15 DAT of 9 plants per dose rate and line; ED_{50} value of NC_{LRS} was 105 ± 54 g ae ha^{-1}; ED_{50} value of NC_{LRR} was 235 ± 168 g ae ha^{-1}; the standard error indicated by the error bars.

The dose response results showed differences between both accessions. The ED_{50} value of NC_{LRS} was 105 ± 54 g ae ha^{-1} (P = 0.05) glyphosate (ED_{90}: 169 g ae ha^{-1} glyphosate), while the ED_{50} value of NC_{LRR} was 235 ± 168 g ae ha^{-1} (P = 0.05) glyphosate (ED_{90}: 552 g ae ha^{-1} glyphosate). Despite the shifting in the glyphosate tolerance, both values are not significantly different (P = 0.05), which might be explained by the amount of individuals in NC_{LRR} bearing a low *EPSPS* gene copy number. The latter was supported by the high standard deviation in the ED_{50} value of NC_{LRR}. Therefore, even if the results are not significantly different, the higher *EPSPS* gene copy numbers in NC_{LRR} provoke a higher glyphosate tolerance in comparison to close related individuals of NC_{LRS}. However, the glyphosate resistance is lower than expected by the number of *EPSPS* gene copies.

4.1.7 *A. palmeri* EPSPS Enzyme Activity

The *EPSPS* is strongly amplified in the genome and transcriptome of glyphosate resistant *A. palmeri* individuals. The constants of the EPSPS to PEP, K_M(app.), V_{max} and K_{cat} were measured for two reasons: (1) to test whether the higher amount of EPSPS protein is actively translated in the protein pool of plant cell and (2) if any mutations in the EPSPS protein sequence occur changing the K_M value to PEP as the counter part to glyphosate. To determine the constants of the NC_S and NC_R EPSPS enzyme, the crude enzyme extracts were incubated with increasing PEP concentrations to determine the K_M(app.),

Tab. 11: Enzymatic characteristics of the native *EPSPS* protein of plants of the *A. palmeri* populations NC_R and NC_S, determined in crude protein extracts; values as mean of three biological with 2 technical replicates each; * indicates significant differences between both populations (P=0.05); n.s. indicates no significant differences; protein concentration measured as protein in μg BSA equivalent.

	NC_S	NC_R
K_M(app.) (Pep)[μM]	$70.75^{n.s.}$	$108.2^{n.s.}$
V_{max}[μM s^{-1}]	0.069*	0.78*
K_{cat} [μM P_i μg^{-1} protein]	6.35*	66.32*

V_{max} and the turnover rate of the enzyme in a Lineweaver-Burk diagram. The apparent K_M value of the EPSPS in the sensitive plants was 70.75 μM PEP without significant differences to the EPSPS of the resistant plants with an apparent K_M value of 108.2 μM PEP (Tab. 11). The affinity of the EPSPS to PEP is therefore not significantly different between both populations indicating no alterations in the enzyme sequence affecting the enzyme affinity to PEP, the counter part to glyphosate.

Significant differences were detected between the V_{max} values of the sensitive enzyme extracts of 0.069 μM s^{-1} to the resistant with 0.78 μM s^{-1}. The same significant differences were found for the K_{cat} values with a turn over rate in sensitive individuals of 6.35 μM P_i μg^{-1} crude protein extract and 66.32 μM P_i μg^{-1} crude protein extract in resistant plants. This indicates a 10 - 11 fold higher amount of active translated EPSPS in the protein pool of glyphosate resistant NC_R plants in comparison to the sensitive plants of NC_S.

Due to supplemented $NaMoO_4$ and NaF in the assay the phosphatase activity was inhibited. The controls either exclusively with PEP or S3P showed no P_i release during the measuring period. In conclusion they had no influence on the results. The EPSPS protein activity was measured at a constant crude protein concentration without a direct measurement of the EPSPS protein concentration. The increased K_{cat} and V_{max} values indicated therefore, no increase in the catalytic efficacy of the enzyme but an increased concentration of EPSPS in the crude protein extract of the glyphosate resistant NC_R plants. Despite the high variation in the obtained K_m values (PEP) no significant modifications in the EPSPS protein sequence affecting the affinity to PEP should occur, and indirectly the same related to glyphosate. With respect to these results a target site based resistance of the investigated *A. palmeri* population can be excluded since the K_M values show no significant differences.

This shows that the high genomic *EPSPS* gene copy number is translated into active protein but not exceeding about 10x the activity found in sensitive plants of NC_S. However, these results confirm in a second population that the *EPSPS* gene amplification is a mechanism conferring glyphosate resistance to *A. palmeri* plants (Gaines et al., 2010).

4.1.8 Relationship among the Different *A. palmeri* Accessions

The glyphosate resistance of the most *A. palmeri* populations is based on EPSPS gene amplification and a higher EPSPS enzyme concentration in the protein pool of resistant plant. But nothing is known about the development of glyphosate resistance in *A. palmeri*. In principle there are two possible explanations for the development: (1) the resistance was developed separately at several spots across the U.S. or (2) the resistance was developed at a single spot and than had spread throughout the whole infestation area, or a combination of both. To attempt to answer to this question, the relationship between the populations was studied based on the random amplified polymorphic DNA (RAPD) technique.

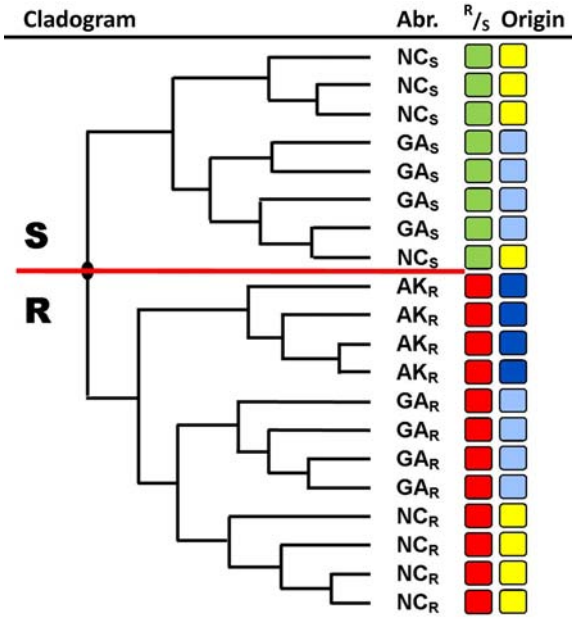

Fig. 23: Relationship of glyphosate resistant and sensitive *A. palmeri* populations NC_S, GA_S, NC_R, GA_R and AR_R; based on 4 individuals each accomplished with 107 polymorphic RAPD markers based on 8 different RAPD primers (Tab. 12), the color code on the graphic is related to the glyphosate response (left side) and to the geographical origin (right side, according to Tab. 2) of the populations, green color on the left side indicates glyphosate sensitive populations, red color indicates glyphosate resistant populations.

The available *A. palmeri* populations for this study were collected between 2005 - GA_S & GA_R - and 2009 - AR_R & TN_R - in different states of the U.S. (Tab. 2). GA_R and GA_S were collected in locations approximately 50 kilometers away from each other, whereas the sensitive and resistant populations, collected in North Carolina, were sampled in a few kilometers distance. The mean distance between the sampling places in Georgia, U.S. and North Carolina was about 300 kilometers. The origins of AR_R and NC_R & NC_S have a

Tab. 12: Relationship among *A. palmeri* populations; individuals of each population tested ("Ind."); "P_L" polymorphic markers within each population; "Total marker" gives the total amount of polymorphic marker analyzed in each population; gene diversity provides the "Mean" and standard deviation ("SD") of genetic diversity within each of the *A. palmeri* populations GA_S, NC_S, NC_{LRR}, NC_R, GA_R, AR_R and TN_R computed according to Nei et al., (1973) and calculated with POPGENE 32

Pop.	Ind.	P_L	Total marker	gene diversity	
				Mean	SD
GA_S	4	51	113	0.17	0.2
NC_S	4	52	113	0.16	0.19
NC_R	4	48	113	0.15	0.19
GA_R	4	48	113	0.15	0.19
AR_R	4	43	113	0.13	0.18

mean distance of around 1200 kilometers including the Eastern Continental Divide, the Appalachian mountains and the Mississippi river in between.

To determine the relationship among *A. palmeri* populations by RAPD technique 40 different RAPD primer were first evaluated for a reproducible marker pattern in replicates of the same DNA sample and also in different DNA extractions of the same plant of the populations NC_S and NC_R. This first test resulted in 16 oligonucleotides which were selected to determine the relationship of individuals. To avoid biased results only jointly amplified and separated marker were evaluated together. A comparison between sets of individuals based on a length determination of marker was for the same reason not performed. The comparison of populations was performed with 4 - 7 individuals of each population. Due to technical limitations the comparison within a set was restricted to 34 individuals which were tested and evaluated at the same time and the same gel. Nevertheless, the most important factor for a proper marker pattern was the DNA quality, integrity and purity.

In Fig. 23 eight RAPD oligonucleotides were used - OPW 2; OPW 4; OPW 5; OPW 6; OPW 7; OPW 16; OPW 18 & OPW 20 - to determine the relationship of NC_S, NC_R, GA_S, GA_R and AR_R based on 107 polymorphic marker and 6 monomorphic marker. In Fig. 25 202 marker were used, amplified with the RAPD oligonucleotides OPW 4; OPW 5; OPW 6; OPW 7; OPW 16; OPW 20; OPN 2; OPN 6; OPN 11; OPN 12; OPN 15; OPN 16 & OPN 18 - to produce 196 polymorphic marker and 6 monomorphic marker. The RAPD oligonucleotides OPW 2; OPW 18 and OPN 4 failed due to an unsatisfying gel separation of fragments and were not repeated in this set. To determine the cladogram of Fig. 24 243 marker were evaluated, resulting in 240 polymorphic and 3 monomorphic marker (RAPD oligonucleotides: OPW 2; OPW 4; OPW 5; OPW 6; OPW 7; OPW 16; OPW 18; OPW 20; OPN 2; OPN 4; OPN 6; OPN 11; OPN 12; OPN 15; OPN 16 & OPN 18). The amount of polymorphic marker found in each population, displayed in Fig. 25 &

Tab. 13: Genetic divergence of the *A. palmeri* populations GA_S, NC_S, NC_R, GA_R, AR_R and TN_R computed among each individual and given as average (M) of each population together with the standard deviation (SD) according to Nei & Li et al. (1979) calculated with Phylip 3.69; H_S provides the heterozygosity of population according to Nei et al. (1973) calculated with POPGENE 32; P_L indicates the amount of polymorphic marker found in a populations.

	GA_S		NC_S		NC_R		GA_R		AR_R		TN_R			
	M	SD	M	SD	M	SD	M	SD	M	SD	M	SD	H_S	P_L
GA_S	0.0230	0.0062											0.15	82
NC_S	0.0399	0.0053	0.0304	0.0045									0.17	109
NC_R	0.0431	0.0065	0.0406	0.0052	0.0275	0.0052							0.18	107
GA_R	0.0460	0.0078	0.0386	0.0082	0.0353	0.0064	0.0316	0.0099					0.16	93
AR_R	0.0460	0.0077	0.0397	0.0062	0.0400	0.0048	0.0378	0.0064	0.0316	0.0061			0.15	97
TN_R	0.0439	0.0073	0.0386	0.0041	0.0384	0.0048	0.0412	0.0063	0.0375	0.0064	0.0283	0.0051	0.16	105

Tab. 14: Genetic divergence of the *A. palmeri* populations GA_S, NC_S, NC_{LR}, NC_R, GA_R and TN_R computed among each individual and given as average (M) of each population together with the standard deviation (SD) according to Nei & Li et al. (1979) calculated with Phylip 3.69; H_S provides the heterozygosity of population according to Nei et al. (1973) calculated with POPGENE 32; P_L indicates the amount of polymorphic marker found in a populations

	GA_S		NC_S		NC_{LR}		NC_R		GA_R		TN_R			
	M	SD	M	SD	M	SD	M	SD	M	SD	M	SD	H_S	P_L
GA_S	0.0205	0.0045											0.13	89
NC_S	0.0457	0.0113	0.0414	0.0103									0.14	113
NC_{LR}	0.0449	0.0042	0.0538	0.0099	0.0138	0.0024							0.12	86
NC_R	0.0437	0.0036	0.0505	0.0062	0.0383	0.0034	0.0238	0.0043					0.15	108
GA_R	0.0509	0.0056	0.0511	0.0072	0.0510	0.0057	0.0437	0.0055	0.0299	0.0043			0.10	78
TN_R	0.0416	0.0043	0.0479	0.0082	0.0419	0.0067	0.0399	0.0050	0.0502	0.0071	0.0335	0.0054	0.18	164

Tab. 15: Analyses of the influence of geographical distance and glyphosate appearance (Gly app.) on the genetic variation of the investigated populations; hierarchical analysis of variance on *A. palmeri* populations of Fig. 24 & 25 in the F-statistic based on euclidean distance matrices of the RAPD marker according to Excoffier et al. (1992).

	Populations of Fig. 25						Populations of Fig. 24					
Source of variation	*df*	MSD	*P*	σ	% Total	Φ	*df*	MSD	*P*	σ	% Total	Φ
Variations due to geographic distance												
Among regions	3	5.95	≤ 0.001	-0.17	-4.33	Φ_{RT} = -0.043	2	7.10	≤ 0.001	-0.19	-4.48	Φ_{RT} = -0.045
Among populations / region	2	6.55	≤ 0.001	0.62	15.76	Φ_{SR} = 0.151	3	8.39	≤ 0.001	0.89	21.01	Φ_{SR} = 0.201
Within populations	26	3.47	≤ 0.001	3.47	86.86	Φ_{ST} = 0.114	29	3.54	≤ 0.001	3.54	83.47	Φ_{ST} = 0.165
Total variation	31	3.87	≤ 0.001	3.91	100.00		34	4.18	≤ 0.001	4.24	100.00	
Variations due to glyphosate sensitivity												
Among glyphosate tolerance	1	7.20	≤ 0.025	0.12	2.97	Φ_{GT} = 0.030	1	8.36	≤ 0.01	0.06	1.54	Φ_{GT} = 0.015
Among populations / Gly app.	4	5.63	≤ 0.001	0.41	10.17	Φ_{SG} = 0.105	4	7.75	≤ 0.001	0.71	16.52	Φ_{SG} = 0.168
Within populations	26	3.47	≤ 0.001	3.47	86.86	Φ_{ST} = 0.131	29	3.54	≤ 0.001	3.54	81.95	Φ_{ST} = 0.181
Total variation	31	3.87	≤ 0.001	3.99	100.00		34	4.18	≤ 0.001	4.32	100.00	

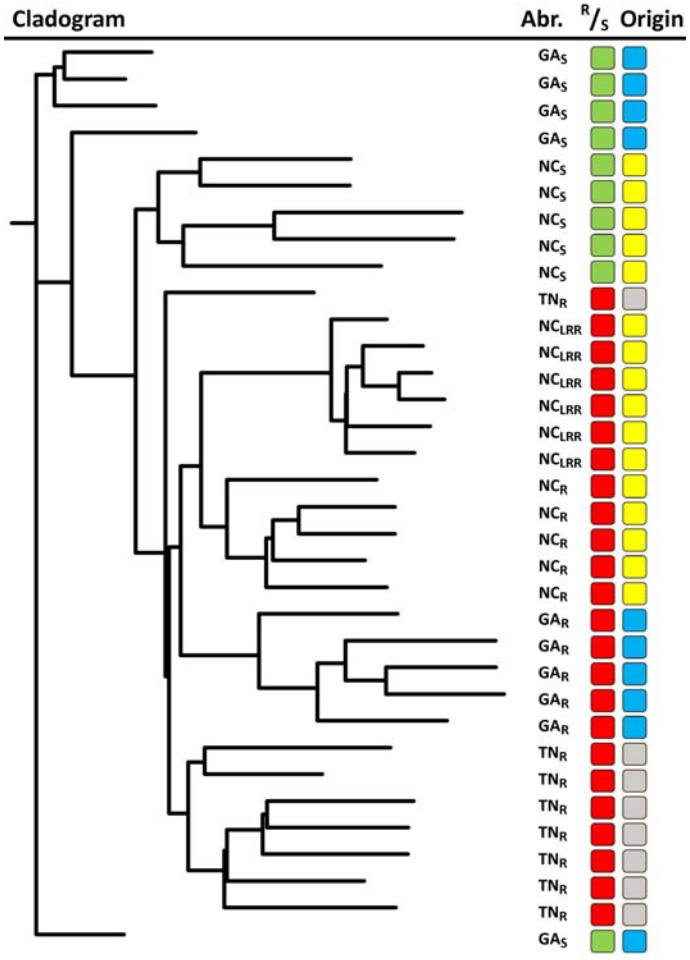

Fig. 24: Cladogram of *A. palmeri* populations (1); relationship of the populations GA_S, NC_S, NC_{LRR}, NC_R, TN_R and GA_R based on 243 RAPD-markers; calculation of the unrooted phylogenetic tree based on Nei's distance matrix, the color code on the graphic is related to the glyphosate response (left side) and to the geographical origin (right side, according to Tab. 2) of the populations, green color on the left side indicates glyphosate sensitive populations, red color indicates glyphosate resistant populations.

24 is given in Tab. 13 & 14, respectively. With exception of the population GA_R, showing 78 polymorphic marker, in all cases more than 80 polymorphic marker were found, which is described as the necessary amount for meaningful results (Staub et al., 2000). None of the evaluated marker was uniquely present either in the glyphosate resistant or sensitive group of plants which support that the populations were grouped according to their origin and not to the presence or absence of EPSPS gene amplification. However, the mean heterozygosity of all tested populations was $H_S = 0.15$ with values between $H_S = 0.10$, GA_R, and $H_S = 0.18$, TN_R & NC_R, whereas the mean genetic divergence was 0.0387 ± 0.008 - Tab. 13 and 0.0432 ± 0.010 - Tab. 14.

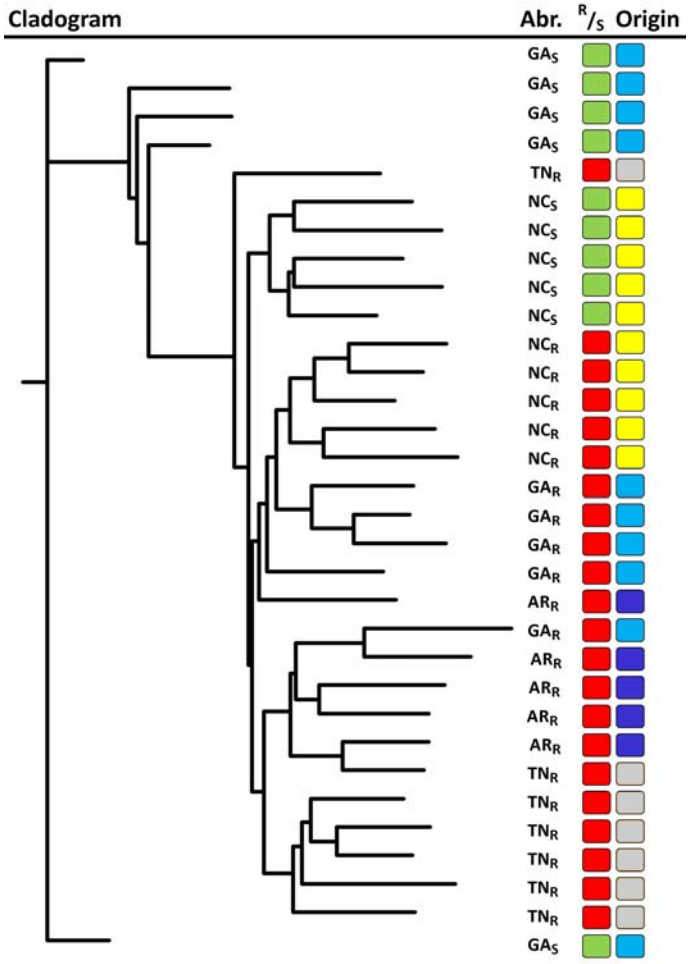

Fig. 25: Cladogram of *A. palmeri* populations (2); relationship of the populations GA_S, NC_S, NC_R, GA_R, AR_R and TN_R based on 202 RAPD-markers; calculation of the unrooted phylogenetic tree based on Nei's distance matrix, the color code on the graphic is related to the glyphosate response (left side) and to the geographical origin (right side, according to Tab. 2) of the populations, green color on the left side indicates glyphosate sensitive populations, red color indicates glyphosate resistant populations.

In Fig. 23 four individuals of each of the populations GA_S, NC_S, GA_R, NC_R and AR_R were compared with 113 marker. The 20 individuals were first clustered into the population they belong to, which supports the meaning of the results. Only a single individual of NC_S was close related to the GA_S individuals. However, the glyphosate sensitive individuals of NC_S and GA_S were as close related as the glyphosate resistant populations NC_R, GA_R and AR_R were. Especially the at close locations sampled populations NC_R and NC_S were clustered according to their glyphosate tolerance and not to their geographic distance into two different groups. Within the glyphosate resistant group GA_R and NC_R were closer related than AR_R which was more distantly related. In summary the five populations com-

pared in Fig. 23 were grouped according to their glyphosate tolerance and not according to the geographic distance as expected.
The second comparison in Fig. 25 with individuals of GA_S, NC_S, GA_R, NC_R, AR_R and TN_R shows the same picture. The sensitive individuals of NC_S and GA_S clustered close together, separated from the glyphosate resistant individuals, as in the previous comparison. The resistant individuals itself were roughly separated into each population, but the separation was not as sharp as in the sensitive. For example, six of the seven individuals of TN_R clustered close together, but a single plant clustered together with the AR_R population. At all GA_R and NC_R had a close relation, as AR_R and TN_R had, but these four individuals together were clearly separated from the sensitive populations NC_S and GA_S. This comparison shows therefore also a clear separation in the relationship between resistant and sensitive *A. palmeri* populations.
In the third comparison in Fig. 24, accomplished out of 243 markers, individuals of GA_S, NC_S, GA_R, NC_R and TN_R were compared and in addition individuals of NC_{LRR} were included. The individuals of the different populations clustered together as found in the previous study and were also in their relationship divided into a glyphosate resistant and a sensitive group. The order of relation was the same as in the previous cladogramms. NC_R and GA_R had the closest relation, TN_R was less related and the glyphosate resistant individuals were separated from sensitive individuals.
However, the populations were grouped together without significant differences due to the clustering methode used. The cladogramms based on genetic distances in Fig. 24 & 25 and the cladograms based on the parsimony methode in Fig. 49 & 47 showed an equal relationship, solely the relations between individuals within a population were slightly shifted. All of them showed a clear clustering into glyphosate sensitive and glyphosate resistant populations and none of them gave incidence on a clustering according to the geographic distance. To support these findings an analysis of molecular variance (AMOVA) according to Excoffier et al. (1992) was performed and the results are displayed in Tab. 15. The comparison showed that the most genetic diversity with 81.95 and 86.86 % was found within each population, as expected in an obligate outcrossing specie. A variation of 15.76 % and 21.01 % could be found between populations within a region. But the variation between populations with an equal glyphosate tolerance level was 5.6 % and 4.5 % lower in comparison to the populations grouped by region (Tab. 15). About 2.97 % and 1.54 % of the variations were only based on the different glyphosate tolerance level, while no direct influence of the geographic distance could be observed between the *A. palmeri* populations tested (Tab. 15). These results were statistically significant at a P value of at least $P = 0.025$ based on F-statistic (Tab. 15).
These results showed, that the relation of the investigated populations was not, as expected, depending on the geographic distance they grew, they rather clustered together, according to their response to glyphosate.

4.2 Investigations into the *Amaranthus tuberculatus* Glyphosate Resistance

The aim was to examine the glyphosate resistance mechanism of the north American weed species *A. tuberculatus*. Six different *A. tuberculatus* populations collected in Missouri and Illinois (Tab. 3) were used to determine and compare their behavior towards glyphosate. Five of them - IL_1, Mo_{13}, Mo_{14}, Mo_{15} Mo_{16}, Mo_{17} - were difficult to control in field, while the sensitive population, Mo_{18}, was well controlled by glyphosate applications in field. The 7^{th} population, CO_1, was included into the dose response study as a second sensitive standard since it was not in contact with any glyphosate resistant *Amaranthus* spp. plants before and belongs to the species *Amaranthus blitum* var. *emarginatus*. This *Amarantus blitum* population was collected 2005 in Colorado and is a close relative to *Amaranthus tuberculatus*.

In the following, the populations Il_1, Mo_{13} and Mo_{18} were evaluated concerning their glyphosate uptake and translocation, possible target site mutations, *EPSPS* gene amplification and the expression of *EPSPS* after glyphosate treatment. The K_m and V_{max} values of the native EPSPS were determined to investigate and confirm the *EPSPS* gene amplification influences at the protein level and to detect possible further mutations in the protein sequence due to alterations in the kinetic properties of the EPSPS enzyme. The EPSPS K_{cat} values of IL_1, Mo_{13}, Mo_{15}, Mo_{16} and Mo_{18} were in addition determined to confirm that *EPSPS* gene amplification affected the EPSPS in the plant protein pool in a larger number of populations. In a first approach to understand the evolution of glyphosate resistance in *A. tuberculatus* across the U.S.A. the relationship of the populations Il_1, Mo_{13} and Mo_{18} was checked by using RAPD markers.

4.2.1 Response of the *A. tuberculatus* Populations to Glyphosate

During the whole cultivation period no difference among the different populations was observed related to germination, plant shape, or plant height regarding their response to glyphosate. However, two days after the application of 12 increasing glyphosate dose rates the first herbicidal injury symptoms were macroscopic visible in glyphosate resistant and sensitive plants as droopy, followed by chlorotic and later stunted young leaves. In resistant plants leaf necroses, probably due to the high surfactant content of the spraying solution at higher doses rates, were observed approximately 6 - 8 days after treatment (DAT). These symptoms were comparable to the previous described symptoms in *A. palmeri*, Section 4.1 and Fig. 45.

Comparable glyphosate injury symptoms in sensitive plants appeared in sensitive *A. tu-*

berculatus and *A. blitum* populations at less than 45 g ae ha^{-1} glyphosate. In resistant populations a similar response and comparable symptoms appeared but starting at a dose rate, higher than 180 g ae ha^{-1} glyphosate. Thus, similar injury symptoms were observed in the glyphosate sensitive and resistant populations, but at a much higher rate in the resistant plants.

The shikimic acid content after glyphosate treatment in plant tissue was used as an early

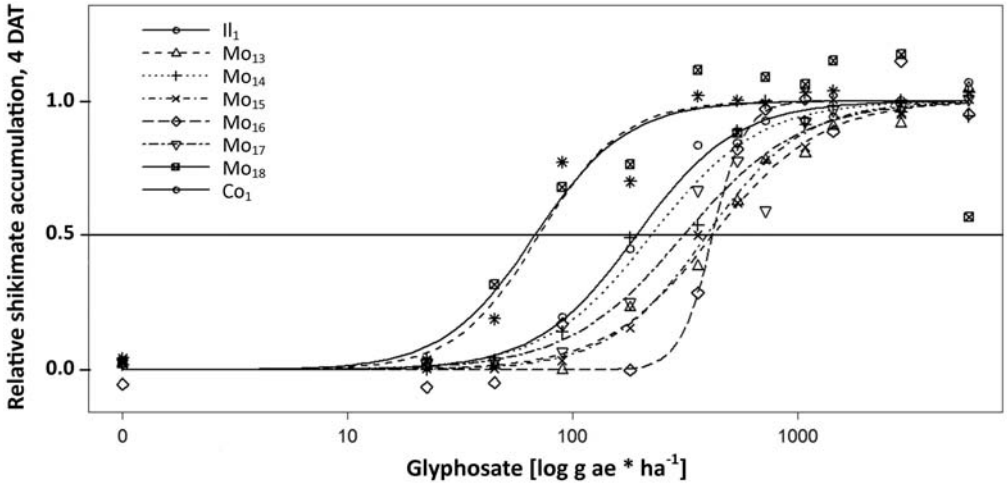

Fig. 26: Relative shikimic acid accumulation in the youngest fully expanded leaf of *A. tuberculatus* and *A. blitum* plants 4 days after glyphosate treatment; plants belong to the *A. tuberculatus* populations IL_1, Mo_{13}, Mo_{14}, Mo_{15} Mo_{16}, Mo_{17}, Mo_{18} and the *A. blitum* population Co_1.

indicator for glyphosate mediated plant injury (Shaner et al., 2005) and was therefore used to characterize the glyphosate resistance of the *A. tuberculatus* populations. Since *A. tuberculatus* and *A. palmeri* are closely related species a similar shikimic acid content in function of glyphosate treatment in the different parts of plant was expected. In *A. tuberculatus* also the youngest fully expanded leaf was used to measure the increase in the shikimic acid content after glyphosate treatment. The measurement 4 DAT in the youngest fully expanded leaf, given in Fig. 26, was only observed in plants treated with 5760; 2880; 1440; 1080; 720; 540; 360; 180; 90; 45; 22.5; 0 g ae ha^{-1} glyphosate. At the highest dose rate of 11520 g ae ha^{-1} glyphosate, the measurement of the shikimic acid content was due to strongly stunted leaves not possible. The shikimic acid content allowed to group the populations according to their response to glyphosate into three significant different groups. The sensitive *A. tuberculatus* population Mo_{18} and the *A. blitum* population Co_1 showed the same response concerning the shikimic acid content in leaf tissue and their ED_{50} values were not significantly different, 68 g ae ha^{-1} and 71 g ae ha^{-1} glyphosate for Mo_{18} and Co_1 respectively (Tab. 16). The relative and absolute increase of shikimic acid in leaf tissue was comparable between both in relation to the applied dose and supports

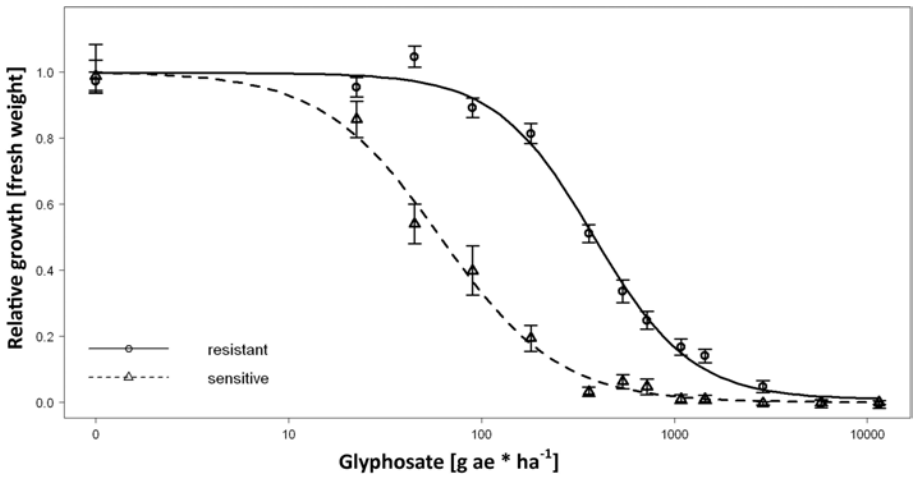

Fig. 27: Relative fresh weight of entire above ground foliage 19 DAT of the *A. tuberculatus* populations IL_1, Mo_{13}, Mo_{14}, Mo_{15} Mo_{16}, Mo_{17}, Mo_{18} and the *A. blitum* population Co_1; plants treated with increasing glyphosate dose rates; the sensitive group contains the populations Mo_{18} and Co_1; the resistant group includes the populations IL_1, Mo_{13}, Mo_{14}, Mo_{15} Mo_{16} and Mo_{17}; the standard error is indicated by the errorbars.

the sensitivity of the *A. tuberculatus* population Mo_{18}. The variation of the shikimic acid content in the sampled leaves was higher among the resistant populations than among the sensitive. The resistant populations could therefore be divided into two resistant groups. Il_1 belonged to the first resistant group and showed already at lower glyphosate dose rates an increase of shikimic acid with a calculated ED_{50} value of 192 g ae ha^{-1} glyphosate.
Mo_{13}, Mo_{15} and Mo_{16} belong to the second resistant group and showed an shikimic acid increase at higher doses rates in comparison to IL_1. Their ED_{50} value was in average 410 g ae ha^{-1} glyphosate. The populations Mo_{14} and Mo_{17} collected in Calloway- and Pettis-county, Missouri, U.S. had in average a calculated ED_{50} value of 267 g ae ha^{-1} glyphosate and were not significant different from both resistant groups. Thus, the ED_{50} values obtained by shikimic acid assessment in glyphosate resistant *A. tuberculatus* populations were between 192 - 419 g ae ha^{-1} glyphosate.
The fresh weight of treated plants was assessed 19 DAT to determine the influence of increasing glyphosate dose rates on plant growth (Fig. 27). The plant growth variations among resistant populations were smaller than expected by shikimic acid assessment. They allowed a separation into a sensitive and a resistant group of populations.
The ED_{50} values of the sensitive populations Mo_{18} and Co_1 were between 61 and 82 g ae ha^{-1} glyphosate as indicated in Tab. 16 and showed therefore greater differences than obtained by shikimic acid measurements. At all, glyphosate seemed to have a faster action in the *Amarantus blitum* population in comparison to *A. tuberculatus* population Mo_{18}, indicated by a sharper slope in the dose response relation 19 DAT (data not shown). Nev-

ertheless, both ED_{50} values were not significant different and none of the sensitive plants survived a dose rate of 540 g ae ha^{-1} glyphosate, as their ED_{90} values were below 300 g ae ha^{-1} glyphosate.

In contrast to the previous shikimic acid assessment the resistant populations were in their growth not dividable into different categories of resistance. They were clustered together in a single group of glyphosate resistance. The ED_{50} values were in comparison to the shikimic acid data higher and reached in average 396 g ae ha^{-1} with a variation between 320 g and 460 g ae ha^{-1} glyphosate. These plants survived dose rates of 720 g ae ha^{-1} glyphosate as their ED_{90} values were between 1350 - 3000 g ae ha^{-1} glyphosate. The population Il_1 belonged in the first characterization of the glyphosate response based on the shikimic acid content to the first and less glyphosate resistant group of populations. In the fresh weight assessment Il_1 was not significantly different to the other populations Mo_{13}, Mo_{14}, Mo_{15} Mo_{16} and Mo_{17} and did not showed the lowest sensitivity towards glyphosate within the group of resistant populations.

However the resulting resistance factor at 50 % plant growth reduction between the most sensitive population Mo_{18} and the most resistant population Mo_{17} was RF = 7.5. In average the resistance factor between the grouped sensitive and grouped resistant populations was RF = 5.7. However, the populations IL_1, Mo_{13}, Mo_{14}, Mo_{15} Mo_{16} and Mo_{17} were glyphosate resistant according to both, to shikimic acid and to fresh weight assessment.

Tab. 16: ED_{50}, ED_{90} values and RF of *A. tuberculatus* & *A. blitum* glyphosate dose response relation in shikimic acid concentration 4 DAT and fresh weight assessment 19 DAT ; letters indicate significant differences among populations.

Pop.	Shikimate acc., 4 DAT ED_{50} [g ae ha^{-1}]		R/S ratio	Fresh weight, 19 DAT ED_{50} [g ae ha^{-1}]	95 % confidence interval		R/S ratio	ED_{90} [g ae ha^{-1}]
Il_1	**192**	a	2.8	**420**	349.4 - 490.7	A	6.8	1363
Mo_{13}	**419**	b	6.2	**317**	234.2 - 398.6	A	5.2	1728
Mo_{14}	**224**	ab	3.3	**361**	334.9 - 385.3	A	5.9	485
Mo_{15}	**393**	b	5.8	**411**	294.3 - 524.2	A	6.7	2021
Mo_{16}	**419**	b	6.2	**405**	306.1 - 502.7	A	6.7	1420
Mo_{17}	**310**	ab	4.6	**460**	334.5 - 583.6	A	7.5	2969
Mo_{18}	**68**	c	1	**61**	46.2 - 76.3	B	1	291
Co_1	**71**	c	1	**82**	71.3 - 92.5	B	1.3	117

4.2.2 Glyphosate Uptake and Translocation in *A. tuberculatus*

Differences in absorption and translocation are described to be one of the main glyphosate resistance mechanism in weeds (Shaner, 2009). In *A. palmeri* these mechanisms had

no contribution to glyphosate resistance as previously shown. However, to exclude these mechanisms in the glyphosate resistant *A. tuberculatus* populations the absorption and translocation of ^{14}C-glyphosate throughout the entire plant and within single organs were tested and quantitatively and qualitatively compared among the populations Il_1, Mo_{13} and Mo_{18}. In average a recovery of 90 % of the applied 11500 Bq radioactivity was measured

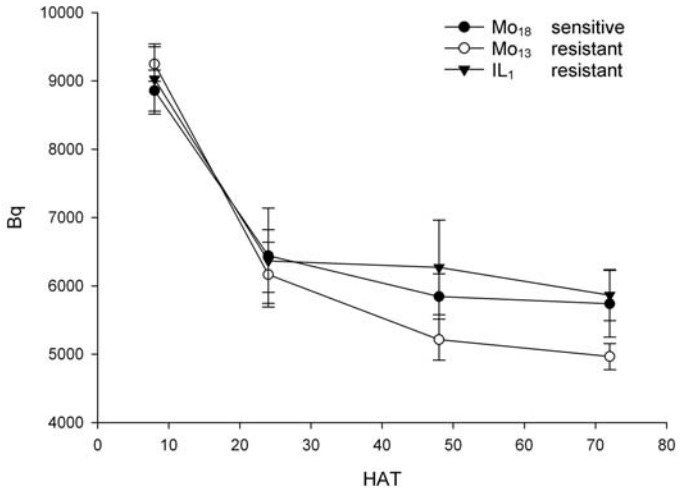

Fig. 28: Glyphosate leaf uptake in *A. tuberculatus* plants; comparison between the resistant populations IL_1 and Mo_{13} and the sensitive population Mo_{18} at BBCH 15; ^{14}C-glyphosate deposit on the leaf surface of the youngest fully expanded leaf was determined at 8, 24, 48 and 72 HAT; the standard error is indicated by the errorbars.

in the leaf washings of all time points tested, assuming it as ^{14}C-glyphosate. Indeed the recovery was decreasing with time. Eight hours after treatment a recovery of 97 % of the applied radioactivity was achieved, but decreased to 86 % after 72 HAT without differences between populations. The decreasing recovery might be caused by remaining root tips or a secretion of radioactivity into the soil, which was not determined.

To measure the glyphosate leaf uptake, remaining radioactivity on the applied leaf surface was washed off and measured. Thus, 46 % of the total applied radioactivity was absorped during the first 24 HAT. Afterwards this rate was decreasing untill a total uptake of about 53 % radioactivity 72 HAT (Fig. 28). The highest uptake occured during the first 24 HAT and no significant difference was observed between glyphosate sensitive and resistant populations at any time points analyzed.

To determine changes in glyphosate translocation, the plant was divided at harvest time into applied leaf, shoot below and above treated leaf and into root. Remaining radioactivity on the surface of the applied leaf was washed off as previously described to determine the absorption. The radioactivity inside the plant tissue was determined separately for each individual and for each plant part in a scintillation counter after combustion of the

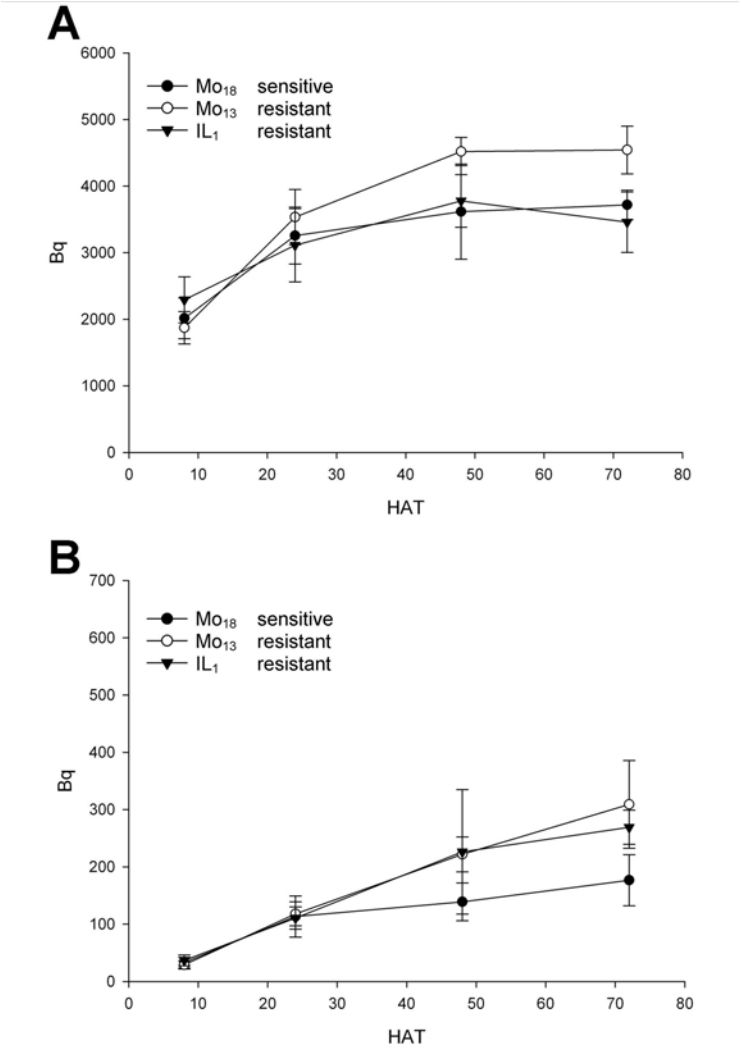

Fig. 29: Glyphosate translocation in *A. tuberculatus* plants; comparison between the resistant populations IL_1 and Mo_{13} and the sensitive population Mo_{18} at BBCH 15; applied leaf (**A**); shoot tip (**B**); measurement at 8, 24, 48 and 72 HAT; measurement after combustion of dried plant material and $^{14}CO_2$ determination; the standard error is indicated by errorbars; statistically significant differences are indicated by the asterix.

dried plant material.

Already 8 HAT radioactivity was detected in all plant parts (Fig. 29 & 30). The amount of radioactivity in the different plant sections was increasing with the time of treatment. The translocation especially into the root was at a nearly constant rate over the time frame of 72 h, without significant differences between populations (Mo_{18}: 2.3 %; Mo_{13}: 2.2 %; IL_1: 3.7 % of absorpt radioactivity). The translocation into the shoot was the highest during the first 24 HAT and showed a slower rate afterwards until a total of absorbed radioactivity of 11 % in Mo_{18}, 8 % in Mo_{13} and 9.5 % IL_1 72 HAT. The highest amount

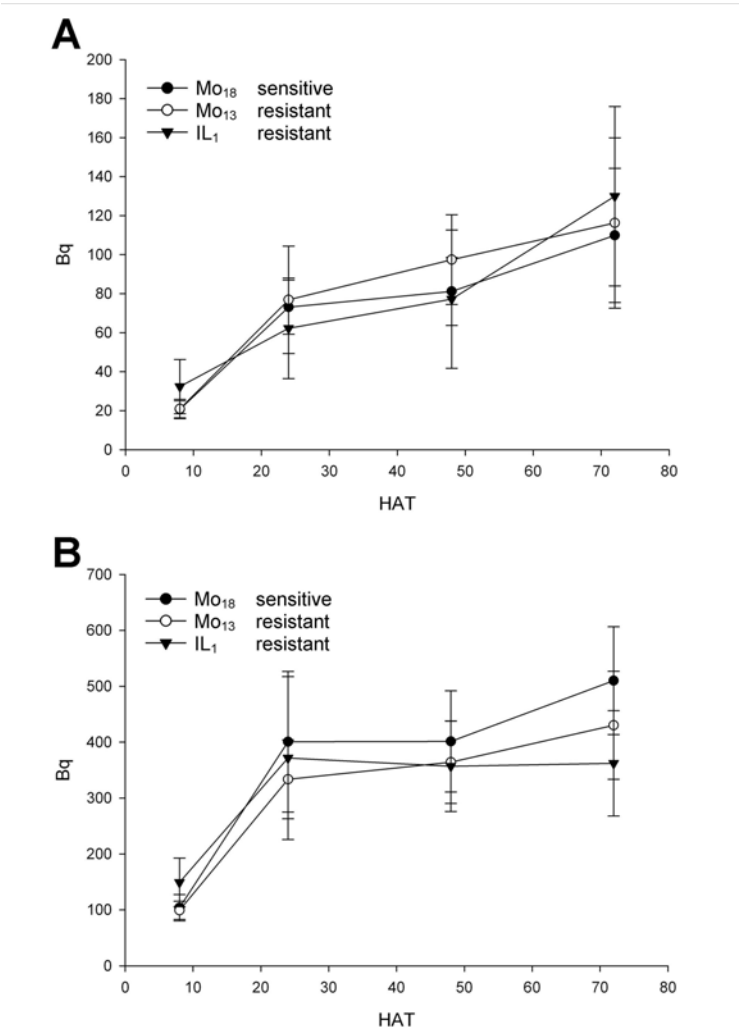

Fig. 30: Glyphosate translocation in *A. tuberculatus* plants; comparison between the resistant populations IL_1 and Mo_{13} and the sensitive population Mo_{18} at BBCH 15; in root (**A**) and shoot (**B**); measurement at 8, 24, 48 and 72 HAT; measurement after combustion of dried plant material and $^{14}CO_2$ determination; the standard error is indicated by errorbars.

of radioactivity was detectable in the applied leaf with Mo_{18}: 82.7 %; Mo_{13}: 84 %; IL_1: 80.6 % of absorbed radioactivity 72 HAT. Significant differences were only found in the shoot tip 72 HAT between the glyphosate sensitive population Mo_{18} with 4 % of absorbed radioactivity and the resistant populations Mo_{13} and IL_1 with 5.7 % and 6.0 % of absorbed radioactivity, respectively. The decreased translocation into the shoot tip of glyphosate sensitive plants was probably related to the apparition of the first herbicidal injury symptoms. Comparable results were previously found in the *A. palmeri* populations.

The autoradio-grams in Fig. 31 illustrate the translocation of ^{14}C-labeled glyphosate throughout the sensitive and resistant *A. tuberculatus* plants. Already 8 HAT radioac-

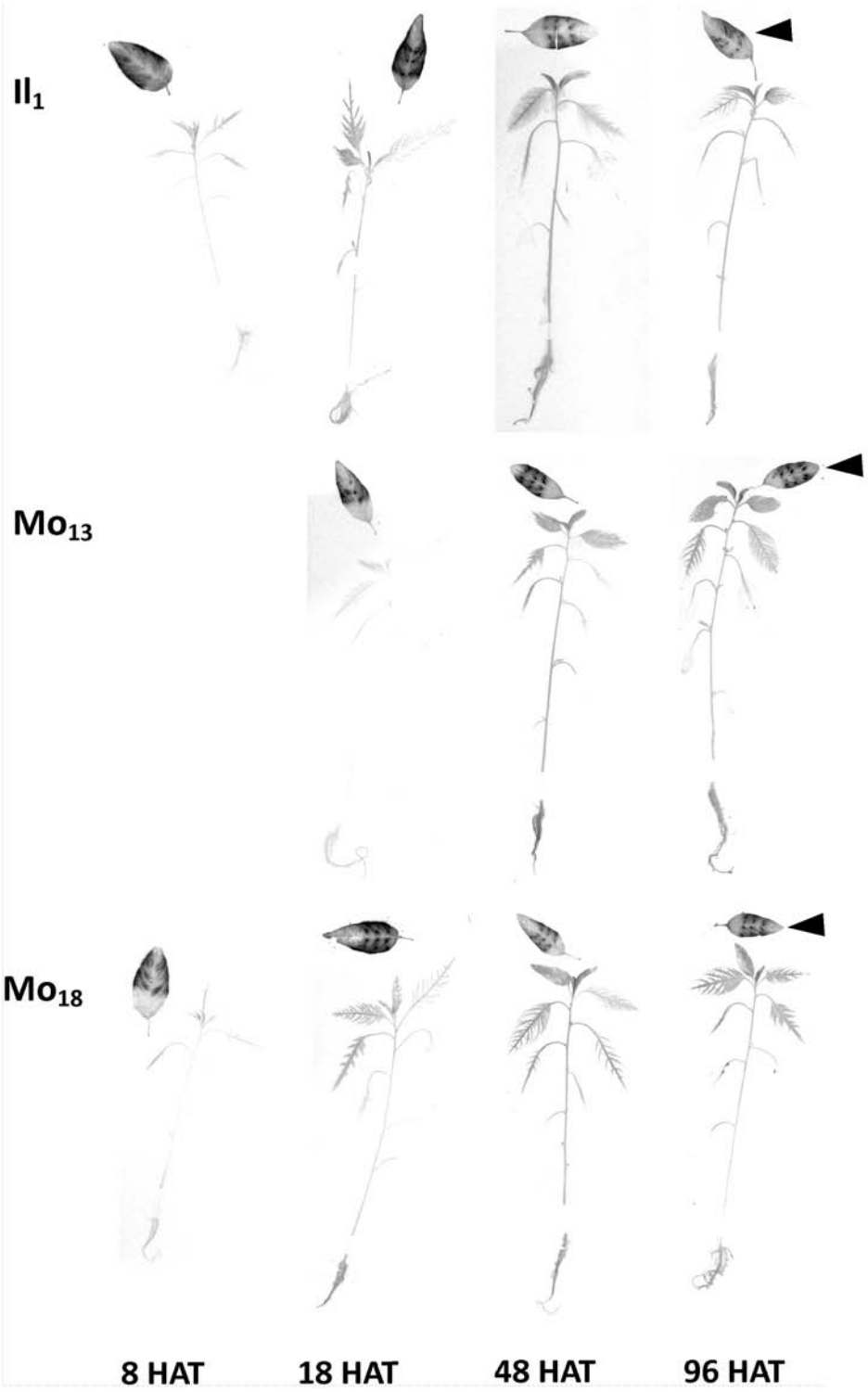

Fig. 31: Phosphorimaging visualization of ^{14}C-glyphosate translocation in *A. tuberculatus* plants of the populations IL_1, Mo_{13} and Mo_{18} at 8; 18; 48; and 96 HAT; arrow heads indicate the treated leafs at 96 HAT, rinsed at harvest time and dried separated from plant; each plant as representative of 5 plants tested in each time-point; increasing color intensity indicates a higher amount of radioactivity within the plant material.

tivity was detectable in the whole plant, with the highest activity found in the youngest and most active plant parts. The amount of radioactivity in older and less active plant parts and the overall amount in each tissue was increasing with time. A sequestration or a directed translocation into the leaf borders or specific plant organs was not observed in the plant populations analyzed in this study. Thus, alterations of glyphosate uptake or translocation cannot be detected and therefore does not contribute to the glyphosate resistance observed in the *A. tuberculatus* populations at the experimental conditions used.

4.2.3 Alterations in the *A. tuberculatus EPSPS* gene sequence

For several glyphosate resistant weeds like *Lolium* spp., *Eleusine indica* or the transgenic and glyphosate resistant corn variety GA21, the target site mutations in the *EPSPS* amino acid sequence G101, T102 or P106 are described to confer glyphosate resistance. Thus, the *A. tuberculatus EPSPS* DNA sequence encoding for the amino acid positions G101, T102 and P106 was tested for single nucleotide polymorphisms (SNP) using pyrosequencing. No DNA sequence alterations were found. Therefore, the *A. tuberculatus* glyphosate resistance was not based on the known mutations encoding the amino-acids G101, T102 or P106 in the EPSPS coding sequence.
An horizontal resistance trait transfer between weeds and herbicide resistant crop cultivars was excluded in the glyphosate resistant *A. tuberculatus* populations using a commercially available test kit for the CP4 EPSPS protein. The test reveals the absence of the RR-gene-construct in all glyphosate resistant *A. tuberculatus* plants. Therefore, the glyphosate resistance in *A. tuberculatus* is not due to an horizontal gene transfer between Roundup Ready®crops and weeds. Neither the target site mutations G101, T102 or P106 nor an horizontal gene transfer between crop cultivars and weeds is responsible for the observed glyphosate resistance in *A. tuberculatus*.

4.2.4 *EPSPS* Gene Amplification in *A. tuberculatus*

EPSPS gene amplification is responsible for the glyphosate resistance observed in *A. palmeri* plants as shown in the previous chapter and as reported by Gaines et al. (2010). *A. palmeri* and *A. tuberculatus* are closely related species and are able to produce fertile crosses. The *EPSPS* gene amplification was therefore tested as a possible glyphosate resistance mechanism in *A. tuberculatus*. Due to the high homology of *EPSPS* and *ALS* genes of both species the same quantitative PCR (qPCR) method was used to determine the relative *EPSPS* gene copy number in 13 individuals per population.
The sensitive *A. tuberculatus* population Mo_{18} contained a comparable amount of *EPSPS* and *ALS* genes in the genome with 1 ± 0.13 relative *EPSPS* gene copy (Fig. 32). In

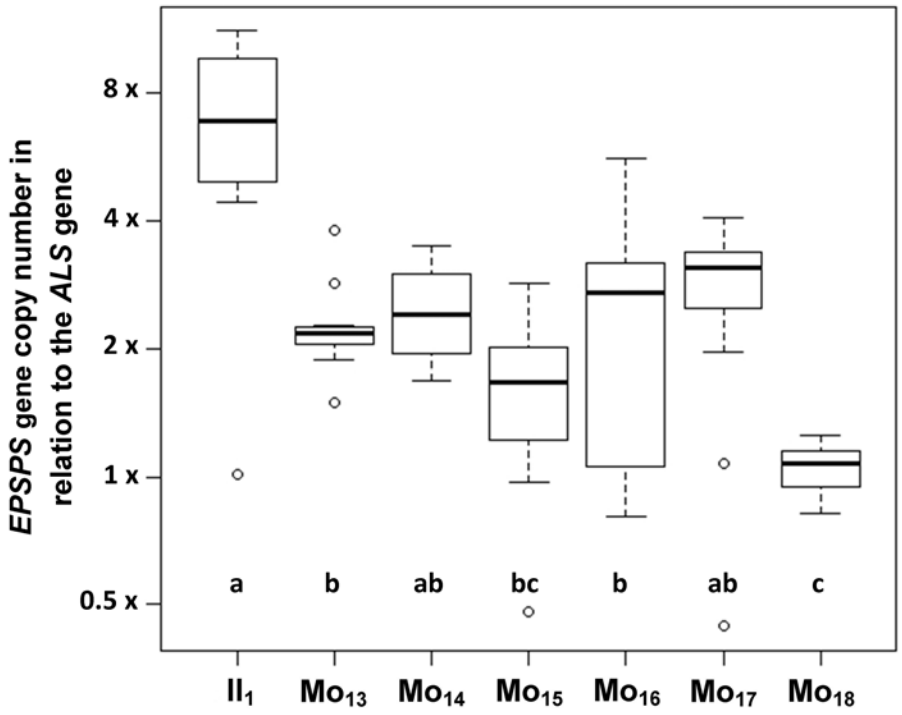

Fig. 32: Genomic *EPSPS* gene copy number in plants of the *Amarantus tuberculatus* populations Il_1, Mo_{13}, Mo_{14}, Mo_{15},Mo_{16}, Mo_{17} and Mo_{18} in relation to *ALS* gene; Il_1 = 7.05 ± 3.03 relative *EPSPS* genes, Mo_{13} = 2.26 ± 0.55, Mo_{14} = 2.52 ± 0.6, Mo_{15} = 1.62 ± 0.64, Mo_{16} = 2.67 ± 1.58, Mo_{17} = 2.75 ± 1.06, Mo_{18} = 1.06 ± 0.13; different letters inside the graph provide significant differences among populations (P=0.05).

contrast, the glyphosate resistant plants had in average a higher amount of *EPSPS* genes in their genome. Among the resistant populations, IL_1 had the highest *EPSPS* gene amplification with in average 7.5 ± 3 fold more *EPSPS* genes in the genomic sequence than the *ALS* genes. The plant with the highest gene amplification showed an *EPSPS* gene content of about 11.7 times the *ALS* gene. The populations collected in Missouri had in average 2.4 ± 1 fold more *EPSPS* than *ALS* genes in the genome. The highest gene amplifications found in a single resistant plant of these populations was 6 times the *ALS* gene, but each population contained also some individuals bearing an equal *EPSPS* and *ALS* amount in the genome. The population with the lowest variation in *EPSPS* gene copies was Mo_{13} with in average 2.3 ± 0.6 fold more *EPSPS* than *ALS* genes. This population was grown from seeds collected from a single resistant mother plant in field. The plants tested for the other populations were grown from field collected seeds, randomly sampled from several plants. A preselection for glyphosate resistance before copy number determination was not performed. This might explain the high *EPSPS* gene copy number variations and also the occurrence of plants bearing a low amount of *EPSPS* gene copies. Nevertheless, these *A. tuberculatus* populations were less glyphosate resistant than the glyphosate resistant *A.*

palmeri populations and also the *EPSPS* gene amplification was lower than those observed in *A. palmeri*.

4.2.5 *EPSPS* Gene Expression and Effects of a Glyphosate Treatment

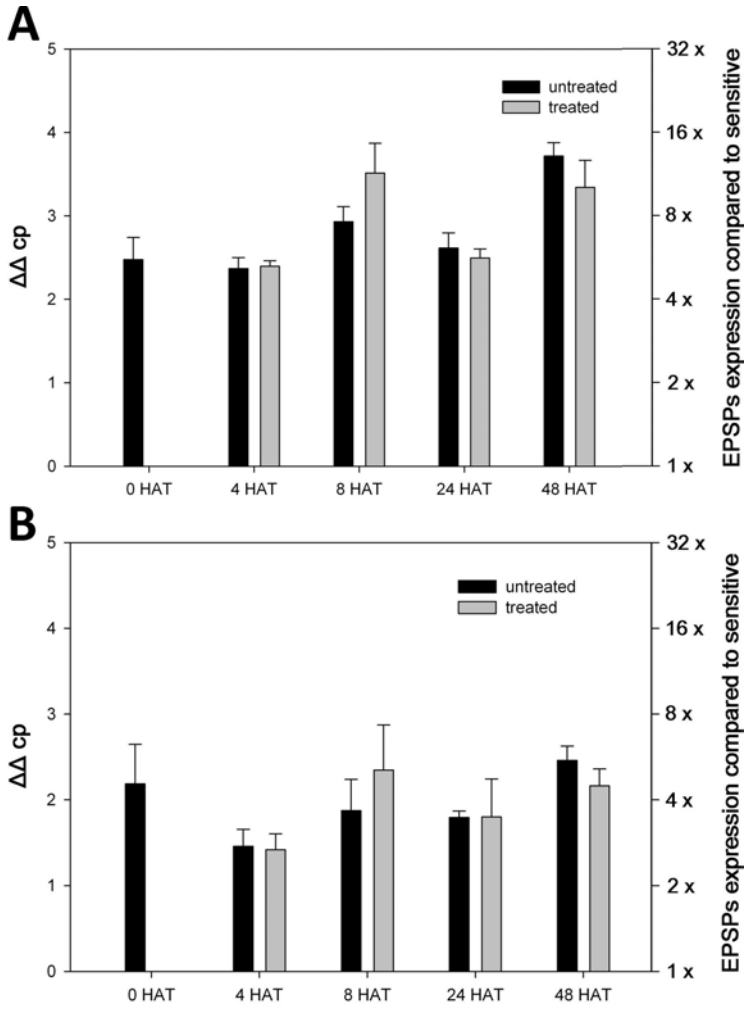

Fig. 33: Comparison of the *EPSPS* expression of plants of the glyphosate resistant *A. tuberculatus* populations IL_1 & Mo_{13} in relation to the *EPSPS* expression of plants of the glyphosate sensitive population Mo_{18} after a treatment with 720 g ae ha^{-1} glyphosate; expression normalized to the *Actin* gene; **A** indicates the expression values of IL_1 plants; **B** gives the values for Mo_{13} plants in relation to the *EPSPS* expression of glyphosate sensitive Mo_{18} plants; the standard error is indicated by the error bars.

The glyphosate resistant *A. tuberculatus* plants showed a higher *EPSPS* gene copy number in their genome than glyphosate sensitive *A. tuberculatus* plants. To study whether amplified *EPSPS* genes are silenced or induced by glyphosate application, the *EPSPS* mRNA

expression in untreated plants was compared to those of plants treated with the recommended field rate of 720 g ae ha^{-1} glyphosate. The *Actin* gene and the *ALS* gene were tested to be used as reference genes.
Significant differences in the *Actin* mRNA expression between treated and untreated plants were not observed. The 188 bp long *Actin* gene fragment was stable expressed at a constant rate in function of time and glyphosate treatment. The expression rate of the *ALS* gene fragment was in comparison to the *Actin* gene also constant, but with a higher variation among biological replicates and treatments within a population and was therefore not used to normalize the *EPSPS* expression data. Thus, the *EPSPS* gene expression was normalized to the *Actin* gene expression.
The *EPSPS* mRNA expression among the *A. tuberculatus* populations Mo_{18}, Mo_{13} and IL_1 was significant different (P = 0.05), but there were no significant differences between treated and untreated plants at any time point within a population (Fig. 33). The genomic *EPSPS* content and the *EPSPS* expression were not measured in the same individuals. A direct comparison of the genomic and expressed *EPSPS* amount was therefore not possible. Nevertheless, within a population no significant differences between genomic and mRNA *EPSPS* expression were observed. The *EPSPS* was in comparison to the *Actin* gene stable expressed and the treatment of 720 g ae ha^{-1} glyphosate was not inducing any alterations in the *EPSPS* expression. This data suggest in addition that all genomic copies are transcribed. Glyphosate treatments seem to not influence the *EPSPS* expression in both, in sensitive and resistant *A. tuberculatus* plants.

4.2.6 EPSPS Enzyme Activity in *A. tuberculatus*

Tab. 17: Native EPSPS protein activity of plants of the *A. tuberculatus* populations Mo_{18}, IL_1, Mo_{13}, Mo_{15}, Mo_{16}; values as mean of 2 biological with 2 technical replicates each; small letter indicate significant differences between values (P = 0.05); *n.s.* not significant; crude protein given in μg BSA equivalent.

	Mo_{18}	IL_1	Mo_{13}	Mo_{15}	Mo_{16}
K_M(app.) (Pep)[μM]	$58.49^{n.s.}$	$59.92^{n.s.}$	-	-	-
V_{max}[μM s^{-1}]	0.064^a	0.15^b	-	-	-
K_{cat} [μM P_i μg^{-1} protein]	11.43^a	41.25^b	22.5^c	22.75^c	21.75^c
K_i (Gly) [μM]	0.88	-	-	-	-

Alterations in the EPSPS at the amino acid position G101, T102 and P106 were not present in glyphosate resistant *A. tuberculatus* plants as shown in chapter 4.2.3. To compare the catalytic active EPSPS enzyme amount in the protein pool of *A. tuberculatus* plants of the populations Mo_{13}, Mo_{15}, Mo_{16}, Mo_{18} and IL_1 the catalytic efficacy (K_{cat}) of

native EPSPS was determined. To further test the EPSPS amino acid sequence for other alterations than the previous mentioned, the K_m and V_{max} values of the extracted EPSPS were compared between Il_1 and Mo_{18}. The EPSPS activity was measured in the extracted, fractionated and desalted crude proteins of both populations. The crude protein extract was fractionated with ammonium sulfate and in a prescreen fractions were tested for their EPSPS activity. Only the 40 - 70 % ammonium sulfate fraction contained EPSPS activity and was used for further testes.

The EPSPS K_m (app.) (Pep) values of Mo_{18} and IL_1 were 58.49 μM and 59.92 μM PEP (Tab. 17). This suggests that no alterations in the EPSPS amino acid sequence influencing the enzyme activity occured. But significant differences were found in the V_{max} values with a 2.4 times higher P_i release in IL_1 with a V_{max} of 0.15 μM s^{-1} than in Mo_{18} with a V_{max} of 0.64 μM s^{-1}. Differences were also obtained in the catalytic efficacy of extracted EPSPS enzymes. The K_{cat} value of the resistant population IL_1 was 3.7 times higher than the K_{cat} value of the sensitive population Mo_{18}, whereas the K_{cat} values of Mo_{13}, Mo_{15}, Mo_{16} were about 2 times higher. Since the V_{max} and K_{cat} values were measured at an equal protein concentration but not at an equal EPSPS concentration among populations the higher V_{max} and K_{cat} values indicate a higher proportion of EPSPS in the protein pool of resistant plants. The K_m values between the protein extracts of plants of IL_1 and Mo_{18} were not significantly different, the EPSPS enzyme sequence contained therefore no alterations influencing the affinity to PEP, the counterpart of glyphosate. The K_i value was only obtained in the sensitive population, because the variations in the resistant population at different inhibitor concentrations did not allow a proper determination of the K_i value.

Thus, the *EPSPS* gene copy number, the *EPSPS* transcript level and the active EPSPS protein concentration was higher in glyphosate resistant than in glyphosate sensitive *A. tuberculatus* plants.

4.2.7 *A. tuberculatus* Population Study using RAPD Markers

The relationship of IL_1, Mo_{13} and Mo_{18} individuals was investigated to get a hint on the development and the spread of glyphosate resistance in and among *A. tuberculatus* populations in the total grown area. The comparison was based on the RAPD technology performed with 9 RAPD primer leading to 117 markers in each of the 5 individuals per population. The comparison among individuals in Fig. 34 was therefore based on 105 polymorphic and 12 monomorphic markers. Within population IL_1 61 polymorphic markers were found, within Mo_{13} 43 polymorphic markers and within Mo_{18} 59 polymorphic markers were found. Based on these data, Nei's gene diversity index is 0.18 $\pm$ 0.19; 0.12 $\pm$ 0.18 and 0.19 $\pm$ 0.2 within each population, respectively (Nei, 1973). According to the

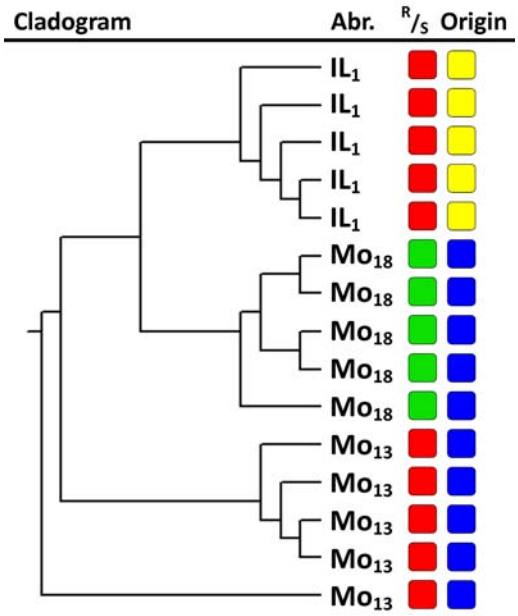

Fig. 34: Relationship of the *A. tuberculatus* populations IL_1, Mo_{13} and Mo_{18}; cladogram based on 9 RAPD primers and 117 evaluated markers for each individual; clustering based on the most parsimoneous tree, performed in Phylip 3.69.

RAPD markers the individuals showed a clear clustering according to the population they belong to. On the population level IL_1 and Mo_{18} were closer related to each other than to the third population Mo_{13}. No relation among populations depending on glyphosate appearance or geographical distance was found.

4.3 Herbicide Resistance in *Sorghum halepense.*

Sorghum halepense is an important weed species, which is causing significant losses in crops such as corn cotton or soybean and also in pastures or fallows. In addition *S. halepense* can propagate by rhizomes which make it even more troublesome and difficult to control it in those cropping systems.

Since the use of glyphosate resistant crops and the intensive use of this herbicide, the high selection pressure has lead to the evolution of glyphosate resistant *S. halepense* populations (Binimelis et al., 2009; de Carvalho et al., 2012).

The study of the resistance mechanisms to glyphosate and to ACCase inhibitors in this weed species offers us the unique opportunity to compare not only two species as previously, but two genera, *i.e. Amaranthus* spp. and *S. halepense.*

4.3.1 Biological Behaviour of the *S. halepense* Populations to Glyphosate

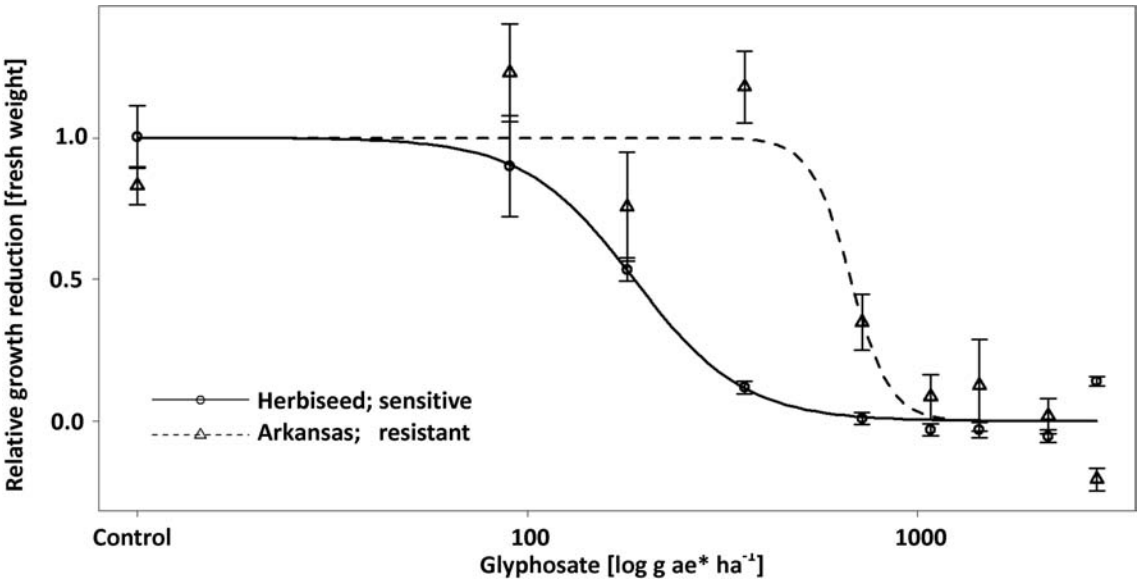

Fig. 35: Relative fresh weight accumulation of the *S. halepense* populations 14 DAT with increasing glyphosate doses rates (Roundup®UltraMax); GLY_R: ED_{50} 670 g ae ha^{-1}; GLY_S: ED_{50} 190 g ae ha^{-1}; resistance factor: 3.5; standard error indicated by error bars.

To determine the sensitivity of the *S. halepense* population GLY_R to glyphosate the fresh weight was assessed and compared to that of GLY_S at 14 DAT, following the first visible regrowth of treated plants. At 48 DAT a visual rating of the treated plants was performed to determine the effect on plant regrowth. The symptoms observed after glyphosate treatment were similar to those as described by Lorentz et al. (2011). An ED_{50} value of 190 g ae ha^{-1} was obtained for the sensitive population while the ED_{50} value for the resistant

population increased to 670 g ae ha^{-1} glyphosate under the conditions used in the test (Fig. 35). The resistance factor (RF) between both populations was calculated as 3.6 for the fresh weight assessments and approximately 4.0 for the visual ratings. At 48 DAT the sensitive population was completely controlled at rates above 1080 g ae ha^{-1} while the resistant population regrew at all glyphosate rates tested, up to 2880 g ae ha^{-1}. The well described G101, T102 and P106 target site mutations in the EPSPS protein sequence were not detected in any of the tested plants. Therefore point mutations were most probably not the cause of the observed resistance in this *S. halepense* population (Data not shown). However, a sustainable control of this population was no longer possible by glyphosate.

4.3.2 Sensitivity of *S. halepense* to other Herbicides

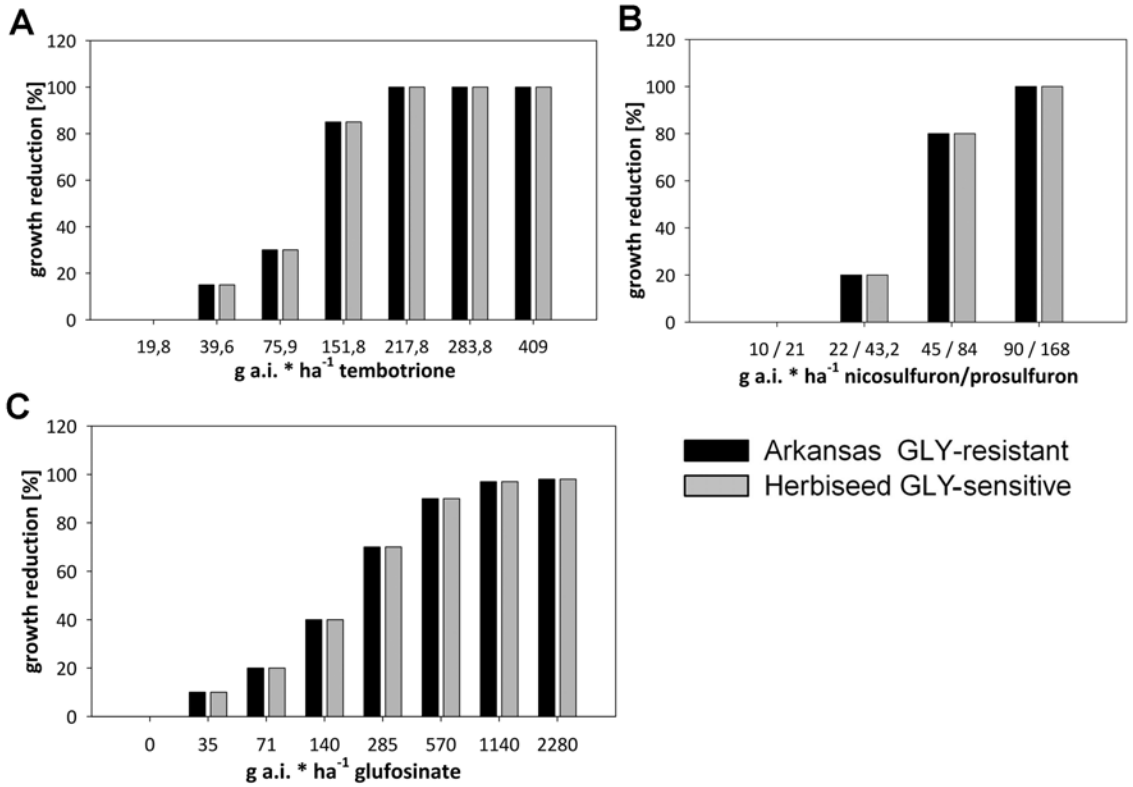

Fig. 36: Visual rating of *S. halepense* 14 DAT relative to untreated control; mature plants (BBCH 52) treated with increasing doses of tembotrione (**A**), nicosulfuron/prosulfuron (**B**) and glufosinate (**C**).

Alternative herbicide options to control the *S. halepense* population GLY_R were evaluated and ALS-, ACCase-, Glutamine synthetase- and HPPD inhibitors were applied. The GLY_R and GLY_S populations were still sensitive to a mixture of nico- and prosulfuron as representatives of the class of ALS inhibitors (HRAC, 2011). There were also no

differences between both *S. halepense* populations to tembotrione and glufosinate; both herbicides were able to effectively control them (Fig. 36). On the other hand ACCase inhibitors were found to poorly control the GLY_R population collected in Arkansas, therefore this population was found to be resistant to both glyphosate and to ACCase inhibitors.

4.3.3 Sensitivity to CHD and APP ACCase Inhibitors and the *ACCase* Zygosity

The different known mutation sites in the eucaryotic *ACCase* are not conferring the same degree of resistance to the different chemical classes of ACCase inhibitors (Powles & Yu, 2010). To evaluate this, APP and CHD ACCase inhibitors were applied to the *S. halepense* populations GLY_S & GLY_R. The plants were therefore treated with fluazifop, quizalofop and clethodim (Fig. 37 & 38).

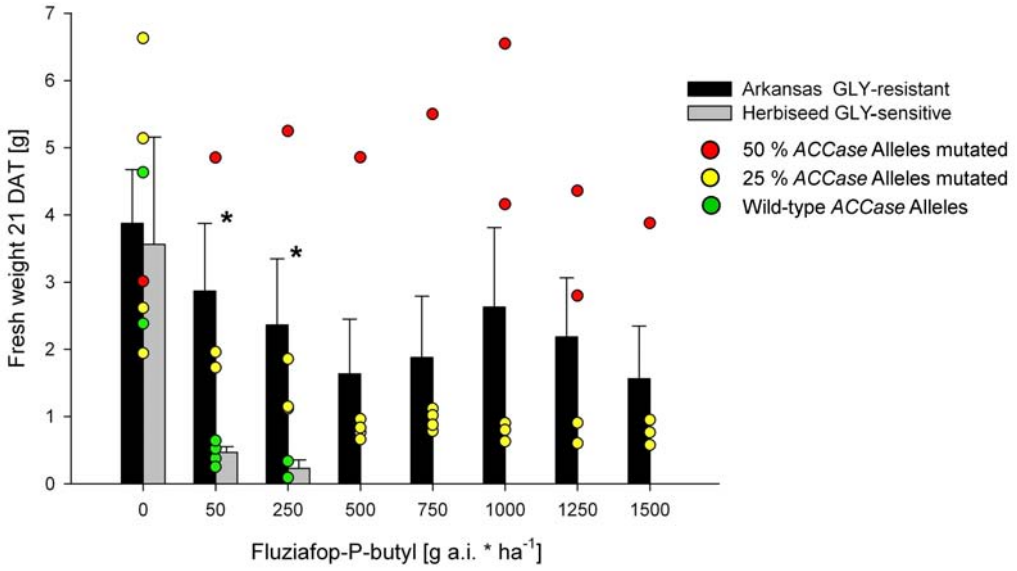

Fig. 37: Plant fresh weight of the glyphosate sensitive and resistant *S. halepense* populations GLY_R and GLY_S 21 DAT in g per tiller; mature plants treated with increasing dose rates of fluazifop-ρ-butyl; "*" indicates statistic significant differences (P = 0.05); colored dots indicate the mean fresh-weight per tiller of each single plant grouped by percentage of mutant *ACCase* alleles; the standard error is indicated by the errorbars.

Dose rates of 50 and 250 g a.i. ha^{-1} fluazifop and 10; 30; and 60 g a.i. ha^{-1} quizalofop showed a complete control of the sensitive plants. These plants were 21 DAT dry and dead and showed no resprouting from the rhizomes. However at these dose rates the effect of fluziafop and quizalofop differed significantly between the resistant and the sensitive

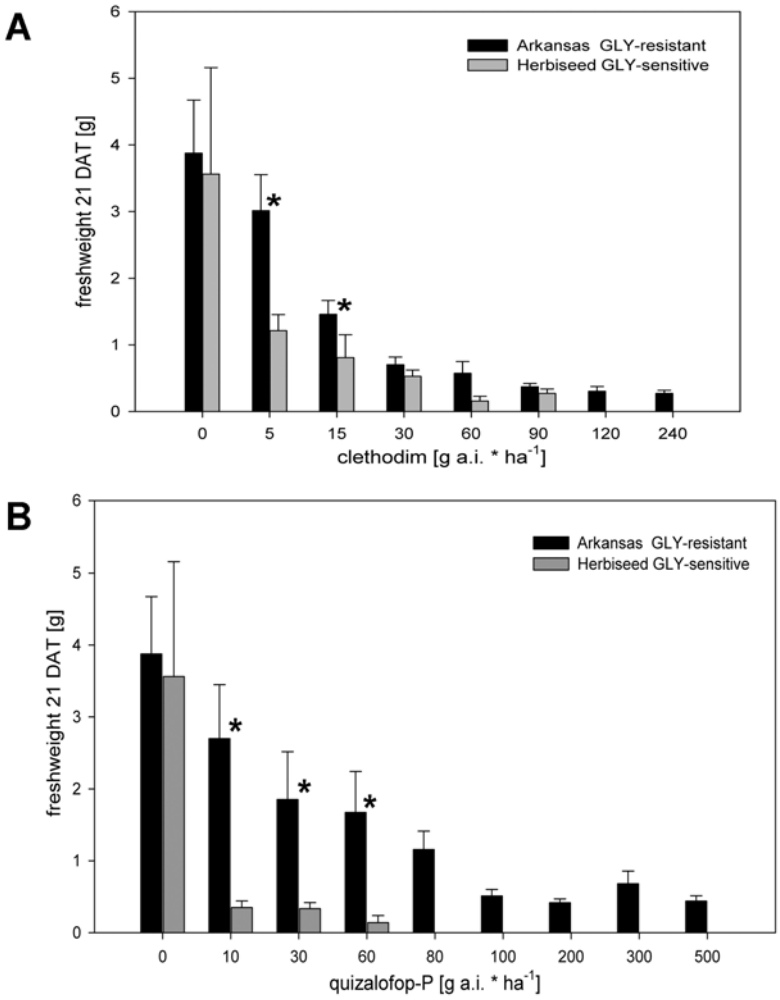

Fig. 38: Assessment of above ground fresh weight of plants of the *S. halepense* populations GLY_S & GLY_R 35 DAT; mature plants treated with increasing dose rates of Targa® (**B**) (quizalofop) and Select® (**A**) (clethodim); the standard error is indicated by the errorbars.

plants. The plants of the resistant population show at these dose rates only weak herbicidal injury symptoms but with variations in the intensity. The plants show in addition a viable regrowth from the rhizomes. The highest rate of 1500 g a.i. ha^{-1} fluazifop tested, gave also an incomplete control of the plants of the GLY_R population. A few plants at this dose were still alive bearing only weak herbicidal injury symptoms. Between plants treated with at 5 and 15 g a.i. ha^{-1} of clethodim significant differences were also observed between the *S. halepense* populations GLY_R and GLY_S. Especially at 5 g a.i. ha^{-1} the sensitive plants were strongly damaged but still alive, while the resistant plants showed only slight herbicide injury symptoms.

4.3.4 Determination of *ACCase* Target Site Mutation W2027C

Pyrosequencing analyses showed the presence of a mutation W2027C in the *ACCase* gene sequence of plants of the *S. halepense* population GLY_R. A single nucleotide exchange in the third position of the codon from guanosine to cytosine (TGG to TGC) (Fig. 39) is conferring resistance mainly to APP ACCase-inhibitors as reviewed by Powels & Yu (2010). *S. halepense* is described as tetraploid (Celarier, 1958). According to the pyrosequencing results we assume that *S. halepense* has 4 alleles of the eucaryotic *ACCase* gene in its genome. Therefore a total of 86 % of the individual *S. halepense* plants were found to bear at least one mutant allele (Tab. 18). In 30 % of the plants 2 mutated alleles were found, whereas in 56 % of them only one mutant allele was observed. Finally 14 % of the individuals were found to be sensitive with no mutation in ACCase amino acid position 2027. No individual plant was found to have more than 50 % of the *ACCase* alleles mutated.

These results were first proofed in the above described treatment of GLY_R with different

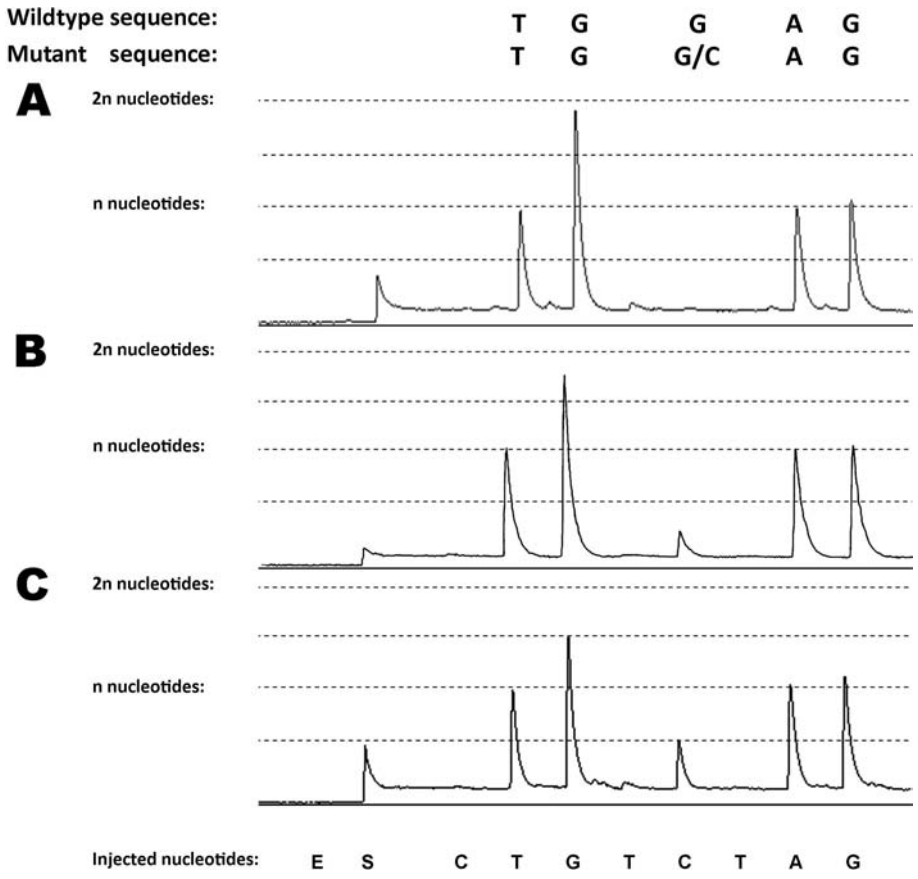

Fig. 39: Mutation site W2027C of the *S. halepense ACCase* gene; "A" pyrogram of the wild type *ACCase* gene; "B" pyrogram of a plant owning 25 % mutant and 75 % wild type *ACCase* gene sequence and "C" pyrogram *ACCase*, 50 % wild type and 50 % mutant genes.

Tab. 18: Distribution of the W2027C *ACCase* target site mutation in the S.halepense populations GLY_S and GLY_R; "S" indicates no mutation at position W2027C; "r" describes a single and "R" a plant bearing two W2027C resistant ACCase alleles.

		GLY_S Sensitive		GLY_R Resistant	
2027S	(G/G)	100 %	n = 17	14 %	n = 15
2027r	(G/C)	0 %	n = 0	56 %	n = 72
2027R	(C/C)	0 %	n = 0	30 %	n = 31

rates of fluazifop (Fig. 37). After the determination of the resistant allele frequency of each single plant, the evaluation of the herbicide effects showed that plants bearing 2 mutant alleles were even less damaged than plants bearing only a single mutant *ACCase* allele. The strongest damage was observed in sensitive plants, bearing no mutant allele. Additional *ACCase* mutations in position I1781, I2041, and G2096 known to confer resistance to ACCase inhibitors were not found in the plants of GLY_R (data not shown).

To confirm the influence of the number of alleles bearing the W2027C *ACCase* target

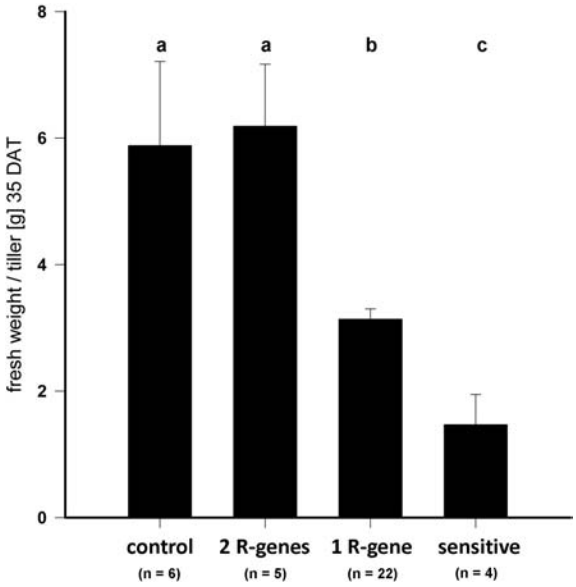

Fig. 40: Sensitivity of *S. halepense* plants bearing different numbers of W2027C resistant *ACCase* allels treated with 100 g a.i. ha^{-1} fluazifop-ρ-butyl compared to untreated control plants bearing no (n = 2), a single (n = 2) or two mutant (n = 2) *ACCase* alleles; letters indicate statistically significant differences between the different groups (P = 0.05). "n" indicates the number of mature plants tested; the standard error is indicated by the error bars.

site mutation, untreated plants were compared with plants bearing one or two resistant *ACCase* alleles or to sensitive, wild type plants, by the application of 100 g a.i. ha^{-1}

fluazifop. At that rate the sensitive plants were controlled 35 DAT, while resistant plants bearing 2 resistant *ACCase* alleles show only weak injury symptoms not affecting the overall plant growth. Significant differences in the fresh weight 35 DAT were not found between these highly resistant plants and the untreated control plants. Plants bearing one resistant *ACCase* allele showed after the fluazifop treatment stronger herbicide injury symptoms, significantly different (P = 0.05) from both, the untreated control and treated sensitive plants (Fig. 40).

The same results but with stronger herbicide injury symptoms were also visible in the plants treated with quizalofop (Fig. 38 B). The activity of CHD-ACCase inhibitors is known to be only weakly affected by the *ACCase* mutation W2027C as previously shown for the treatments with different dose rates of clethodim (Fig. 38 A). Therefore clethodim was still able to control both populations at rates of 30 g a.i. ha^{-1} and above. Field rates of clethodim and quizalofop showed therefore a growth reduction of more than 90 % (Fig. 38) for both the sensitive and the resistant population.

5 Discussion

In the decades prior to the commercialization of glyphosate resistant crops, glyphosate was used just before crop seeding for non-selective weed control. This practice was conducted for decades without any significant resistance development (Powles, 2008). Glyphosate has no soil activity and therefore exerts a short and intense selection pressure only on emerged plants, which some weeds have been able to circumvent by emerging throughout the entire growing season (Powles, 2008). A noticeable amount of individuals remained therefore unselected, reducing the glyphosate selection pressure on the whole weed population. Other weed control options like tillage, mowing or herbicides with other modes of action were often used additionally to diversify the selection pressure on the weed community enabling the use of glyphosate over decades without any obvious resistance problems (Powles, 2008). Finally, some researchers expressed the opinion that the evolution of glyphosate resistant weeds had a very low risk or even was practically not possible (Bradshaw et al., 1997).

With the introduction of glyphosate resistant crops (GRC), beginning in 1996, glyphosate became a selective herbicide in these corn, cotton and soybean cultivars. Their advantages for those cropping systems, simplicity, cost and convenience, unleashed the rapid adaptation of glyphosate resistant corn, cotton and soybean in the USA, with these crops representing up to 60 %, 91 % and 90 % of the total grown areas, respectively, in 2007 (Powles, 2008). Following the introduction of GRCs other selective herbicides, herbicide mixtures and mechanical weed control options were largely replaced by glyphosate (Owen, 2008). Consequently, the high reliance on glyphosate as the only weed control measure resulted in nearly uniform intense glyphosate selection pressure in large parts of U.S. corn, cotton and soybean agroecosystems that was unprecedented in scope. This allowed the development of glyphosate resistant weeds such as *Conyza canadensis*, *A. palmeri*, *A. tuberculatus* and *S. halepense* (Powles, 2008; Duke & Powles, 2008). The latter three weed species, their resistance mechanisms to glyhosate or ACCase inhibitors and the relationship among sensitive and resistant populations are presented in this work. Because of the severity of the current situation with these resistant species, it is therefore important to learn more about the mechanisms, the evolution and the dispersal of these weed resistance phenotypes to help preserve the utility of glyphosate and to protect the diversity of available chemical weed control options for a sustainable future food, feed and fiber production.

To our knowledge for the first time several glyphosate resistant *A. palmeri* and *A. tuberculatus* populations of various regions of the southeast U.S. cropping area have been compared with one another. In several of the populations, glyphosate resistance was either confirmed or has been described for the first time. The EPSPS activity and alterations in glyphosate absorption or translocation were also tested in some populations of both species. The results allowed to exclude these important resistance mechanisms as the cause of the

observed glyphosate resistance in these populations (Tab. 1). We were able to show that *EPSPS* gene amplification is not only a unique event in a single *A. palmeri* population. It also occurs in several *A. palmeri* and *A. tuberculatus* populations from the areas most affected by resistance but it is not the only glyphosate resistance mechanism found in both species. Increased *EPSPS* gene copy number is not the resistance mechanism of the *A. palmeri* population collected in Tennessee, TN_{LR}, other, as yet unknown mechanisms may be present. Finally, neutral markers were used as an approach to investigate the evolution and spread of glyphosate resistance in different populations with the aim of studying glyphosate resistance trait dispersal in common weed species of these agroecosystems.

5.1 Response to Glyphosate of the *A. palmeri* and *A. tuberculatus* Populations

Tab. 19: Glyphosate resistance of different weed species; GR_{50}, LD_{50} and resistance factors (RF) of different glyphosate resistant and sensitive species; a selection of values published in literature; determination based on "[1]": fresh weight; "[2]": mortality rate; "[3]": not mentioned as resistant; "[4]": measured at rosette stage; "[5]": mean values of replicates published.

Species	ED_{50} [g ae ha^{-1}]			Reference
	Sensitive	**Resistant**	**RF**	
A. palmeri	90	560	1.5 - 6.2[1;2]	Steckel et al., 2008; Culpepper et al., 2006
A. tuberculatus	120-240	620-2300	2.6 - 19.2[1;3]	Zelaya & Owen, 2005; Legleiter & Bradley, 2008
C. canadensis	340-530	1360-2110	4 - 4.7[2;4]	Zelaya et al., 2004; Dinelli et al., 2006
S. halepense	470-500	1263-2350	- [2]	Vila-Aiub et al., 2008; Riar et al., (2011)
L. multiflorum	44-290	408-1635	2.2-12.6[1;5]	Perez & Kogan, 2003; Jasieniuk et al. (2008)
L. rigidum	102-103	967	9.5[2]	Pratley et al., 1999
E. indica	231-322	492-1531	2.1-6.3 [1]	Ng et al., (2004a); Ng et al., (2004b)

It is the first time that the sensitivity of glyphosate resistant and sensitive *A. palmeri* and *A. tuberculatus* populations collected across the southeastern U.S. was compared (Tab. 2). The glyphosate sensitivity of the *A. palmeri* and *A. tuberculatus* populations was assessed by dose response studies. Since both species were not tested at the same time, it is more difficult to compare the absolute values of their response to glyphosate. Nevertheless, the most important agronomically relevant point is to compare the sensitive with the resistant

populations within each species and to characterize their response to glyphosate.
The shikimic acid and fresh weight data were fitted to the log-logistic dose response model (Seefeldt et al., 1995). For the data evaluation the 4 parameter sigmoidal and log-logistic model developed by Ritz & Streibig (2005) and Knezevic et al. (2007) was used to determine the ED_{50} values, the resistance factors and the graphical presentation of plant glyphosate response. A good calculation of the log-logistic dose response curves requires a wide range of doses, ideally covering rates causing no visible effect to rates providing complete plant control (Seefeldt et al., 1995). These requirements were fulfilled for *A. palmeri* dose response curves - Fig. 11 & 9 and *A. tuberculatus* dose response curves - Fig. 26 & 27, with the exception that the highest rates of 11560 g a ha^{-1} did not provide complete control of the tested resistant *A. palmeri* populations. Nevertheless, the measured plant response was sufficient to ensure meaningful results for the calculation of the ED_{50} values (Seefeldt et al., 1995).
The herbicide rate is not the only factor influencing plant growth in dose response studies. Environmental conditions and plant development stage at the time of treatment are also important factors. Technical questions like harvest time, nutrient supply or application quality are also important factors influencing the results. Variations among studies can generally be attributed to (1) different environmental conditions like humidity, UV-radiation or temperature at different test locations, (2) different growth or developmental stages and plant fitness at application time, (3) a poor coverage of the entire herbicide response range to determine ED_{50} values with certainty, (4) utilization of different dose response evaluation parameters like fresh weight, dry weight, mortality rate or visual assessments and (5) a poor coverage of plant or soil surface (Muzik & Mauldin, 1964; Hammerton, 1967; Ahmadi et al., 1980; McWorter, 1980; Seefeldt et al., 1995).
The comparison of selected *A. palmeri* and *A. tuberculatus* populations was performed in the same greenhouse in the dry climate of Ft. Collins, Colorado, U.S. at an elevation of 1525 m above sea level with a corresponding high solar radiation in early spring 2009. These environmental conditions enabled a comparison of common glyphosate response as indicated by the low variation in the ED_{50} values based on plant fresh weight of the sensitive *A. palmeri* and *A. tuberculatus* populations GA_S, NC_S, Co_1 and Mo_{18} with an ED_{50} value of 80.6 $\pm$ 13.6 g ae ha^{-1} glyphosate. Although, these populations are members of the 3 different *Amaranthus* ssp., *A. palmeri*, *A. tuberculatus* and *A. blitum*, the mean ED_{50} value of sensitive populations corresponds approximately to one-tenth of the recommended field rate of 720 g ae ha^{-1} glyphosate and underlines the success of glyphosate in controlling sensitive *Amaranthus* species in field (Tab. 10 & Tab. 16).
The sensitive *A. palmeri*, *A. tuberculatus* and the *A. blitum* populations were examined as a reference for the evaluation of resistant populations and to allow a comparison with published values of other populations. Culpepper et al. (2006) reported an ED_{50} value of 90 g ae ha^{-1} glyphosate in the sensitive *A. palmeri* population GA_S, which is in line

with our results. The same was found in a sensitive *A. tuberculatus* population collected in Barton County, Missouri with an ED_{50} value of 120 g ae ha^{-1} glyphosate compared to an ED_{50} value of 61 g ae ha^{-1} in Mo_{18} in the present work (Legleiter & Bradley, 2008). But the probable influence of natural or biological variations and the influence of environmental or test conditions has been described in literature. Patzoldt et al. (2002) compared 16 *A. tuberculatus* populations, randomly collected across Illinois, in a greenhouse study. They found that 75 % of these were at least 90 % controlled by application of 210 g ae ha^{-1} glyphosate. In contrast Zelaya & Owen (2005) obtained an ED_{50} value of 210 g ae ha^{-1} with glyphosate in a sensitive *A. tuberculatus* population. This difference shows the impact af the experimental conditions on the results of such studies.
With respect to the variation of published values our data are in the range of those described previously and the difference between resistant and sensitive populations was well established in the set of seeds analyzed and can be used to further characterize the glyphosate tolerance of the investigated populations.

Glyphosate Resistant *A. palmeri* Populations

Tab. 20: Comparison of GA_R glyphosate resistance between locations; ED_{50} and RF-values obtained in present work and by Culpepper et al. 2006.

Location	Culpepper et al., 2006		Present work	
	ED_{50} [g ae ha^{-1}]	R/S ratio	ED_{50} [g ae ha^{-1}]	R/S ratio
field	6100	-	-	-
greenhouse	560	6.2	2000	22.7

Dose response experiments were conducted for 5 different *A. palmeri* populations with suspected glyphosate resistance: TN_{LR}, NC_{LR}, NC_R, NC_{R1} and GA_R. The resistance factors were determined in relation to the ED_{50} value of the most sensitive population GA_S. The GA_R, NC_{R1} and NC_R populations were found to be highly resistant to glyphosate with a resistance factor (RF) of more than 10 in comparison to the sensitive population (Fig. 9). Whereas the glyphosate resistance in TN_{LR} was less pronounced with an RF of 1.8, but is was even stronger than the resistance found in NC_{LR}, RF of 1.1, which was harvested at a site located between the NC_S and NC_{R1} collection sites. NC_{LR} was described to be inconsistent with its glyphosate response but in the dose response study it approved to be sensitive and the findings are discussed further in chapter 5.6.
The glyphosate resistant *A. palmeri* population GA_R is the result of crossing between highly resistant plants of the first glyphosate resistant *A. palmeri* population found in Georgia done by Culpepper and his collaborators (Culpepper et al., 2006). The field pop-

ulation was controlled 82 % by an application of 10 kg ae ha^{-1} glyphosate. The calculated ED_{50} value was 6.1 kg ae ha^{-1} glyphosate (visual assessment). In a greenhouse experiment they found an ED_{50} value of 560 g ae ha^{-1} glyphosate, corresponding to a resistance factor of RF: 6.2 (Tab. 20). The ten-fold difference between the field and greenhouse experiment ED_{50} values underlines the high level of glyphosate resistance of the NC_R, NC_{R1} and GA_R populations tested in the present study. In one particularly resistant population, an ED_{50} value of 2.0 kg ae ha^{-1} glyphosate and a resistance factor of RF: 22.7 was found in (GA_R). Despite the similarities in the responses between the sensitive populations, the GA_R population is 3.7-fold more resistant than the parental strain used by Culpepper et al. (2006). This higher resistance level might be explained by the selection of highly resistant parental plants used to produce GA_R population. The same effect can be found among the NC_R and NC_{R1} populations. Highly resistant plants of the population NC_{R1} population, showing an ED_{50} of 924 g ae ha^{-1} for glyphosate, were crossed to produce the NC_R population with an ED_{50} of 1416 g ae ha^{-1} to glyphosate a 1.5-fold increase. Thus, the ED_{50} values of the three different glyphosate resistant *A. palmeri* populations GA_R, NC_R and NC_{R1} are well above the recommended field rate of 720 g ae ha^{-1} and therefore no longer controlled by labeled glyphosate rates in field (page 24).

The TN_{LR} population was described by Steckel et al. (2008) as having low level resistance with a RF of 5. The ED_{50} value was not mentioned. An ED_{50} of 156 g ae ha^{-1} for glyphosate - RF 1.8 - was obtained in the present work for the TN_{LR} population. Since the response of TN_{LR} to glyphosate was significantly different from the sensitive populations GA_S and NC_S, this population was classified as having weak glyphosate resistance. Indeed an increased *EPSPS* gene copy number was not found in this population. Steckel et al. (2008) also observed that "The plants wilted and displayed typical chlorosis, and then regained turgor and resprouted from secondary points of growth". These symptoms were only occasionally observed under our experimental conditions. Nevertheless, it indicates the potential for a different glyphosate resistance mechanism in this population. Indeed EPSPS gene amplification was not found in this population, whereas it plays a major role in the glyphosate resistance of the other tested *A. palmeri* populations (Fig. 16). The resistance mechanism in the TN_{LR} population might lead to higher variability in glyphosate response, depending on the environmental conditions. Glyphosate sequestration the resistance mechanism found in *Conyza canadensis* is according to Ge et al. 2011 strongly temperature dependent. Since glyphosate phytotoxicity might, next to the shikimic acid pathway, also to be linked to the decay of vascular tissues and of pith the surviving of meristematic tissues surrounding the vascular bundles could also be responsible for the TN_{LR} glyphosate resistance (Lorentz et al., 2011). This would also provide an explanation for the atypical symptoms after glyphosate treatment observed by Steckel et al. (2008). No evidence for alterations of the meristematic tissues or alterations in glyphosate translocation in relation to sensitive plants were not found in the TN_{LR} population. How-

ever, the presence of different resistance mechanisms within this weed species provides *A. palmeri* with an opportunity to adapt further to glyphosate resistance through passing on resistance traits through genetic exchange between populations.
The *A. palmeri* RF of 22.7 in GA_R, 10.3 in NC_{R1} and 15.7 in NC_R, Tab. 10, are higher than the values published for most other glyphosate resistant species including *Eleusine indica*, *Lolium rigidum*, *L. multiflorum*, *Sorghum halepense*, *Ambrosia artemisiifolia* or *Conyza canadensis* (Feng et al., 2004; Perez et al., 2004; Wakelin et al., 2004; Koger & Reddy, 2005; Michitte et al., 2007; Perez-Jones et al., 2007; Dinelli et al., 2008; Preston & Wakelin, 2008; Shaner, 2009; Ge et al., 2010; Riar et al., 2011). The exceptions are the high resistance factor published by Legleiter & Bradley (2008) for *A. tuberculatus* and *Conyza canadensis* by VanGessel (2001), RF 19.2 and RF 13, respectively. The high resistance factor found in *A. palmeri* populations is not only the result of the high level of glyphosate resistance in the NC_R, GA_R and NC_{R1} populations, it is also partially the result of the high degree of sensitivity of NC_S and GA_S to glyphosate (ED_{50} average 80.6 $\pm$ 13.6 g ae ha^{-1}). Nevertheless, the glyphosate RF resulting from *EPSPS* gene amplification is thus far higher than those reported for other weeds showing different glyphosate resistance mechanisms (Tab. 19).

Glyphosate Resistance of the *A. tuberculatus* Populations

Glyphosate resistance was also confirmed in the tested *A. tuberculatus* populations IL_1, Mo_{13}, Mo_{14}, Mo_{15} Mo_{16} and Mo_{17}, but it was at a lower level than found in *A. palmeri* (Tab. 16). The average ED_{50} value of resistant populations was 396 g ae ha^{-1} glyphosate, while the average RF was 6.5. This value is lower than the values published by Legleiter & Bradley (2008) for the same populations. In our study the ED_{50} value of Mo_{13} was 317 g ae ha^{-1} glyphosate in comparison to 2300 g ae ha^{-1} glyphosate found by Legleiter & Bradley (2008). Also the resulting RF of 5.2 is lower than their published value of 19.2. The sensitive population Mo_{18} used in our studies, with an ED_{50} of 61 g ae ha^{-1} was twofold more sensitive to glyphosate than the population used by Legleiter & Bradley (2008) with an ED_{50} of 120 g ae ha^{-1}. The differences in response to glyphosate could be partially based on the environmental conditions, which can result in stronger activity on the sensitive population. Legleiter & Bradley, (2008) treated 15 cm tall plants in their dose response study but did not describe the BBCH stage or a comparable measurement unit of the plants. We also found that the growth rate of *A. tuberculatus* plants is strongly influenced by the environmental conditions. Well watered and fertilized plants, exposed to weak light, will show rapid growth that are etiolated and are easy to kill with herbicides, whereas well watered and fertilized plants exposed to a high light intensity will show a more compact growth and require higher rates of herbicide to kill. Therefore a

more detailed description of the experimental conditions and the developmental stage of the treated plants would have greatly helped to compare the results between the different studies.
Nevertheless, with an ED_{90} value of 485 - 2969 g ae ha^{-1} the populations Mo_{13}, Mo_{14}, Mo_{15}, Mo_{16}, Mo_{17} and IL_1 survive the recommended field rate of 720 g ae ha^{-1} glyphosate and will be able to set viable seeds (Tab. 16). An ongoing glyphosate selection pressure will most probably provoke the further development of higher resistance factors in *A. tuberculatus*.

5.1.1 Fitness Costs and *A. palmeri* and *A. tuberculatus* Dose Response Studies

The fitness cost of herbicide resistance traits are an important factor contributing to the longevity of the trait within weed populations. Fitness costs are difficult to measure and are strongly influenced by the biotic, abiotic and genetic environment (Vila-Aiub et al., 2009). Important contributors to plant fitness are plant growth characteristics and sensitivity to pathogens. The claims are sometimes contradictory. According to Johal & Huber (2009) teatments with glyphosate result in a higher plant susceptibility towards biotic infections. On the other hand Velini et al. (2008) observed a stimulation of plant growth at low glyphosate rates, generally known as hormesis effects, in *Glycine max*, *Commelia benghalensis*, *Eucalyptus grandis* and *Pinus caribea* plants treated with 10 and 30 g ae ha^{-1} glyphosate. Increased growth of root, stem and leaf, but also the overall increase in fresh weight were observed. Thus an important question is whether resistance can indirectly contribute competitiveness of a population through enhanced plant growth. One important aspect to be clarified in the glyphosate resistant populations tested herein is, whether glyphosate induces an hormesis effect and if so, whether the rate approaches labeled glyphosate field rates. Hormesis would have a negative impact on the inter-specie weed-crop competition if this phenomenon would occur in resistant populations at dose rates close to labeled field rates. Further work is necessary to determine this effect in the investigated *A. palmeri*, *A. tuberculatus* or *A. blitum* populations. The dose range just below the onset of glyphosate injury should be adequately tested with sufficient glyphosate dose rates to determine this effect properly in highly heterogeneous field populations.

5.2 The Rise of Shikimic Acid in Treated Plants

Glyphosate acts on EPSPS, the sixth enzyme in the plant shikimic acid pathway. The shikimic acid pathway produces the precursors of the aromatic amino acids and many secondary aromatic metabolites (McCue & Conn, 1990; Weaver & Herrmann, 1997;

Herrmann & Weaver, 1999). Therefore EPSPS is indirectly involved in plant stress response (Chaves et al., 2011; Sánchez-Rodríguez et al., 2011). EPSPS is most active in the youngest and actively growing plant parts. It converts S-3-P together with PEP into EPSP, a precursor in the formation of aromatic amino acids. Due to a missing feedback inhibition at this pathway step, the S-3-P concentration in plant increases. An increase of shikimic acid concentration is generally observed and is to the best of our knowledge only observed after treatment with glyphosate or related compounds (Metraux, 2002). With its energy rich bonds, the phosphate group S-3-P is rather unstable in plant cells and is rapidly degraded into shikimic acid. Shikimic acid can be detected in plant extracts by a colour reaction method and can be used as an early indicator of glyphosate activity in plants (Singh & Shaner, 1998; Cromartie & Polge, 2000; Pline et al., 2002). Plants resistant to glyphosate can therefore be characterized by analyzing its shikimic acid content (Singh & Shaner, 1998; Koger et al., 2005). The highest shikimic acid increase can be measured after glyphosate treatment in the most active growing plant parts, particularly in the meristematic tissues (Shaner et al., 2005). According to Harring et al., (1998) the highest shikimic acid content was found in leaf tissue between 4 and 5 DAT and then decreased. The same was found by Huangfu et al. (2007) in *Brassica juncea* plants treated with 169 g ae ha^{-1} glyphosate, whereas in other studies plants treated with higher glyphosate dose rates had a steady shikimic acid increase over the test period (Huangfu et al., 2007; Jasieniuk et al., 2008). The shikimic acid content in plant tissues after glyphosate treatment is a readily available diagnostic tool that can be used to classify and characterize sensitive and resistant weed populations.

To compare the consequences of a glyphosate treatment in *A. palmeri* the shikimic acid content throughout the entire plant was first compared between glyphosate treated and untreated plants (Fig. 10). The highest shikimic acid increase in untreated plants was found in the shoot tip and the youngest leaf and decreased with the age of plant tissue. Shikimic acid concentrations 4 DAT allowed a clear separation of treated and untreated plants as shown in Fig. 10. Up to 4 μg shikimic acid per mg plant fresh weight was found in the analyzed tissue of treated plants, which is comparable to the maximum concentrations found by Mueller et al., (2003) in *Conyza canadensis*. Therefore, the youngest fully expanded leaf was chosen to assess and characterize glyphosate effects in *A. palmeri* and *A. tuberculatus* plants.

The shikimic acid content in plant tissue has been determined either in excised leaf discs incubated in solutions containing glyphosate or by direct measurement of shikimic acid concentrations in glyphosate treated plants, including *C. canadensis*, *Ambrosia artemisiifolia*, *Brassica juncea* and *Lolium* spp. (Singh & Shaner, 1998; Mueller et al., 2003; Koger et al., 2005; Shaner et al., 2005; Culpepper et al., 2006; Huangfu et al., 2007; Perez-Jones et al., 2007; Jasieniuk et al., 2008; Steckel et al., 2008; Brewer & Oliver, 2009; Yu et al., 2009; Gaines et al., 2010). The investigators were all able to distinguish

between resistant and sensitive plants by shikimic acid measurements. The same results were obtained in this work for *A. palmeri* in Fig. 11 and for *A. tuberculatus* in Fig. 26. Sensitive plants show a shikimic acid increase at low glyphosate doses rates, whereas resistant plants have to be treated with higher rates to show the same shikimic acid increase as in sensitive plants. Culpepper et al. (2006) incubated excised leaf discs of glyphosate sensitive and resistant parental populations of GA_S and GA_R in glyphosate solutions. They found a good correlation between increasing glyphosate dose rates and shikimic acid content in leaf discs of sensitive but not in resistant plants. Gaines et al. (2010) later discovered the glyphosate resistance mechanism based on *EPSPS* gene amplification in this same population. They found the *EPSPS* gene copies were amplified in glyphosate resistant plants by up to 160 times. The protein pool of cell in these plants was also found by immunoblotting and enzyme activity measurements to contain a higher EPSPS enzyme concentration. The tolerance of plants o glyphosate was tested by measuring the shikimic acid concentration in excised leaf discs that had been incubated in glyphosate solutions. Even in leaf discs of plants bearing 4 relative genomic *EPSPS* copies, only a slight increase of shikimic acid concentration of 250 μM glyphosate was detected. Plants containing between 4 and 160 relative *EPSPS* gene copies were tested and compared to sensitive plants, containing a single relative *EPSPS* gene copy. The data in Fig. 11 show a shikimic acid increase at low glyphosate dose rates in GA_S, but not in GA_R. The shikimic acid content in GA_R began to increase at dose rates where GA_S had already reached the highest detected shikimic acid content. These results indicate that even an amplified *EPSPS* can be inhibited depending on the applied glyphosate rate. The results in the work herein are comparable to the results found by Culpepper et al. (2006) and Gaines et al., (2010) but are based upon a wider range of doses. Steckel et al. (2008) determined the shikimic acid content in the entire above ground biomass of sensitive in comparison to resistant TN_{LR} plants. They found an equivalent shikimic acid increase in both at a dose rate of 840 g ae ha^{-1} glyphosate. The results presented in this work show the same picture at dose rates above 360 g ae ha^{-1} glyphosate *e.g.* in TN_{LR} and NC_S. Thus, since higher glyphosate concentrations were used by Steckel et al. (2008), the discriminating, lower dose rates were probably missed.

The data presented in this study confirm previous findings that the shikimic acid content at 4 DAT can be used to distinguish between sensitive and resistant *A. palmeri* and *A. tuberculatus* plants. On the other hand they also suggested that this assay gave an early and reliable approximation of ED_{50} values in relation to the fresh weight data obtained at 16 or 19 DAT (Tab. 10 & 16). These results corroborate those by Harring et al. (1998) who evaluated the activity of different glyphosate formulations in plants by comparing the shikimic acid content in plant tissue 5 and 48 hour after treatment (HAT) to visual plant death assessment 14 DAT. Our data supports their proposal of using shikimic acid content to give an early approximation of glyphosate efficacy, especially considering that the plant

shikimic acid content seems not to be induced by other stresses or compounds other than glyphosate and closely related compounds (Metraux, 2002).

5.3 Absorption & Translocation of ^{14}C-Glyphosate

Glyphosate is amphimobile in plants and is highly translocated from the site of application through the phloem and xylem to shoot and root sink tissues, following the plant carbon flow. This behavior helps to explain the good activity of the herbicide (Dewey & Appleby, 1983; Preston & Wakelin, 2008). The target of glyphosate is the EPSPS enzyme, which is mostly expressed and active in the meristematic and actively growing plant tissues (Weaver & Hermann, 1997). These tissues are also the main sink tissues for the carbon flow of non-fruiting plants. Alterations in glyphosate absorption and translocation throughout the entire plant, play an important role in the glyphosate resistance of several plant species as reviewed by Shaner (2009) and described for *Conyza canadensis*, *C. bonariensis*, *Lolium multiflorum*, *L. rigidum*, *Digitaria insularis* and *Sorghum halepense* (Feng et al., 2004; Perez et al., 2004; Wakelin et al., 2004; Koger & Reddy, 2005; Michitte et al., 2007; Perez-Jones et al., 2007; Dinelli et al., 2008; Preston & Wakelin, 2008; Shaner, 2009; Ge et al., 2010; de Carvalho et al., 2012; Riar et al., 2011).

5.3.1 Glyphosate Uptake

Glyphosate resistance due to altered uptake rates is present in fewer weed populations than resistance based on altered translocation. Glyphosate resistance described in a *Lolium multiflorum* population was found to be partly based on this mechanism (Michitte et al., 2007). Despite these findings, differences in absorption were not observed between resistant and sensitive *A. palmeri*, Fig. 12, or *A. tuberculatus*, Fig. 28, populations within the first 48 HAT. Glyphosate resistant and sensitive *A. palmeri* populations NC_S and NC_R absorbed 38.8 % and 42 % of the applied radioactivity, respectively, during the first 48 HAT. Glyphosate resistant *A. tuberculatus* populations IL_1, Mo_{13} and Mo_{18} absorbed 49 %, 54 % and 41 % of applied radioactivity, respectively, during the first 48 HAT. Culpepper et al. (2006) found 31.2 % and 36.4 % absorption in the parental populations of GA_S and GA_R and Li et al. (2005) found 53 % uptake during the first 50 HAT in an *A. tuberculatus* population. The absorption rates obtained in the present work are comparable to those described earlier, especially with respect to variations in the environmental conditions. Furthermore the observed absorption rate seems to be within the common range of rates of plant glyphosate uptake, as for example that found in *C. canadensis* (Koger & Reddy, 2005). Cruz-Hipolito et al. (2009) studied glyphosate resistance in the South American weed species *Canavalia ensiformis* and showed the potential environmental im-

pact on glyphosate uptake rates. In their studies they reached maximum uptake rates of around 93 % of applied radioactivity 48 HAT in *Amaranthus hybridus* plants grown solely in climate chambers. Thus, the environmental conditions used herein, were adequate to test ^{14}C-glyphosate uptake in both species and likely reflect glyphosate absorption rates in field. Our data clearly demonstrated that glyphosate resistance is not based on this mechanism in the investigated *A. palmeri* and *A. tuberculatus* populations.

5.3.2 Translocation of Glyphosate

Alterations in the translocation of glyphosate have been found to be the main mechanism contributing to the glyphosate resistance in certain populations of *C. canadensis*, *S. halepense*, *L. multiflorum* and *L. rigidum* (Feng et al., 2004; Wakelin et al., 2004; Michitte et al., 2007; Perez-Jones et al., 2007; Riar et al., 2011). This phenomenon is, at least in *C. canadensis*, most probably caused by temperature dependent glyphosate sequestration into vacuoles (Ge et al., 2010; Ge et al., 2011).
Since alterations in glyphosate translocation are a common glyphosate resistance mechanism in some weeds, its translocation pattern were checked in further glyphosate resistant *A. palmeri* and *A. tuberculatus* populations (Shaner, 2009). They attempted to clarify, (1) whether translocation changes contribute to glyphosate resistance in other *A. palmeri* populations originating elsewhere from GA_R and (2) whether this mechanism contributes to the glyphosate resistance of *A. tuberculatus* populations. The translocation of ^{14}C labeled glyphosate was observed throughout the entire plant.
No spatial and temporal differences in the glyphosate translocation were observed in *A. palmeri* populations in Fig. 13 & 14 and *A. tuberculatus* populations in Fig. 29 & 30 until 48 HAT when the compound reached all plant tissues. The only differences found were after long incubation times following observation of the first herbicidal injury symptoms in sensitive populations. Therefore these differences are most probably caused by herbicide injury and are not the reason for resistance.
Glyphosate translocation changes within a single plant organ were intensively tested in the *A. palmeri* and *A. tuberculatus* populations. Glyphosate translocation was qualitatively visualized by autoradiograms. Different glyphosate translocation patterns were not detected between resistant and sensitive *A. palmeri* populations NC_R and NC_S, in Fig. 15 nor were differences found between resistant and sensitive *A. tuberculatus* populations IL_1, Mo_{13} and Mo_{18} (Fig. 31). The rate of transport and the main glyphosate sink in plant were the same in glyphosate resistant and sensitive plants. Differences in uptake or translocation like those described in resistant *C. canadensis*, *S. halepense*, *Lolium multiflorum* and *L. rigidum* populations were not found. The glyphosate translocation found herein approached the same rate and went to the same sink as described in other plant

species or populations by Li et al. (2005), Cruz-Hipolito et al. (2009) or by Lorentz et al. (2011).

The resistance in the investigated *Amaranthus* populations was therefore not based on significant differences in glyphosate uptake or translocation under the tested conditions. These result raises the question whether the *EPSPS* gene was modified in the resistant *A. palmeri* or *A. tuberculatus* plants.

5.4 EPSPS Gene Amplification

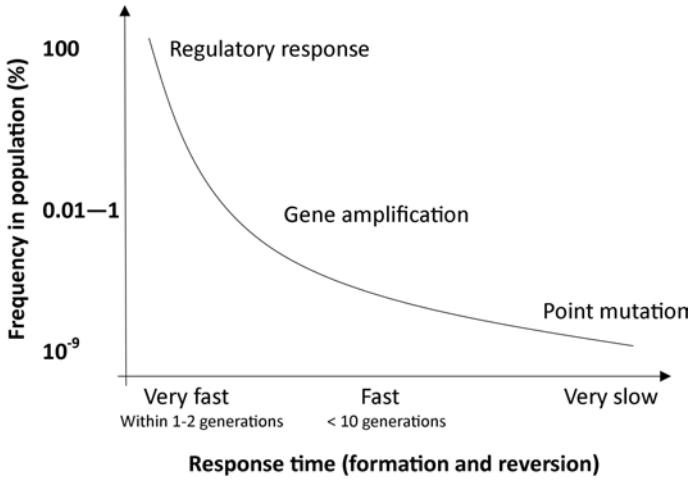

Fig. 41: Frequency of point mutations, gene amplification and regulatory response to selection pressure in a population; model based on the example of the bacteria *Salmonella typhimurium* according to Sandegreen & Andersson (2009).

"Gene amplification is found in all three kingdoms of life (Sandegreen & Andersson, 2009)" and is an important contributor to genomic variability, caused *e.g.* by transposons (Li et al., 2007; Sandegreen & Andersson, 2009). Resistance against many stresses is often caused or associated with gene amplification. Powdery mildew resistance in barley and several cases of insecticide resistance are examples of gene amplification mediated stress response (Hemingway & Ranson, 2000; Piffanelli et al., 2004). Gene amplification is also a significant contributor to the rising problem of antibiotic resistance in bacteria (Sun et al., 2009; Watanabe et al., 2011; Sandegreen & Andersson, 2009). In bacteria, the wide spread development of antibiotic resistance has been well studied in the recent years and can be probably used as a prediction model of resistance development in higher organisms. The typical bacterial adaptation towards selection pressure appears following two steps (Sandegreen & Andersson, 2009). The first evolved resistance mechanism leads to reduced bacterial fitness, followed by an adaptation to compensate the fitness cost. This compensation can be due to gene amplification of unregulated target genes. The occur-

rence of gene duplications was estimated in *Salmonella typhimurium* to be between 10^{-2} to 10^{-5} events per cell and generation depending on the amplified fragment (Fig. 41). Gene amplification can occur in all chromosome regions, with size ranging from a few kilobases to several megabases. A correlation of the amplified fragment size and fitness cost in bacteria was not found. In addition, target gene amplification increases the probability of the occurrence of mutations, which might confer strong target site resistance. Finally, gene amplification in bacteria is mostly unstable over the long term and has to be stabilized through ongoing selection pressure (Sandegreen & Andersson, 2010).

Despite the early detection of the importance of gene amplification in conferring insecticide or antibiotic resistance, the amplification of the *EPSPS* gene in a single *A. palmeri* population was the first case of a naturally occurring herbicide resistance induced by this mechanism (Gaines et al., 2010). Up to 160 times more *EPSPS* gene copies were found in the genome of resistant in comparison to sensitive plants. Mutations in the *EPSPS* mRNA sequence were not detected (Gaines et al., 2010). The *EPSPS* transcript abundance in untreated resistant plants is positively correlated with the genomic *EPSPS* gene copy number, indicating no significant gene silencing of amplified genes. Amplified *EPSPS* genes are also actively translated into protein (Gaines et al., 2010). Glyphosate resistance based on gene amplification has been found in *in vitro* cell cultures reared on glyphosate containing media, in *Daucus carota* L., *Petunia hybrida*, *Nicotiana tabacum*, *Glycine max* and *Medicago sativa* (Nafziger et al., 1984; Shah et al., 1986; Goldsbrough et al., 1990; Widholm et al., 2001). In addition, a higher level of EPSPS activity was found in *Corydalis sempervirens*, probably caused by a higher *EPSPS* expression or reduced EPSPS enzyme turnover (Smart et al., 1985).

Before this study, *EPSPS* gene amplification has only been found in the single glyphosate resistant population GA_R. To test if *EPSPS* gene amplification is a wide-spread glyphosate resistance mechanism in *A. palmeri* populations, the genomic *EPSPS* gene copy number was determined in different glyphosate resistant populations. *A. palmeri* and *A. tuberculatus* are closely related plant species, and are able to produce fertile crosses albeit at very low frequencies, therefore this resistance mechanism was also studied in the tested *A. tuberculatus* populations (Steinau et al., 2003). In addition, *EPSPS* expression and enzyme activity were measured in order to determine influence of the *EPSPS* gene amplification on the glyphosate resistance of various *A. palmeri* and *A. tuberculatus* populations.

5.4.1 *EPSPS* in *A. palmeri*

The resistance factor (RF) found in the *A. palmeri* population GA_R demonstrated an RF of 22.7 and 21.7, either by fresh weights 16 DAT or shikimic acid content measured 4 DAT, respectively, in comparison to the sensitive population GA_S (Tab. 10). Glyphosate sensi-

tive individuals of GA_S and NC_S contained the same number of *EPSPS* and *ALS* genes (reference gene). The sensitive individuals of GA_S contained an average of 1.2 ± 0.5 *EPSPS* copies relative to the *ALS* gene, whereas the *EPSPS* gene in the glyphosate resistant individuals of GA_R was 30 to 196 times amplified relatively to the *ALS* gene. These plants contained an average of 65 ± 49 relative *EPSPS* gene copies, as measured by quantitative or real time PCR. Real-time PCR is described as the best method to measure the gene copy number in genomes (Sandegreen & Andersson, 2009).

These data corroborate the level of *EPSPS* gene amplification found in the glyphosate resistant *A. palmeri* population GA_R found by Gaines et al. (2010). *EPSPS* gene amplification was also found in the glyphosate resistant populations NC_R ($RF_{freshweight}$: 15.8) and NC_{R1} ($RF_{freshweight}$: 10.3). NC_R contained an average of 45 ± 11 relative *EPSPS* gene copies, while NC_{R1} contained in average 51 ± 18.3 relative *EPSPS* gene copies (Fig. 16). *EPSPS* gene amplification is also present in the glyphosate resistant population AR_R collected in Arkansas, with an average of 11.5 ± 12.1 more relative *EPSPS* gene copies and in the population collected in Tennessee, TN_R with in average of 65.6 ± 32.7 relative *EPSPS* gene copies. Both populations are described to be glyphosate resistant in field (Fig. 16).

All *A. palmeri* populations were sampled across the Southeastern U.S. and the results indicate, that *EPSPS* gene amplification was the most common glyphosate resistance mechanism found in these populations. Nevertheless, gene amplification is not the only glyphosate resistance mechanism in *A. palmeri*. The population TN_{LR} has a weak glyphosate resistance ($RF_{freshweight}$: 1.8) but no significant increase in the *EPSPS* gene copy number. This population has an average of 1.3 ± 0.12 *EPSPS* genes in comparison to the *ALS* gene. Significant differences in *EPSPS* gene copy number with the glyphosate sensitive populations GA_S and NC_S were not found.

Gaines et al. (2010) found in GA_R that the expressed level of *EPSPS* genes in plant corresponds to the number of *EPSPS* gene copies present in the genomic sequence. In NC_R it was possible to show that there are also no differences in the *EPSPS* expression among glyphosate treated and untreated plants at different time points after treatment (Fig. 19 & 19). This indicates that glyphosate is not able to induce or to silence significant numbers of the *EPSPS* gene copies neither in the resistant nor in the sensitive *A. palmeri* plants. These results are comparable to the EPSPS gene expression data found by Sammons et al., (2011).

Southern blot analyses, confirmed by a higher signal intensity the *EPSPS* gene amplification, and shows that the obtained *EPSPS* fragment has an equal size in both the resistant and the sensitive plants (Fig. 18). In all Southern blots the *EPSPS* of sensitive individuals was only barely visible, as similarly described by Widholm et al. (2001) and Gaines et al. (2010). This is probably due to the weak emitted signal of the short probe.

The single signal in the Southern blots of resistant plants indicates no length differences

between amplified EPSPS sequences within a resistant individual. The equal fragment size of resistant and sensitive plants indicates a comparable length and structure between the *EPSPS* of the sensitive and resistant individuals.
However, these results clearly show the importance of EPSPS gene amplification for the *A. palmeri* glyphosate resistance in the Southeast U.S.. Nevertheless, other glyphosate resistance mechanisms in *A. palmeri* exist as shown in the population TN_{LR}.
This raises the question about the development of EPSPS gene amplification. *EPSPS* is scattered throughout the whole genome and present on several chromosomes of *A. palmeri* (Gaines et al., 2010). The same results could be obtained by rolling circle gene amplification as a mechanism used by some plant viruses and transposons like gemini viruses or *Helitron* transposons, which might be interesting to follow up with further work (Gutierrez, 1999; Kapitonov & Jurka, 2001; Piffanelli et al., 2004; Watanabe et al., 2011), especially since gene amplification is an important response of organisms to environmental stress (Naito et al., 2009; Sandegreen & Andersson, 2010). Gene amplification is thus one of the main mechanisms of evolution and adaptation to environmental stresses in pro- and eukaryotes. It is estimated that the gene birth and death frequency is approximately 10 - 40 % less per locus than nucleotide substitutions per nucleotide (Demuth & Hahn, 2009). Naito et al.(2009) recently found a stress inducible transposon in rice which shows a higher preference for intron than for exon sites in the genome. This preference will greatly reduce the detrimental effects of gene amplification for affected organisms (Naito et al., 2009). If such an transposon would by chance be glyphosate or at a minimum be stress-inducible, gene amplification would have an only modest impact on plant fitness without selection pressure, especially considering that amplification itself has low fitness costs (Sandegreen & Andersson, 2009). Antibiotic resistance in bacteria might be a simple but useful model for glyphosate resistance based on EPSPS gene amplification. Hence, within a few generations, gene amplification could be rapidly removed out of the population in the absence of any selection pressure, but this will most probably not solve the problem of gene amplification mediated glyphosate resistance, if the ability for EPSPS gene amplification is still present within the population. This thought raises again a key question related to the resistance mechanism: is it the EPSPS gene amplification itself or is it solely the ability for EPSPS gene amplification the most important?

5.4.2 *EPSPS* in *A. tuberculatus*

The *A. tuberculatus* populations Mo_{13}, Mo_{14}, Mo_{15}, Mo_{16}, Mo_{17} and IL_1 were found to be resistant to glyphosate in the dose response study performed. In comparison to glyphosate resistant *A. palmeri* populations they are less glyphosate tolerant (lower RF values). Since *A. palmeri* and *A. tuberculatus* are closely related species and are able to produce fertile

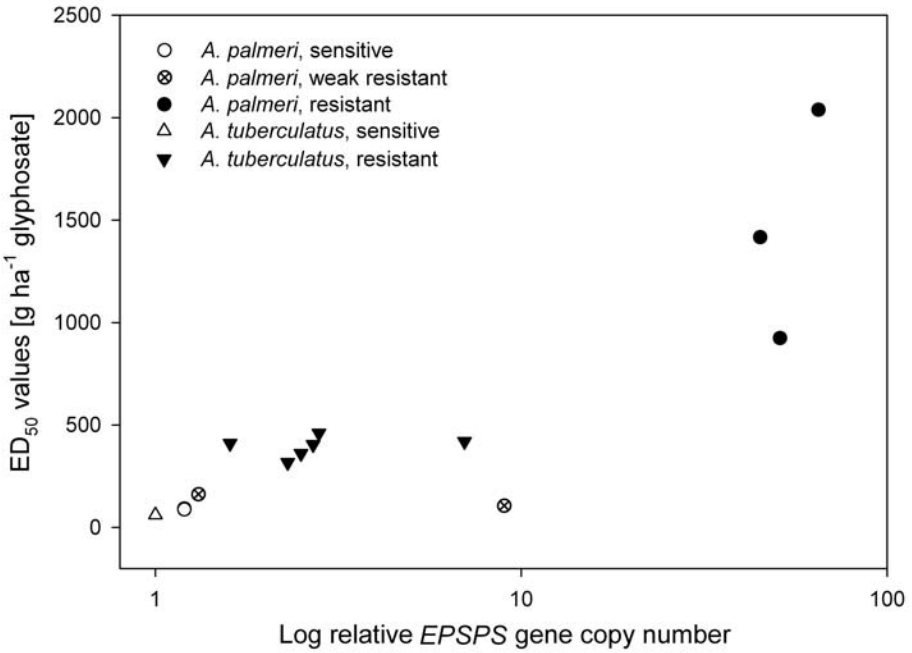

Fig. 42: Relation among the glyphosate sensitivity displayed as ED_{50} values based on fresh weight assessment and the *EPSPS* gene copy number of the *A. palmeri* and *A. tuberculatus* populations; ED_{50} values as indicated in Tab. 10 & 16; *EPSPS* gene copy number as indicated in Fig. 16 & 32, respectively.

crosses, *EPSPS* gene amplification was also checked in these populations (Franssen et al., 2001; Steinau et al., 2003; Gaines et al., 2011). It reveals that the glyphosate resistant *A. tuberculatus* populations contained an EPSPS gene amplification as found in glyphosate resistant *A. palmeri* plants. Nevertheless, the *EPSPS* gene copy number in the glyphosate resistant *A. tuberculatus* populations is lower than that detected in *A. palmeri* and bear in their genome up to 11.2 relative *EPSPS* gene copies in comparison to sensitive plants. The resistant population IL_1 contains in average 7 ± 3 relative *EPSPS* gene copies, while an average of 2.4 relative *EPSPS* gene copies were found in the populations collected in Missouri. They have a lower EPSPS gene amplification than IL_1. Nevertheless, all glyphosate resistant *A. tuberculatus* populations have the same glyphosate tolerance in the dose response based on fresh weight at 16 DAT. However, EPSPS expression either in IL_1, Mo_{13} or Mo_{18} was not induced or silenced by a glyphosate treatment as was also found in the *A. palmeri* populations NC_S and NC_R (Fig. 33).

These findings support the thesis of Zelaya & Owen (2005) of the occurrence of a polygenetic based glyphosate tolerance mechanism in *A. tuberculatus*. Based on these results we propose that *A. tuberculatus* glyphosate resistance is based on the amplification of the *EPSPS* gene.

5.5 EPSPS - Enzyme Kinetics

Gaines et al. (2010) showed by immunoblotting the higher content of the EPSPS protein in the resistant *A. palmeri* population GA_R and a higher activity while determining the I_{50} values to glyphosate.
To test for higher EPSPS enzyme content in *A. tuberculatus* and in a second glyphosate resistant *A. palmeri* population, the EPSPS enzyme activity was measured in both species. The first step was to determine based on the appK_{cat} values if the EPSPS is present in its active form and if it was present at a higher content in the glyphosate resistant plants of other *A. palmeri* and *A. tuberculatus* populations other than GA_R. Glyphosate inhibits EPSPS as a competitive inhibitor of PEP (Pline-Srnic, 2006). Higher appK_m[PEP] values indicate a lower enzyme affinity to its substrate PEP and decreases EPSPS activity at lower PEP concentrations (Pline-Srnic, 2006). Glyphosate resistant plant EPSPS enzymes typically have increased appK_m[PEP] values in comparison to wild type enzymes (Pline-Srnic, 2006). Therefore the appK_m[PEP] of sensitive and resistant plants were measured to determine additional changes within the EPSPS enzyme sequence. Several hundreds of time and money consuming sequencing reactions would have been necessary to exclude additional changes within the EPSPS DNA sequence with respect to the high *EPSPS* gene amplification.
The appK_m[PEP] values were determined in the *A. palmeri* populations NC_S and NC_R and in the *A. tuberculatus* populations Mo_{18} and IL_1 (Tab. 11 & 17). No evidence was found for alterations in the EPSPS sequence. The values although measured in a crude enzyme extract are comparable to those published *e.g.* for *Corydalis sempervirens* and *Zea mays* (Smart et al., 1985; Forlani et al., 1994). They are about 10-fold higher than those published for *Sorghum bicolor*, *E. coli*, *Pseudosomonas aeruginosa*, and also for the EPSPS of *Eleusine indica* and *Zea mays* expressed in *E. coli* (Ream et al., 1988; Baerson et al., 2002; Vaithanomsat & Brown, 2007). While no significant differences were obtained between the appK_m[PEP] values between resistant and sensitive populations, the V_{max} and K_{cat} values were significantly different between resistant and sensitive plants. In *A. palmeri* an increase between sensitive and resistant plants of about 11 times was measured in the V_{max} and K_{cat} values, whereas in *A. tuberculatus* a much lower increase in V_{max} and K_{cat} values was measured between resistant and sensitive plants (2.34 and 3.67 times, respectively). These values developed naturally are lower than those reported for the same mechanism developed in cell culture, *e.g.* a 40-fold increase in EPSPS activity in *C. sempervirens* (Smart et al., 1985). However, the obtained values are already high enough to mediate plant glyphosate resistance in field. The V_{max} and K_{cat} values of resistant plants are also lower than expected by the qPCR results for both species and are probably caused by regulatory mechanism of translation or protein turnover.
In plants the variability of protein levels in relation to the genome or the transcriptome is

widely unknown (Muers, 2011; Schwanhäusser et al., 2011). Schwanhäusser et al. (2011) found that mRNA levels can explain about 40 % of the variability in the protein level of mouse cells. In *A. palmeri* and *A. tuberculatus* this relation is unknown. A further EPSPS regulation of the protein pool in response to severe selection pressure might be possible and needs further investigations. Also measurement of EPSPS activity at different time points after glyphosate treatment might be helpful to determine further regulatory processes in plant cells. Nevertheless, the different V_{max} and K_{cat} values indicate an increased amount of active EPSPS in the protein pool of resistant plants and underline gene amplification as glyphosate resistance mechanism in *A. palmeri* and *A. tuberculatus* populations.

5.5.1 EPSPS in *A. palmeri* and *A. tuberculatus*

The results of this work strongly suggest that *EPSPS* gene amplification and the higher content and activity of the EPSPS enzyme in glyphosate resistant plant are the main mechanism involved in the glyphosate resistance in the tested populations of both *A. palmeri* and *A. tuberculatus* populations. The comparison of dose response data to the EPSPS gene amplification of both plant species indicates the same resistance mechanism. The glyphosate resistant *A. palmeri* populations developed an average $RF_{freshweight}$ of 16.3, corresponding to an *EPSPS* gene amplification of 53.6 times relative to the *ALS* gene. Glyphosate resistant *A. tuberculatus* populations showed an $RF_{freshweight}$ of 6.5 compared to an average of 3.2 times of amplified *EPSPS* genes (all populations considered). The relationship of the ED_{50} values based on fresh weight assessment and the EPSPS gene copy number in both species gave a correlation coefficient of $R^2 = 0.8516$ (Fig. 42). The correlation coefficient based on shikimic acid assessment was $R^2 = 0.9076$.
However, a high level of *EPSPS* gene amplification is not exclusively linked to a high degree of glyphosate resistance as documented in the comparison of NC_R and NC_{R1}. NC_R has an ED_{50} value (fresh weight evaluation) of 1416.1 g ae ha^{-1} related to a 45 $\pm$ 11 relative *EPSPS* gene copies, whereas NC_{R1} showed an ED_{50} value (fresh weight evaluation) of 924 g ae ha^{-1} glyphosate, *i.e.* a lower glyphosate resistance, but related to 51 $\pm$ 18.3 relative *EPSPS* gene copies. The relationship of the resistance ratio with the *EPSPS* gene copy number seems thus to follow a curve with a plateau. Further cell regulatory mechanisms might be involved in the expression of glyphosate resistance, like the amount of expressed genes, enzyme translation or turn over.
This is especially supported by the 11-fold higher amount of active EPSPS protein in resistant plants bearing approximately 45 $\pm$ 11 relative *EPSPS* genes. In *A. palmeri* the gene - enzyme ratio is therefore $^1/_4$, whereas *A. tuberculatus* with a ratio of $^1/_2$ has a significantly higher ratio. A 7 $\pm$ 3 relative *EPSPS* gene copies in *A. tuberculatus* will lead to an EPSPS K_{cat} increase of about 3.7-fold. Thus, lower gene amplification that is fully expressed and

translated might provide the same degree of resistance as found in the investigated plants.

5.6 A Sensitive Population Bearing EPSPS Gene Amplification.

The *A. palmeri* population NC_{LR} was harvested at a site located between the NC_S and NC_{R1} harvesting sites and was described to be inconsistent to them in its glyphosate response. This particular population was tested with less plants and doses in the dose response study mainly because of its poor germination rate and a higher sensitivity to fungal infections. In comparison to the other populations, only a quarter of plants germinated, yielding barely enough homogeneous plants for a proper tolerance test, to fulfill the requirements of a dose response study (Seefeldt et al., 1995). With an ED_{50} value of 101 g ae ha^{-1}, NC_{LR} was not significantly different to the sensitive populations and to the low level resistant population TN_{LR} (Tab. 10).
In the present study *EPSPS* gene amplification is confirmed to be the main glyphosate resistance mechanism of the tested *A. palmeri* populations. In contrast to the other populations, *EPSPS* gene amplification was bisferriously distributed within the NC_{LR} population (Fig. 20). Individuals can be found bearing the same EPSPS gene copy number as sensitive plants but also as found in some glyphosate resistant plants. This potentially indicates an inbreeding of resistant individuals into a sensitive population or *vice versa*. If this inbreeding is contributing to the poor germination rate and high sensitivity to fungal infections stays in question, but it would provide an explanation of the separation of resistant and sensitive individuals in the later phylogentic studies.
To clarify the influence of *EPSPS* gene amplification on NC_{LR}, the population was split into the two daughter populations NC_{LRS} and NC_{LRR}. Both daughter populations were generated with untreated plants bearing either a low amount of EPSPS gene copies (NC_{LRS}) or a high amount of *EPSPS* gene copies (NC_{LRR}). The NC_{LRS} individuals have therefore a single relative EPSPS gene as found in sensitive plants of the populations GA_S or NC_S. The opposite was found in individuals of NC_{LRR}, which contained either a low or a high amount of *EPSPS* gene copies (Fig. 21).
Both populations were not significantly different in a subsequent dose-response study. Nevertheless, they show a shift in their ED_{50} values. NC_{LRR} is more glyphosate tolerant with an ED_{50} value of 235 $\pm$ 168 g ae ha^{-1} glyphosate based on in average 14 relative *EPSPS* gene copies. NC_{LRS} has on average a single *EPSPS* gene copy and an ED_{50} value of 105 $\pm$ 54 g ae ha^{-1} glyphosate. With respect to the heterogeneous population NC_{LRR} these results confirm that the *EPSPS* gene amplification is the glyphosate resistance mechanism in the two genetically identical *A. palmeri* populations.
On the other hand the low difference between NC_{LRS} and NC_{LRR} raises the question about further regulatory mechanisms involved in glyphosate resistance based on *EPSPS* gene

amplification. Such regulatory mechanisms might be involved in EPSPS translation and protein turn over, as mentioned above and might also contribute to the extent of glyphosate resistance expressed in *A. palmeri* populations.

5.7 Phylogenetic Studies

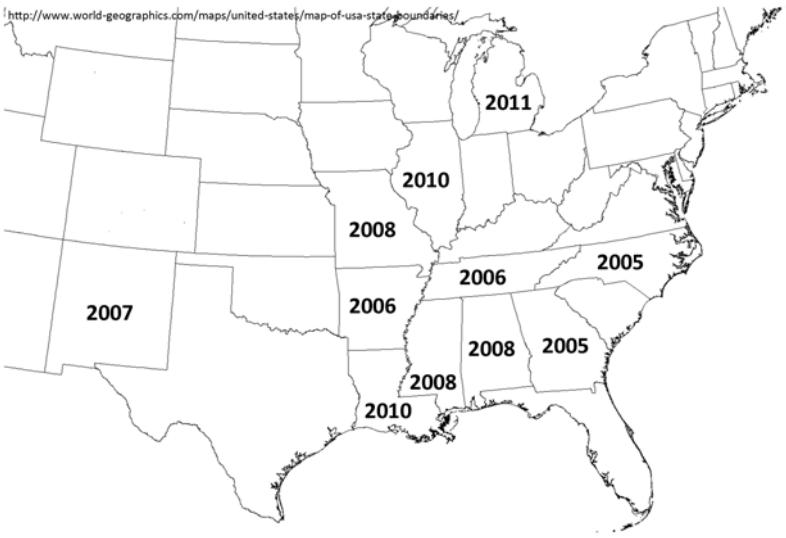

Fig. 43: Reported first cases of glyphosate resistance in *A. palmeri* in the different States of of the U.S. in North America. Data mentioned as reported by Heap (2011) to be the first confirmed cases of glyphosate resistance in each particular State, the color code on the graphic is related to the glyphosate response (left side) and to the geographical origin (right side, according to Tab. 2) of the populations, green color on the left side indicates glyphosate sensitive populations, red color indicates glyphosate resistant populations.

The first case of glyphosate resistance in *A. palmeri* was found in 2005 in a Georgia cotton field (Culpepper et al., 2006). In the same year another case of glyphosate resistance was detected in North Carolina, followed by cases in Arkansas and Tennessee one year later (Fig. 43) (Heap, 2011). Resistance due to a similar mechanism occurred nearly simultaneously at several locations. This development raises the question on the relationship between glyphosate resistant and sensitive populations to provide a better knowledge on the infestation or settlement of new areas by glyphosate resistant species or populations (Slotta, 2008; Jasieniuk & Maxwell, 2001).

The relationship and genetic variation among individuals and populations can be characterized in various ways, including allozymes, AFLP-, RFLP-, RAPD-, VNTR-marker or by DNA sequence comparisons (Jasieniuk & Maxwell, 2001). RAPD technique has been widely used in plant to differentiate relationships among individuals, accessions and populations (Jasieniuk & Maxwell, 2001; Heslop-Harrison & Schwarzacher, 2011). The

RAPD technique or AP-PCR is based on PCR amplification of random DNA segments with short, usually 10bp long oligonucleotide primers of arbitrary sequence (Williams et al., 1990; Welsh & McClelland, 1990). The RAPD-marker technique has also been demonstrated in plants to provide results similar to comparisons based on AFLP-markers, microsatellites or allozymes (Nybom, 2004). The RAPD technique is simple and requires less time and money than other techniques and allows a random sampling of the whole genomic DNA without further DNA sequence information (Jasieniuk & Maxwell, 2001). According to Jasieniuk & Maxwell (2001) RAPD markers give a greater resolution and a more definitive separation of taxa than other molecular markers and have been shown to group populations or varieties according to their origin and morphological characteristics (Huff et al., 1993; Sitthiwong et al., 2005). Nevertheless RAPD markers seem to be less reliable when estimating interspecific relationships (Thormann et al., 1994; Powell et al., 1996). The RAPD markers in the present study were only used to determine intraspecific genetic relationships.

A further disadvantage of this technique is the reproducibility between different labs and conditions (Jones et al., 1997). This is probably also the reason for the different opinions on the number of individuals and markers needed to obtain reliable results. Lynch & Milligan (1994) proposed a 2 - 10-fold higher number than necessary for other marker types, but recommended using at least 100 individuals. Huff et al., (1993) achieved a significant separation of the origin of 48 *Buchoë dactyloides* individuals by using 98 polymorphic RAPD markers. Staub et al. (2000) calculated 80 polymorphic markers as sufficient and found no further improvements on the accuracy of genetic distances by using a higher amount of polymorphic markers. Excoffier et al. (1992) estimated that about 62 restriction sites were needed to come to a confidence interval of 99 % reliability. This estimate should be transferrable to RAPD markers due to the similar size of the recognition site of restriction enzymes and RAPD oligonucleotide primers with usually 2 x 10 bp which is almost equal to a 20 bp recognition site for restriction enzymes.

In the present work between 7 and 14 individuals from each of the populations NC_{LRR}, NC_R, NC_S, TN_R, GA_R and GA_S were tested based on 113, 202 and 243 markers. In all cases, with the exception of GA_R with 78 polymorphic markers, more than 80 polymorphic markers were found, *i.e.* the number was generally considered to be sufficient to obtain significant results (Tab. 13 & 14). The number of individuals per population is rather low, especially with respect to the proposed number of around 100 individuals necessary to obtain reliable test results (Lynch & Milligan, 1994). But as shown by Huff et al. (1993), who used 48 individuals, the number of individuals and polymorphic markers used in this work is adequate to obtain statistically significant results on the relationship between the individuals.

Gaines et al. (2010) suggested that transposable elements are the source of EPSPS gene amplification. Transposable elements are widely present in the plant kingdom. In the study

presented herein no marker was found to be exclusively present in glyphosate resistant or sensitive individuals, supporting the conclusion that the RAPD results are not only based on the amplified sequences. Therefore, the influences of the amplified sequence affecting the relationship results between the different *A. palmeri* populations can be excluded.
In the *A. palmeri* relationship analysis, seven different populations with 102 individuals were analyzed by using the RAPD marker technique. The relationship was assessed in 4 separated runs avoiding any length determination of markers based on nucleotide base pairs to reduce the incidence of technical failure.
The majority of individuals clustered with their original population. This indicates that the RAPD markers were able to group the individuals according to their true relationship, since some populations belong to F_1 generations of field collections. This explains the close grouping of individuals within a given population. Exceptions were found only in AR_R and TN_R, which were grown directly from field collected seeds. The fact that some individuals are more closely related to individuals of foreign populations reflects the high degree of plant heterogeneity, even in F_1 generations of this obligate outcrossing or allogam species. The high degree of heterogeneity reflects on the other hand the probable migration of genes from the source population bearing the first case of glyphosate resistance to the investigated population.

The populations used in this comparison were collected in North Carolina, Georgia,

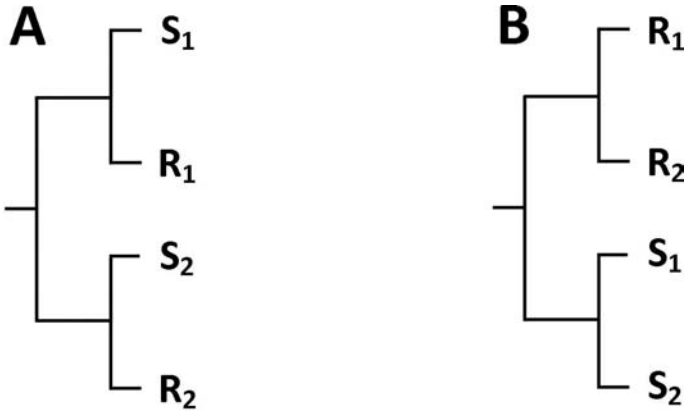

Fig. 44: Schematic relationship among herbicide resistant and sensitive weed populations; **A** expected situation if resistance will be developed as a single event in a sensitive population or by inbreeding *e.g.* via pollen; **B** actual situation in the sensitive and resistant populations in Georgia and North Carolina, probably conferred due to gene flow by seeds; R_1; R_2; S_1 and S_2 are theoretical populations; the same area of seed collection is indicated by the same index.

Arkansas and Tennessee (Tab. 2). The populations NC_S, NC_R and NC_{LR} were collected close to each other, only a few kilometers apart, whereas GA_R and GA_S were sampled approximately 40 kilometers from of each other. It was therefore expected that the populations inside each group - 1^{st} group: NC_S, NC_R and NC_{LR} and the 2^{nd} group: GA_R

and GA_S - would be closely related, assuming that the individuals represent a propagation community and have a low genetic distance within the groups. The genetic relationship among plants is, according to Hamrick & Godt (1990), mainly depending on the geographic distance from one another. However, the different populations were in fact more closely grouped grouped together according to their level of glyphosate tolerance. They clustered into a families of glyphosate sensitive and resistant populations (Fig. 23; 25 & 24). As shown in Tab. 15 the contribution of geographic distance to the overall genetic variability between individuals and populations is rather low - 1.54 % and 2.97 % of the genetic variability can be explained by the glyphosate resistance or sensitivity. The most genetic diversity was found within each population with values between 81.95 and 86.86 % of the total variation, which reflects the outcrossing prevalent in this dioecious weed species (Kaundun & Park, 2002). The close relationship of glyphosate resistant individuals is one potential explanation for the small differences found between NC_R and GA_R in the dose response study and the *EPSPS* gene amplification study in the present study and previously published results (Culpepper et al., 2006; Gaines et al., 2010).

In the *A. tuberculatus* populations IL_1, Mo_{13} and Mo_{18}, however, no population clustering connected to glyphosate tolerance was detected (Fig. 34). The glyphosate sensitive and resistant populations IL_1 and Mo_{18} showed the closest relation, whereas Mo_{13} was more distantly related. However, the Mo_{18} and IL_1 collection sites are the furthest from each other, while the Mo_{13} collection site was located between them. In addition, IL_1 has significantly higher EPSPS gene amplification than Mo_{13}, providing the first hint of a looser relationship between them. These data were obtained in only a few individuals, therefore it might be interesting to test a higher amount of individuals collected at different locations. Since glyphosate and ALS-resistance can be transferred from *A. palmeri* to *A. tuberculatus* by interspecific hybridization and both are able produce fertile hybrids, it might be even more interesting to compare the relationship of several glyphosate resistant and sensitive populations of both species and populations to clarify if none, a single or multiple inbreeding processes are responsible for glyphosate resistance (Franssen et al., 2001; Steinau et al., 2003; Gaines et al., 2011). However, further work using different marker techniques is necessary to answer this question, as might be answered by a comparison of *EPSPS* intron sequences of both species.

These overall results on the genetic similarity of populations collected far apart from one another on the basic of glyphosate resistance were unexpected and are contrary to the main opinion of resistance development in weeds. If we consider that RAPD-markers reflect the true genetic relationship among the *A. palmeri* populations, then our findings raises several further questions on herbicide resistance development and especially on the development of glyphosate resistance in *A. palmeri*. The development of herbicide resistance over recent years was mostly seen as a series of single events occurring separately in each location and limited to a field or on a regional scale (Delye et al., 2010). Thus, Warwick (1991), for

example, explained the development of triazine resistance in weeds as an isolated event occurring at several locations in parallel. Based on this view all individuals of a region would be in a propagation community and should share a distinct part of their genome, or rather, of their marker pattern. Different geographic populations should have a high genetic distance and geographically neighboring sensitive and resistant populations should show a low genetic distance (Hamrick & Godt, 1990). An example on the expected cladogram, based on this view, is given in Fig. 44 **A**. The cladograms obtained in this work, however, differ completely from the expected pattern and are comparable to the scheme in Fig. 44 **B**.

The implied distribution of resistant weed populations over distances of several hundreds of kilometers within approximately 2 - 6 years is not easy to understand. Glyphosate resistance in *A. palmeri* is not inherited through plastids (Gaines et al., 2010). The *EPSPS* gene copies are scattered throughout the whole plant genome and are therefore propagated either by seeds or pollen and are biparentally inherited (Gaines et al., 2010). Pollen mediated spread of an herbicide resistance trait has been reported over a distance of more than 21 km in the prevalent wind direction in Creeping Bentgrass[15] (Watrud et al., 2004). The main wind direction in the Southeastern United States during summer is mostly in a longitudinal direction. But the different collection sites are distributed along both longi- and latitudinal transects. In addition the Appalachian mountains, the Eastern continental divide and the Mississippi river are located between the sampling sites. A pollen mediated spread of glyphosate resistance in *A. palmeri* is therefore rather unlikely. The spread of resistance seems to be more probable due to the spread of seed, as is also presumed for the spread of ACCase resistant *Alopecurus mysuroides* in northern France (Menchari et al., 2006), a much smaller territory than described for *A. palmeri*. Nevertheless, reports about plant seed dispersal are rare and usually based on the majority of seeds from an individual or population (Cain et al., 2000). When driven by wind seeds of *A. palmeri* will scatter mainly only a few meters away from the source plant. The implicated transport distances of several hundreds of kilometers can therefore not be explained by wind mediated transport. An alternative way of long distance weed seed dispersal might be due to animals like birds. Proctor (1968) investigated the retention time of viable weed seeds in the digestive tract of several bird species and found that the retention time of viable *A. palmeri* seeds was between 2 hours in Mockingbirds[16] and 15 hours in Killdeer[17]. A more probable way for wide weed seed dispersal seems to be the distribution by machinery or agricultural products. Norsworthy et al. (2009) published the survival and distribution of viable weed seeds, including *A. palmeri* seeds, through cotton gin trash. This might be a possible route of glyphosate resistant *A. palmeri* seed dispersal, especially

[15] *Agrostis stolonifera*
[16] *Mimus polyglottos*
[17] *Charadrius vociferus*

considering that the first glyphosate resistant *A. palmeri* plants were found in a cotton field (Culpepper et al., 2006). Cotton gin trash is used to increase the organic matter in soil, as a crop fertilizer, or to feed livestock that is usually composted for 1 - 2 years to avoid any weed seed contamination before using it. Nevertheless, Norsworthy et al. (2009) found significant amounts of viable *A. palmeri* and *Sorghum halepense* seeds in the surface of cotton gin trash piles. These seeds were not killed by the composting process and are probably also the reason for reports of high weed infestations after using composted cotton gin trash in field (Norsworthy et al., 2009). The use of cotton gin trash as fodder for livestock will not circumvent the problem of seed distribution because *A. retroflexus* seeds, a close relative of *A. palmeri*, withstand rumen digestion and retain viability after being buried for 21 days in manure (Blackshaw & Rode, 1991; Larney & Blackshaw, 2003)(Chapter 2.1). These might all be possibilities for long distance dispersal of *A. palmeri* seeds, especially with respect to the high seed persistence under unfavorable conditions and the nationwide transport of agricultural products, particularly of livestock and of contracted harvest equipment.

The relationship among different *A. palmeri* populations is not the only example of a relationship among weed populations clustered according to herbicide tolerance. The results of this study are comparable to the results found among ACCase resistant *A. myosuroides* populations in Northern France (Menchari et al., 2006). *A. myosuroides* is a self infertile and allogamous weed species, as the dioecious *A. palmeri*. Both species are therefore obligate outcrossing species (Jasieniuk & Maxwell, 2001). Menchari et al. (2006) found a high relationship between ACCase resistant *Alopecurus myosuroides* populations, which were clustered into families bearing the same ACCase target-site mutation. The different populations of each individual ACCase target-site mutation family can also be spread over the whole sampling region, as found herein to be the case among glyphosate resistant *A. palmeri* populations. Based on AFLP markers Menchari et al. (2007) showed that the total genetic diversity between different *A. myosuroides* populations was high, while the genetic differentiation among populations and geographical distances was low. This indicated extensive gene flow between different *A. myosuroides* populations with no relationship due to geographic distance and a genetic homogeneity of arable weed populations at the landscape level (Menchari et al., 2007; Delye et al., 2010). The reduced importance of distance to explain the genetic variability is probably caused by distribution through agricultural machinery, as presumed by Menchari et al. (2007). It seems that the selection pressure caused by herbicides has a higher effect on genetic weed diversity than geographic distance, especially if geographic distances are effectively reduced through rapid and efficient national or international transportation networks as found in modern agriculture. In bacteria a *resistotype* is defined as the specific genotype and phenotype of a specific antibiotic resistance machanism (Allen et al., 2010). Therefore it might be possible and necessary to divide geographically separated herbicide resistant weed populations

of a single species into *resistotypes* as in antibiotic resistant bacteria.
Only through the widespread and nearly exclusive use of glyphosate and the Roundup Ready®system glyphosate resistance confers such a superior advantage to certain weeds that allow them to successfully compete with crops throughout the US cropping area. This will promote a weed spectrum shift in a field towards less and less herbicide sensitive species, as the herbicide acts as a strong selection pressure on the weed community (Culpepper, 2006; Norsworthy, 2008; Webster & Sosnoskie, 2010). Even small amounts of seeds bearing such a superior advantage as glyphosate resistance will reproduce and settle new areas within a few growing seasons (Fig. 43). Glyphosate resistant weeds have, once they proliferate in a field, no other competitors in field than the RR-crop. *A. palmeri* is usually highly sensitive to glyphosate as shown in Tab. 10 and was in the past very efficiently controlled by glyphosate in fields (Culpepper et al., 2006). The absence of sensitive plants in a field also limits potential introgression of native and sensitive plants into the resistant population. This leads to the observed situation of highly resistant individuals with a close genetic relationship to spread further and to start new resistant populations scattered throughout the Southeast of the United States. More attention should be payed in future to the potential for long distance seed dispersal and the widespread and the sole use of a single herbicidal mode of action. Therefore, field hygiene and alterations in weed management practices by using, for example, integrated weed management approaches are essential for future sustainable pest control.

5.8 Discussion *Sorghum halepense*

Sorghum halepense, or Johnsongrass, is known to be an aggressive and invasive weed species in the U.S.A. (Warwick & Black, 1983). Mature plants with developed viable rhizomes used in this work are known to be more difficult to eradicate than seedlings with herbicides or mechanical weed control options (Warwick & Black, 1983). Several herbicides used in the past for *S. halepense* control, for example glufosinate, weaken plants by burning off mainly the treated foliage, but they are generally not translocated basipetally and thus do not kill them. Several applications are therefore necessary to achieve a sustainable level of control of Johnsongrass. In contrast, not only the foliage, but also roots and rhizomes of *S. halepense* are reliably controlled by glyphosate and ACCase inhibitors (Banks & Tripp, 1983; Carter & Keeley, 1987; Smeda et al., 1997). The introduction of glyphosate resistant crops and the exclusive use of glyphosate resulted often in a need to switch to another mode of action due to resistance brought upon through an intensive use of ACCase inhibitors. This intensive selection pressure and reliance on only a few or even a single mode of action has often resulted in emerging control problems with some weed species. Following the success of the ACCase herbicides, the first case of resistant *S. halepense* was found in 1991 in Mississippi, U.S.A. (Smeda et al., 1997). Following the introduction of glyphosate tolerant crops in 1996 and adoption of glyphosate, the control of *S. halepense* became simple and effective. Extensive use of glyphosate as the sole herbicide led also to the development of resistance. Glyphosate resistance in the U.S.A. was first detected in 2007 in a *S. halepense* population from Arkansas (Riar et al., 2011). The plants used in the present work were grown from rhizomes of plants originating from this population.
In addition to the glyphosate resistance in the *S. halepense* population GLY_R, a decrease in the sensitivity to ACCase inhibitors was detected. These results, obtained under greenhouse conditions, confirmed therefore the glyphosate resistance data published by Riar et al. (2011) and describe the first multiple herbicide resistance of a *S. halepense* population, which was resistant to both ACCase inhibitors and glyphosate. To the best of our knowledge this report described for the first time a specific target site mutation in the *ACCase* gene of *S. halepense*. The data showed a correlation between the number of mutated alleles and the level of resistance to an herbicide treatment. This suggests that the ratio between the mutated protein and the wild type protein is correlated with the number of mutant alleles and through that to the level of expressed ACCase resistance.

Glyphosate tolerance of mature *S. halepense* plants was tested under greenhouse conditions, which were different from those used by Riar et al. (2011) (Fig. 35). Under our conditions a growth reduction of 50 %, ED_{50} in the sensitive *S. halepense* population GLY_S was observed at 190 g ae ha^{-1} glyphosate, whereas 670 g ae ha^{-1} glyphosate was necessary to reduce the growth of the resistant population GLY_R by 50 %. These rates are about 2.5- and 3.5-fold lower, respectively, than the glyphosate ED_{50} values obtained

Tab. 21: Glyphosate resistance of GLY_R in the present work compared to the results of Riar et al. (2011)

	Riar et al. (2011)		Present work	
	ED_{50} (g ae ha^{-1})	R/S ratio	ED_{50} (g ae ha^{-1})	R/S ratio
Sensitive	470	5.0	190	3.6
Resistant	2350		670	

by Riar et al. (2011). The resistance factor (RF) of 3.6, in our studies is similar to the resistance factor of 5 observed by Riar et al. (2011). The plants of the resistant population GLY_R survived a glyphosate treatment in field at the recommended field rate of 720 g ae ha^{-1}. These results confirm the previous findings and strongly suggest that the resistance can be differently expressed under different environmental conditions and might show the heterogeneity among individuals within a weed population. The higher overall sensitivity of both populations to glyphosate, independent of their resistance level, may be explained by differences in the environmental conditions like humidity, UV-radiation or temperature, or different growth stages and plant fitness at application time (Muzik & Mauldin, 1964; Hammerton, 1967; Ahmadi et al. 1980; McWorter et al., 1980), especially since the plants in the present study were approximately 20 - 30 cm smaller than the plants used by Riar et al. (2011).

Glyphosate resistance in *S. halepense* in the population tested in our studies has been shown to be caused by differences in glyphosate translocation (Riar et al., 2011). According to these results a higher proportion of glyphosate remains in the treated leaves of resistant plants than is distributed into the rest of the plant. A comparable glyphosate resistance mechanism was found in *Lolium rigidum* and in *Lolium multiflorum* populations (Perez-Jones et al., 2007; Yu et al., 2007). These studies also reported an EPSPS target site mutation at proline to serine (P106S) and a proline to alanine (P106A) at position 106 in the EPSPS amino acid sequence, responsible for a decreased sensitivity to glyphosate (Perez-Jones et al., 2007; Yu et al., 2007). The proline to serine (P106S) substitution was also found in a Malaysian glyphosate resistant *Eleusine indica* population (Baerson et al., 2002). Mutations in positions G101 and T102 are also known to confer glyphosate resistance (Kishore et al., 1986; Lebrun et al., 1997; Pline-Srnic, 2005). However, no target site mutations in amino acid positions (AA) P106 or in G101 and T102 were detected in the *S. halepense* plants studied here. Therefore, EPSPS target site mutations at these positions are not contributing to the glyphosate resistance detected in the *S. halepense* population GLY_R. The resistance seems to be solely based on differences in the glyphosate translocation as found by Riar et al. (2011).

The second type of herbicide resistance in the *S. halepense* population GLY_R is the resistance to AOPP ACCase inhibitors. Several resistance mechanisms have been described

that confer resistance to ACCase inhibitors. They are divided into target site based and non target site based resistance mechanisms (Delye, 2005). Within target site based resistance, a preliminary estimation on the prevalent resistance mechanism can be accomplished by comparing differences in the plant response to the AOPP and CHP subgroups of the ACCase inhibitors (Delye, 2005). Seven different amino acid positions in the ACCase sequence that confer resistance to ACCase inhibitors in monocotyledonous plants have been described, including amino acid positions I1781, W1999, W2027, I2041, D2078, C2088 and G2096 (Yu et al., 2007; Powles & Yu, 2010). In addition to these target site mutations, an overproduction of the ACCase enzyme can be also responsible for ACCase resistance in *S. halepense* (Bradley et al., 2001).

According to Burke et al. (2006) each of the five reported ACCase resistant *S. halepense* populations in the U.S. are resistant to different individual or groups of AOPP and CHD ACCase herbicides indicating the occurence of different target site mutations or resistance mechanisms within a single population. Delye et al. (2005) determined the I_{50} values to different ACCase inhibitors for purified *A. myosuruides* ACCase enzyme bearing the W2027D target site mutation. In their study, high I_{50} values of the ACCase enzyme were obtained by using AOPP ACCase inhibitors while CHD ACCase inhibitors led to a only weak enzyme inhibition, which was additionally shown by Liu et al. (2007). In our study GLY_R plants showed comparable behavior after herbicide treatment and are strongly resistant to fluazifop, while the sensitivity to quizalofop and clethodim is only slightly reduced (Fig. 37 & 38). In *Alopecurus myosuroides* 3 different ACCase target site mutations have been found: tryptophan to cysteine at position 2027 (W2027C), isoleucine to asparagine at position 2041 (I2041N) and glycine to alanine at position 2096 (G2096A) (Delye, 2005). In *S. halepense* no target site mutation at the ACCase amino acid positions I1781, I2041, D2078, or G2096 was found by PCR/pyrosequencing. However, a target site mutation was found at amino acid position W2027 with an exchange of the DNA codon TGG to TGC resulting in an amino acid change from tryptophan to cystein (W2027C)(Fig. 39). The high resistance to fluazifop (Fig. 37) and the lower clethodim sensitivity found in the greenhouse test (Fig. 38), are therefore in good agreement with the properties of the found target site mutation. The same mutation site also confers resistance to ACCase inhibitors in *Avena sterilis*, *Avena fatua*, *Lolium rigidum*, *Phalaris minor* and *Alopecurus myosuroides* populations (Vila-Aiub et al., 2009). The finding of a target site mutation in the *ACCase* sequence confirms the assumption of Burke et al. (2006) and Burke et al. (2007) of an insensitive form of the ACCase as the main resistance mechanism in *S. halepense* populations based on enzyme activity measurements and by assessing the ACCase resistance expressed in pollen.

The single nucleotide polymorphism (SNP) in position W2027 in the *ACCase* sequence in *S. halepense* was detected using PCR coupled with pyrosequencing technology. The peak heights for a heterozygote base in the DNA in the resulting pyrogramms reflects

the relative proportion of each individual nucleotide in comparison to a homozygote base (Ronaghi, 2001). In the *ACCase* sequence of GLY_R the third position of the codon was mutated from TGG to TGC. In the pyrograms only individuals were found with a signal intensity of about 25, 50 and 0 % in comparison to a single homozygote base pair in the DNA (Fig. 39). *S. halepense* is described as a tetraploid weed species (Celarier, 1958) and according to the proportions of the wild-type nucleotide and the mutated nucleotide in the pyrosequencing results we assume that *S. halepense* contains in total four eukaryotic *ACCase* sequences in the genome. Therefore, only GLY_R individuals were found bearing either 1, 2, or 0 W2027C resistant *ACCase* alleles of the over all 4 alleles present in the plant genome (Tab. 18). No individual in GLY_R was found bearing the W2027C target site mutation in 3 or all 4 *ACCase* alleles.

The fact that no plant was found bearing the W2027C mutation in 3 ACCase alleles or homozygote could be caused by two reasons: (1) be the absence of homologous recombination between the two genomes of *S. halepense* or (2) by a fitness penalty due to the W2027C target site mutation in the *ACCase* sequence.

S. halepense is a allopoly- and tetraploid weed species. The W2027C mutation might be only present in one of the two fused genomes and it may not have been transferred since homologous recombination is a rare event between parental genomes in established allopolyploid plants (Comai, 2000; Price et al., 2005).

The second possible reason might be based on a fitness penalty of the W2027C ACCase target site mutation as described by Delye et al. (2005) for the enzymatic activity of the *A. myosuroides* W2027C mutant ACCase. The mutation site W2027 is located in the CT-domain of the ACCase, but in contrast to the other mutations in the ACCase sequence conferring resistance, the mutation sites W2027 and D2078 are not directly involved in the interference with AOPP inhibitor binding (Delye et al., 2005). It leads to changes in the three dimensional structure of the binding cavity of the ACCase (Delye et al., 2005). This is probably responsible for the 2-fold reduced activity found for the enzymes bearing the W2027 or the D2078 mutation (Delye et al., 2005). In *A. myosuroides* plants homozygous for the mutation site D2078 the plant height, biomass and the seed production were strongly reduced in comparison to wild type plants or plants heterozygous for this mutation site (Menchari et al., 2008). A fitness penalty for *A. myosuroides* plants due to the mutation site D2078 is therefore most probable. The same disadvantages might also explain why the *S. halepense* population GLY_R contained only plants bearing the W2027C mutation in only 1 or 2 alleles of the overall 4 alleles present in plant, especially since the ACCase enzyme activity in *A. myorusuides* was reduced two fold in enzymes bearing either the W2027 or the D2078 mutation (Delye et al., 2005). Assuming the same level of enzymatic activity of the wild type and mutant ACCase in *A. myosuruides* and in *S. halepense*, then the overall ACCase activity in a plant bearing two W2027 mutant alleles would theoretically be reduced by a quarter. This reduction seems to be not significant

enough to affect plant fitness and allows still a relatively normal plant growth.
The different amount of resistant *ACCase* alleles in plant was correlated to the biological data when the plants were treated with a given rate of fluazifop, quizalofop or clethodim. Some plants developed only a few herbicide injury symptoms while others of the same population were highly injured or dead. The fresh weight data were compared with the frequency of the target site mutation W2027 in these plants. A strong correlation was found between the degree of herbicidal injury and the proportion of resistant alleles in plants treated with fluazifop. These results were confirmed by an additional treatment using 100 g a.i. ha^{-1} fluazifop on equal sized *S. halepense* plants. Plants bearing two mutant alleles were undamaged at this application rate, while plants bearing a single mutant allele were more injured and sensitive plants were killed (Fig. 40). Comparable results on the influence of zygosity on herbicide susceptibility were also found in ACCase resistant and diploid *Lolium multiflorum* plants bearing the D2078 ACCase target site mutation either homo- or heterozygous (Kaundun, 2010).
A single or two resistant alleles in plant were enough to allow it to withstand a normal ACCase graminicide treatment in the field and permit it to produce seeds and viable rhizomes. Resistant ACCase alleles were present in 86 % of the collected individuals (Tab. 18). The probable fitness penalty of the target site mutation W2027 seems to be acceptable for overall plant fitness if only present in 1 or 2 of the 4 *ACCase* alleles. *S. halepense* is a predominantly self-pollinating perennial species with a low out-crossing rate of less than 10 % (Burke et al., 2007). If the mutation leads to a decrease in overall plant fitness it would be expected to decline over a series of seasons due to the selective advantage of individuals having less of this mutation. The ability to survive an APP ACCase treatment was still present in a high proportion of individuals in this population even after years of no further ACCase herbicide usage. The survival of the W2027C mutation in the population without selection pressure suggests that there is no significant fitness penalty associated with this mutation at the given proportion of wild type and mutant *ACCase* genes (Riar et al., 2011). This might be different at higher proportions of the mutant to the wild-type allele. Therefore, the ACCase resistance of *S. halepense* plants not only depends on the particular target site mutation itself, but also on the proportion of W2027C mutant to wild-type alleles which might also be an important contributor to the level of resistance and probably to the plant fitness. It would be therefore very informative to further monitor the resistant population without using ACCase inhibitors over the next several years to observe the further development of the occurrence of the W2027C mutation in this *S. halepense* population.
Even if glyphosate and ACCase inhibitors can no longer be used for sustainable control of this weed species, other modes of action are still successfully controlling *S. halepense* plants of these population as shown in the present study. Both populations were controlled at field labeled rates of glufosinate, tembotrione and a mixture of nicosulfuron and prosul-

furon (Fig. 36). Differences between GLY_S and GLY_R at different rates of the previous herbicides were not observed, therefore the population GLY_R is still sensitive to the herbicide classes active on ALS, HPPD or glutamine synthase. These classes of herbicides can still be used as weed control options for this multiple herbicide resistant *S. halepense* population. Furthermore, with respect to the recent resistance development of this population, these modes of action have to be used in addition to other chemical and mechanical weed control options to delay or prevent development of herbicide resistance to another mode of action in this GLY_R population.

In summary, the importance and widespread occurrence of *EPSPS* gene amplification has been shown in the present work for the glyphosate resistance of *Amaranthus palmeri* populations originally collected in the south east U.S.A. In addition, in term of evolution a higher relationship among glyphosate resistant individuals was found whatever their harvesting location, indicating that a common ancestor of the glyphosate resistant *A. palmeri* might have spread. In glyphosate resistant *Amaranthus tuberculatus EPSPS* gene amplification was found to be the most probable resistance mechanism of different origins of the middle east U.S.A. Finally, a W2027C *ACCase* target site mutation was first described in *S. halepense* and the contribution of the W2027C mutant allele number to the ACCase herbicide resistance has been shown *in planta*. For future studies these plant species provide good examples how plants can adapt to changing environments by the evolution of different mechanisms to survive an herbicide treatment. The evolution of herbicide resistance and the ability of weeds to rapidly spread wide areas once a trait conferring an advantage has been developed will be of key interest for future weed management programs and, more important, stresses the need to discover new herbicides and herbicidal mode of action.

6 Outlook

6.1 Outlook *A. palmeri*

The major glyphosate resistance mechanism in *A. palmeri*, so far identified, is based on *EPSPS* gene amplification. This mechanism was found to be widely distributed throughout populations in the mid and southeast of the U.S. *EPSPS* gene amplification is proposed to work as a molecular swam by binding glyphosate and reducing the free glyphosate concentration in plant cell. However, this mechanism is still not completely understood and the variation of *EPSPS* gene amplification among plants within a population is high. More work is necessary to determine more precisely the relationship between the degree of glyphosate resistance and the *EPSPS* gene amplification. In addition, the low glyphosate tolerance of NC_{LR} plants bearing *EPSPS* gene amplification, raises, in addition, the question of the possible role of pre- and posttranscriptional regulation of the *EPSPS* gene among individuals and populations and its contribution to resistance. Gene amplification in bacteria is rapidly lost in absence of any selection pressure, the ability of *A. palmeri* to keep the high *EPSPS* gene copy numbers needs therefore to be further investigated (Sandegren & Andersson, 2009). Also the mechanisms responsible for gene amplification *e.g.* transposons, their regulation and induction in populations are still unknown. This is closely related to the question if *EPSPS* gene amplification evolved by chance and was selected by glyphosate or if it can be induced by glyphosate treatments. Thus, gene amplification might be rapidly removed out of populations, following the bacterial example, but the amplification ability might have a much longer half-life in these populations. For sustainable future weed management programs it is of key importance to further clarify which is the proximate and which is the ultimate glyphosate resistance mechanism - a stochastic *EPSPS* gene amplification or a regulated process to amplify it.

A. palmeri glyphosate resistance in the most investigated populations is mainly based on *EPSPS* gene amplification, although it is not the only resistance mechanism in this weed species. The glyphosate resistance of the population TN_{LR} is based on an other resistance mechanism since *EPSPS* gene amplification was not found. The findings of a rapid glyphosate sequestration, a possible glyphosate metabolism as most recently described, or modifications in the vascular transport system might be interesting starting points for further investigations aiming to clarify the resistance mechanism in this particular population (Ge et al., 2010; Lorentz et al., 2011; de Carvalho et al., 2012).

The relationship analyses among *A. palmeri* populations provide meaningful results, even if it is based on the use of only one technology (RAPD-technology). Another technology, *e.g.* DNA sequence comparisons or AFLP marker, might help to provide additional data to better understand the spreading of the resistance traits and will help to confirm the results based on the RAPD analyses. A relationship analysis of additional resistant and sensitive *A. palmeri* populations, interspecies crosses with *A. tuberculatus* or other important weed

species populations would be also highly informative to better understand the spreading and evolution of herbicide resistant weed populations. In several wild species a relation depending on geographic distance is described. This correlation has to be tested also in agricultural important weed species as the findings in the present work suggest a higher correlation to cultivation practices than to geographic distances, particularly in *A. palmeri*. Since weed seed dispersal is strongly affecting cultivation practices a better understanding will be essential for future sustainable agriculture.

6.2 Outlook *A. tuberculatus*

In the presented work, it is shown for the first time that the *EPSPS* gene amplification is associated with the glyphosate resistance in *A. tuberculatus* and is therefore most probably the main resistance mechanism. Other resistance mechanisms like alterations in the glyphosate absorption or translocation and a glyphosate insensitive EPSPS enzyme can be excluded in the investigated populations. Being found in two *Amaranthus* species, gene amplification as a mechanism conferring herbicide resistance might be much broader distributed in plants than observed so far. Further work using *A. tuberculatus* inbred lines bearing a narrow range of EPSPS gene amplification would be helpful to study if the *EPSPS* gene amplification is the only resistance mechanism conferring the glyphosate resistance in *A. tuberculatus*. The additional use of *A. palmeri* lines bearing a narrow *EPSPS* gene copy number might also help to support this point.
The two glyphosate resistant populations Il_1 and Mo_{13} expressed a comparable glyphosate resistance in the greenhouse, but had strong differences in their *EPSPS* gene amplification and EPSPS enzyme activity. In the conditions used resistance mechanisms others than *EPSPS* gene amplification were not observed. Both populations provide therefore the advantage to further study regulatory mechanisms involved in the translatory and/or post translatory regulation of the *EPSPS* gene expression or protein turn-over, especially since no alterations in the *EPSPS* mRNA transcript level were found between both populations before and after glyphosate treatment. The different levels of *EPSPS* gene amplification between both populations opens, in addition, the possibility to clarify the influence of *EPSPS* gene amplification on the plant fitness at various environmental conditions.
Among the *A. palmeri* populations analyzed using RAPD markers, a strong relationship based on glyphosate tolerance was found, but not among *A. tuberculatus* populations despite that the same glyphosate resistance mechanism is acting in both species. In a first conclusion a single event of resistance development in *A. tuberculatus* might probably be excluded. The possible spatial and temporal evolution of resistance might include therefore several separated events as well as several inbreeding processes between *A. tuberculatus* and glyphosate resistant or sensitive *A. palmeri* populations, especially since *A. palmeri* and *A.*

tuberculatus are able to produce a fertile progeny. More glyphosate resistant and sensitive *A. tuberculatus* populations from different regions will help to define possible geographical or/and agricultural clusters. Also a comparison with neighbored glyphosate resistant *A. palmeri* populations would provide a better picture on the resistance development and trait or population dispersal of *A. tuberculatus*. Collateral knowledge in migration of weeds species, particularly weed seed dispersal, are essential to prohibit in future field infection with resistant weeds to optimize and protect integrated weed management programs.

6.3 Outlook *S. halepense*

The perennial weed *S. halepense* is difficult to control if glyphosate and ACCase inhibitors fail or are not available. In the present study, the investigated population is resistant to both herbicides MoA, showing that a lot of effort has to be invested to find alternative solutions for a sustainable control of *S. halepense* grown from seeds and rhizomes. As shown in the present work resistant traits might have a long halflife in weed populations, even in the absence of a particular selection pressure and an assumed fitness penalty for the specific *ACCase* target site mutation W2027C. A fitness penalty of herbicide resistance traits is always the hope to reduce resistant plants in a weed population. The tetraploid *S. halepense* would provide the opportunity to deeper investigate the contribution of the W2027C resistant ACCase genes to the overall plant fitness especially regarding the proportion of wildtype to mutant genes. This knowledge can contribute to estimate the necessary time of ACCase abdication for a complete removal of this resistance trait out of a *S. halepense* or other grassy weed population, if possible at all (Andersson & Hughes, 2010).
The glyphosate resistance mechanism of *S. halepense* is known to be caused by alterations of glyphosate translocation as found in other weed species too. The genetic of the weed species is widely unknown. The close relationship between *S. halepense* and corn might open the possibility to further investigate the molecular resistance mechanism of an altered glyphosate translocation based on the genetic tools developed for corn, like RNA chips or other genome and transcriptomic based technologies like RNAseq.

7 Conclusion

Due to the limited number of available herbicidal modes of action, herbicide resistance will be one of the major threats during the next decade(s) in sustainable agriculture systems. Thus, the most promising way to select resistant weed populations is the nearly exclusive use of a single herbicide or herbicidal mode of action. In the '80 and early '90 ACCase inhibitors as graminicides were one of the most effective ways to control monocotyledonous weeds in dicotyledonous crops and they were extensively used. With the introduction, of the Roundup®Ready system in several crops beginning in 1996, many farmers relied again exclusively on a single herbicide, *i.e.* glyphosate. The development of glyphosate resistant *A. palmeri* and *A. tuberculatus* populations bearing partly additional PPO, PSII and ALS resistance and also the multiple ACCase and glyphosate resistant *S. halepense* population illustrate therefore the response of nature to this uniform selection pressure by the development of herbicide resistance. Especially glyphosate resistance in weeds relies on several different resistance mechanisms. In the present work this is reflected by the various methods necessary to find the herbicide resistance mechanism of each species. Even a single species, like *A. palmeri* had developed in parallel different resistance mechanisms to a single herbicide.

On the other hand, the influence and importance of target enzyme levels on herbicide resistance was outlined by comparing three different weed species and two different herbicidal modes of action (MoA). A single target site resistant allele was barely able to confer appropriate ACCase resistance to tetraploid *S. halepense* plants at 100 g a.i. ha^{-1} fluazifop-ρ-butyl. Whereas plants bearing 2 resistant alleles survived the treatment undamaged. In the opposite, EPSPS gene amplification and increased EPSPS enzymatic activity in plant protein pool was able to confer appropriate glyphosate resistance in the absence of any target-site mutation. This effect was shown in *A. palmeri* and *A. tuberculatus* plants bearing more than a doubled EPSPS enzyme concentration in their plant protein pool, probably acting as a molecular swam on intracellular glyphosate concentration. Thus, this work demonstrates the importance of sensitive and resistant target enzyme concentration in the cell protein pool in response to xenobiotics. Next to plant fitness this phenomenon might play an important role on the variability of plant xenobiotic response among species, populations or varieties.

The necessity of alterations in weed management practices was not only displayed in the resistance development itself. The rapid spread of glyphosate resistant *A. palmeri* populations throughout the southeast U.S. demonstrates impressively the problem of country wide, uniform selection pressure on weed communities and by that the migration abilities of adapted sessile organisms. The reliance on a single technique during only a few years allowed this development. However, this development underlines the necessity to use integrated weed management technology, including the rotation of diverse cropping systems

together with mechanical weed control and chemical weed control based on different MoAs. The importance of a spatial and temporal diverse cropping system in a regional scale but also country wide is pointed out in addition by the rapid migration of glyphosate resistant *A. palmeri*. Especially the importance of field sanitation aspects to prevent herbicide resistant weed infections were illustrated in that work by the relationship analysis among *A. palmeri* populations. Thus, only a short period of time seems to be necessary to select resistant weed populations as shown in *S. halepense* population. Whereas, a long period of time and abdication of the affected MoA is needed to eliminate herbicide resistance in weed populations, if possible at all.

8 Literature

Ahmadi MS, Haderlie LC & Wicks GA (1980). Effect of growth stage and water stress on Barnyardgrass (*Echinochloa crus-galli*) control and on glyphosate absorption and translocation. *Weed Science* **28**, 277-282.

Alibhai MF & Stallings C (2001). Closing down on glyphosate inhibition - with a new structure for drug discovery. *Proceedings of the National Academy of Sciences* **98**, 2944-2946.

Allen HK, Donato J, Huimi Wang H, Cloud-Hansen KA, Davies J & Handelsman J (2010). Call of the wild: antibiotic resistance genes in natural environments. *Nature Reviews* **8**, 251-259.

Amrhein N, Deus B, Gehrke P & Steinrücken HC (1980). The site of the inhibition of the shikimate pathway by glyphosate: II. Interference of glyphosate with chorismate formation in vivo and in vitro. *Plant Physiology* **66**, 830-834.

Amrhein N, Johänning D, Schab J & Schulz A (1983). Biochemical basis for glyphosate-tolerance in a bacterium and a plant tissue culture. *FEBS Letters* **157**, 191-196.

Anderson LE, Appleby AP & Weseloh JW (1960). Characteristics of Johnsongrass rhizomes. *Weeds* **8**, 402-406.

Anderson KS, Sikorski JA & Johnson KA (1988). Evaluation of 5-Enolpyruvoylshikimate-3-phosphate synthase substrate and inhibitor binding by stopped-flow and equilibrium fluorescense measurements. *Biochemistry* **27**, 1604-1610.

Andersson DI & Hughes D (2010). Antibiotic resistance and its cost: is it possible to reverse resistance? *Nature Reviews* **8**, 260-271.

Anonymous (1998). Herbicide resistance and herbicide tolerance defined. *Weed Technology* **12**, 789.

Anonymous (2011). URL http://www.resistancefighter.com, last accessed 10.6.2011.

Anonymous (2012a). Roundup Ultra®Herbicide; Complete directions for use. *EPA Reg. No.* 524-475.

Anonymous (2012b). Agrochemical Executive Review - 23^{rd} edition, Agranove, UK, March 2012.

Arnaud L, Sailland A, Lebrun M, Pallett K, Ravanel P, Nurit F & Tissut M (1998). Physiological behavior of two tobacco lines expressing EPSP synthase resistant to glyphosate. *Pesticide Biochemistry and Physiology* **62**, 27-39.

Baerson SR, Rodriguez DJ, Tran M, Feng Y, Biest NA & Dill GM (2002). Glyphosate resistant Goosegrass. Identification of a mutation in the Target enzyme 5-enolpyruvylshikimate-3-phosphate synthase. *Plant Physiology* **129**, 1265-1275.

Banks PA & Tripp TN (1983). Control of Johnsongrass (*Sorghum halepense*) in Soybeans (*Glycine max*) with foliar-applied herbicides. *Weed Science* **31**, 628-633.

Barrett SC (1982). Biological control of weeds with plant pathogens; Chaper: Genetic variation in Weeds. *John Wiley & Sons, inc.*

Barry G, Kishore G, Padgette S, Taylor M, Kolacz K, Weldon M, Re D, Eichholtz D, Fincher K & Hallas L (1992). Inhibitors of amino acid biosynthesis: strategies for imparting glyphosate tolerance to crop plants. *Current Topics in Plant Physiology* **7**, 139-145.

Basu C, Halfhill MD, Mueller TC & Steward Jr. CN (2004). Weed genomics: new tools to understand weed biology. *TRENDS in Plant Science* **9** 391-398.

Baylis AD (2000). Why glyphosate is a global herbicide: strengths, weaknesses and prospects. *Pest Management Science* **56**, 299-308.

Beckie HJ (2006). Herbicide-resistant weeds: management tactics and practices. *Weed Technology* **20**, 793-814.

Bertin C, Yang X & Weston LA (2003). The role of root exudates and allelochemicals in the rhizosphere. *Plant and Soil* **256**, 67-83.

Binimelis R, Pengue W & Monterroso I (2009). "Transgentic treadmill": Responses to the emergence and spread of glyphosate-resistant johnsongrass in Argentina. *Geoforum* **40**, 623-633.

Blackshaw RE & Rode LM (1991). Effect of ensiling and rumen digestion by cattle on weed seed viability. *Weed Science*, **39** 104-108.

Bond JA & Oliver RO (2006). Comparative growth of Palmer amaranth (*Amaranthus palmeri*) accessions. *Weed Science* **54**, 121-126.

Borggaard OK & Gimsing AL (2008). Fate of glyphosate in soil and the possibility of leaching to ground and surface waters: a review. *Pest Management Science* **64**, 441-456.

Bradford MM (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry* **72**, 248-254.

Bradley KW & Hagood ES (2001). Identification of a Johnsongrass (*Sorghum halepense*) biotype resistant to aryloxyphenoxypropionate and cyclohexanedione herbicides in Virginia. *Weed Technology* **15**, 623-627.

Bradley KW, Wu J, Hatzios KK & Hagood ES (2001). The mechanism of resistance to aryloxyphenoxypropionate and cyclohexandione herbicides in a johnsongrass biotype. *Weed Science*, **49** 477-484.

Bradshaw LD, Padgette SR, Kimball SL & Wells BH (1997). Perspectives on glyphosate resistance. *Weed Technology* **11**, 189-198.

Brewer CE & Oliver LR (2009). Confirmation and resistance mechanism in glyphosate-resistant Common Ragweed (*Ambrosia artemisiifolia*) in Arkansas. *Weed Science* **57**, 567-573.

Bridghes BA (1997). Hypermutation under stress. *Nature* **387**, 557-558.

Bromilow RH & Chamberlain K (2000). The herbicide glyphosate and related molecules: physicochemical and structural fators determining their mobility in phloem. *Pest Management Science* **56**, 368-373.

Burke IC, Burton JD, York AC, Cranmer J & Wilcut JW (2006). Mechanism of resistance to clethodim in a Johnsongrass (*Sorghum halepense*) biotype. *Weed Science* **54**, 401-406.

Burke IC, Holland JB, Burton JD, York AC & Wilcut JW (2007). Johnsongrass (*Sorghum halepense*) pollen expresses ACCase target-site resistance. *Weed Technology* **21**, 384-388.

Burnside OC, Wilson RG, Weisberg S & Hubbard KG (1996). Seed longevity of 41 weed species buried 17 years in eastern and western Nebraska. *Weed Science* **44**, 74-86.

Busi R & Powles SB (2009). Evolution of glyphosate resistance in a *Lolium rigidum* population by glyphosate selection at sublethal doses. *Heredity* **103**, 318-325.

Cain ML, Milligan BG & Strand AE (2000). Long distance seed dispersal in plant populations. *American Journal of Botany* **87** 1217-1227.

Carter CH & Keeley PE (1987). Selective control of Johnsongrass (*Sorghum halepense*) in Cotton (*Gossypium hirsutum*) with foliar herbicides. *Weed Science* **35**, 418-421.

Caseley J & Copping L (2000). Twenty-five years of increasing glyphosate use: the opportunities ahead. *Pest Managment Science* **56**, 297.

Castle LA, Siehl DL, Gorton R, Patten PA, Chen YH, Bertain S, Cho HJ, Duck N, Wong J, Liu D & Lassner MW (2004). Discovery and directed evolution of a glyphosate tolerance gene. *Science* **304**, 1151-1154.

Celarier RP (1958). Cytotaxonomic notes on the subsection *Halepensia* of the genus *Sorghum*. *Bulletin of the Torrey Botanical Club* **85**, 49-62.

Cerdeira AL & Duke SO (2006). The current status and environmental impacts of glyphosate-resistant crops: A review. *Journal of Environmental Quality* **35**, 1633-1658.

Chaves I, Passarinho JAP, Capitão C, Chaves MM, Fevereiro P & Ricardo CPP (2011). Temperature stress effects in *Quercus suber* leaf metabolism. *Journal of Plant Physiology* **168**, 1729-1734.

Christoffers MJ (1999). Genetic aspects of herbicide-resistant weed management. *Weed Technology* **13**, 647-652.

Claus JS & Behrens B (1976). Glyphosate translocation and quackgrass rhizome bud kill. *Weed Science* **24**, 149-152.

Cromartie TH & Polge ND (2000). An improved assay for shikimic acid and its use as a monitor for the activity of sulfosate. *Proceedings of Weed Science Society of America* **40**, 291.

Comai L, Sen LC & Stalker DM (1983). An altered aroA gene product confers resistance to the herbicide glyphosate. *Science* **221**, 370-371.

Comai L (2000). Genetic and epigenetic interactions in allopolyploid plants. *Plant molecular Biology* **43**, 387-399.

Costea M & Demason DA (2001). Stem morphology and anatomy in Amarantus L. (Amaranthaceae) - Taxonomic significance. *Journal of the Torrey Botanical Society* **128**, 254-281.

Costea M, Weaver SE & Tardif FJ (2005). The biology of invasive alien plants in Canada. 3. *Amaranthus tuberculatus* (Moq.) Sauer var. *rudis* (Sauer) Costea & Tardif. *Canadian Journal of Plant Science* **85**, 507-522.

Cruz-Hipolito H, Osuna MD, Heredia A, Ruiz-Santaella JP & De Prado R (2009). Non-target mechanisms involved in glyphosate tolerance found in *Canavalia ensiformis* plants. *Journal of Agricultural and Food Chemistry* **57**, 4844-4848.

Culpepper AS (2006). Glyphosate induced weed shifts. *Weed Technology* **20**, 277-281.

Culpepper AS, Grey TL, Vencill WK, Kichler JM, Webster TM, Brown SM, York AC, Davis JW & Hanna WW (2006). Glyphosate-resistant Palmer amaranth (Amaranthus palmeri) confirmed in Georgia. *Weed Science* **54**, 620-626.

Darwin C (1859). The origin of species. *John Murray, London, UK.*

Dauer JT, Mortensen DA & VanGessel MJ (2007). Temporal and spatial dynamics of long-distance *Conyza canadensis* seed dispersal. *Journal of Applied Ecology* **44**, 105-114.

De Carvalho LB, da Costa Aguiar Alves PL, Gonzàlez-Torralva F, Cruz-Hipolito HE, Rojano-Delgado AM, R De Prado, Gil-Humanes J & de Castro MDL (2012). Pool of resistance mechanisms to glyhosate in *Digitaria insularis*. *Journal of Agricultural and Food Chemistry* **60**, 615-622.

De Jonge H, de Jonge LW & Jacobsen OH (2000). [^{14}C]Glyphosate transport in undisturbed topsoil columns. *Pest Management Science* **56**, 909-915.

De Prado R, Jorrin J & Garcia-Torres L (1997). Weed and crop resistance to herbicides. *Kluwer Academic Publishers* ISBN: 0-7923-4581-9.

Delye C (2005). Weed resistance to acetyl coenzyme A carboxylase inhibitors: an update. *Weed Science* **53**, 728-746.

Delye C, Zhang XQ, Michel S, Matejicek A & Powles SB (2005). Molecular bases for sensitivity to Acetyl-Coenzyme A carboxylase inhibitors in Black-Grass. *Plant Physiology* **137**, 794-806.

Delye C, Michel S, Berard A, Chauvel B, Brunel D, Guillemin JP, Dessaint F & Le Corre V (2010). Geographical variation in resistance to acetyl-coenzyme A carboxylase-inhibiting herbicides across the range of the arable weed *Alopecurus myosuroides* (black-grass). *New Phytologist* **186**, 1005-1017.

Demuth JP & Hahn MW (2009). The life and death of gene families. *BioEssays* **31**, 29-39.

Dewey SA & Appleby AP (1983). A comparison between glyphosate and assimilate translocation pattern in tall morningglory (*Ipomoea purpurea*). *Weed Science* **31**, 308-314.

Dinelli G, Marotti I, Bonetti A, Minelli M, Catizone P & Barnes J (2006). Physiological and molecular insight on the mechanisms of resistance to glyphosate in Conyza canadensis (L.) Cronq. biotypes. *Pesticide Biochemistry and Physiology* **86**, 30-41.

Dinelli G, Marotti I, Bonetti A, Catizone P, Urbano J M & Barnes J (2008). Physiological and molecular bases of glyphosate resistance in *Conyza bonariensis* biotypes from Spain. *Weed Research* **48**, 257-265.

Dreher K, Khairallah M, Ribaut JM & Morris M (2003). Money matters (I): costs of field and laboratory procedures associated with conventional and marker-assisted maize breeding at CIMMYT. *Molecular Breeding* **11**, 221-234.

Duke SO & Powles SB (2008). Glyphosate: a once in a century herbicide. *Pest Management Science* **64**, 319-325.

Duke SO (2011). Glyphosate degradation in glyphosate-resistant and -susceptible crops and weeds. Journal of *Journal of Agricultural and Food Chemistry* **59** 5835-5841.

Edmondson DE & Gadda G (2009). Guidelines for the functional analysis of engineered and mutant enzymes. pp. 8 *Protein Engineering Handbook* Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim, ISBN: 978-3-527-31850-6

Egley GH & Chandler JM (1983). Longevity of weed seeds after 5.5 years in the Stoneville 50-year buried-seed study. *Weed Science* **31**, 264-270.

Ellis MR, Dennis ES & Peacock J (1999). Arabidopsis roots and shoots have different mechanisms for hypoxic stress tolerance. *Plant Physiology* **119**, 57-64.

Excoffier L, Smouse PE & Quattro JM (1992). Analysis of molecular variance inferred from metric distances among DNA haplotypes: Applications to human mitochondrial DNA restriction data. *Genetics* **131**, 479-491.

FAO (2011). Food and agricultural organization of the United Nations. http://www.fao.org last accessed: 31.12.2011.

Felsenstein J (1978). Cases in which parsimony or compatibility methods will be positively misleading. *Systematic Zoology* **27**, 401-410.

Felsenstein J (1985). Phylogenies and the comparative method. *The American Naturalist* **125**, 1-15.

Felsenstein J (1989). PHYLIP - Phylogeny Inference Package (Version 3.2). *Cladistics* **5**, 164-166. Software free avaliable at: http://evolution.genetics.washington.edu/phylip

Feng PCC, Ryerse JS & Sammons RD (1998). Correlation of leaf damage with uptake and translocation of glyphosate in velvetleaf (Abutilon theophrasti). *Weed Technology* **12**, 300-307.

Feng PCC, Tran M, Chiu T, Sammons RD, Heck GR & CaJacob CA (2004). Investigations into glyphosate-resistant horseweed (*Conyza canadensis*): retention, uptake, translocation, and metabolism. *Weed Science* **52**, 498-505.

Foes MJ, Liu L, Tranel PJ, Wax LM & Stoller EW (1998). A biotype of common waterhemp (*Amaranthus rudis*) resistant to triazines and ALS herbicides. *Weed Science* **46**, 514-520.

Forlani G, Parisi B & Nielsen E (1994). 5-enol-Pyruvyl-Shikimate-3-Phosphate Synthase from *Zea mays* Cultured Cells. Purification and Properties. *Plant Physiology* **105**, 1107-1114.

Franssen AS, Skinner DZ, Al-Khatib K, Horak MJ & Kulakow PA (2001). Interspecific hybridization and gene flow of ALS resistance in *Amaranthus* species. *Weed Science* **49**, 598-606.

Franz JE, Mao MK & Sikorski JA (1997). Glyphosate: A Unique Global Herbicide. ACS Monograph 189, American Chemical Society, Washington DC, USA.

Gaines TA, Zhang W, Wang D et al. (2010). Gene amplification confers glyphosate resistance in Amarantus palmeri. *Proceedings of the National Academy of Sciences* **107**, 1029-1034.

Gaines TA, Ward SM, Bukun B, Preston C, Leach JE & P Westra (2012). Interspecific hybridization transfers a previously unknown glyphosate resistance mechanism in *Amaranthus* species. *Evolutionary Applications* **5**, 29-38.

Garland T, Dickerman AW, Janis CM & Jones JA (1993). Phylogenetic analysis of covariance by computer simulation. *Systematic Biology* **42**, 265-292.

Ge X, d'Avignon DA, Ackerman JJH & Sammons RD (2010). Rapid vacuolar sequestration: the horseweed glyphosate resistance mechanism. *Pest Management Science* **66**, 345-348.

Ge X, d'Avignon DA, Ackerman JJH, Duncan B, Spaur MB & Sammons RD (2011). Glyphosate-resistant horseweed made sensitive to glyphosate: Low-temperature suppression of glyphosate vacuolar sequestration revealed by ^{31}P NMR. *Pest Management Science* **67**, 1215-1221 .

Geiger DR & Bestman HD (1990). Self-limitation of herbicide mobility by phytotoxic action. *Weed Science* **38**, 324-329.

Geiger DR, Shieh WJ & Fuchs MA (1999). Causes of self-limited translocation of glyphosate in *Beta vulgaris* plants. *Pesticide Biochemistry and Physiology* **64**, 241-245.

Gianessi LP (2008). Economic impacts of glyphosate-resistant crops. *Pest Management Science* **64**, 346-352.

Goldsbrough PB, Hatch EM, Huang B, Kosinski WG, Dyer WE, Herrmann KM & Weller SC (1990). Gene amplification in glyphosate tolerant tobacco cells. *Plant Science* **72**, 53-62.

Gower, JC & Legendre, P (1986). Metric and Euclidean properties of dissimilarity coefficients. *Journal of Classification* **3**, 5-48.

Gressel J (2011). Low pesticide rates may hasten the evolution of resistance by increasing mutation frequencies. *Pest Management Science* **67**, 253-257.

Grossbard, E & D Atkinson (1985). The herbicide glyphosate. *Butterworth & Co. Ltd.*, ISBN 0-408-11153-4.

Gutierrez C (1999). Geminivirus DNA replication. *Cellular and Molecular Life Science* **56**, 313-329.

Häfliger E & Brun-Hool J (1969). Ciba-Geigy Weed Tables, A synoptic presentation of the flora accompanying agricultural crops. *CIBA-GEIGY Limited, Agrochemicals Division*

Hall L, Topinka K, Huffman J, Davis L & Good A (2000). Pollen flow between herbicide-resistant *Brassica napus* is the cause of multiple-resistant *B. napus* volunteers. *Weed Science* **48**, 688-694.

Hamill AS & Zhang J (1995). Quackgrass control with glyphosate and SC-0224 in corn and soybean. *Canadian Journal of Plant Science* **75**, 293-299.

Hammerton JL (1967). Environmental factors and susceptibility to herbicides. *Weeds* **15**, 330-336.

Hamrick JL & Godt MJW (1990). Allozyme diversity in plant species. pp. 43-63 in: Brown AHD, Clegg MT, Kahler AL & Weir BS (1990) *Plant population genetics, breeding, and genetic resources*, ISBN 0-87893-117-1.

Hance RJ (1976). Adsorption of glyphosate by soils. *Pesticide Science* **7**, 363-366.

Harlan JR & deWet JMJ (1965). Some thoughts about weeds. *Economic Botany* **19**, 16-24.

Harring T, Streibig JC & Husted S (1998). Accumulation of Shikimic Acid: A Technique for Screening Glyphosate Efficacy. *Journal of Agricultural and Food Chemistry* **46**, 4406-4412.

Hathway, DE (1989). Molecular Mechanisms of Herbicide Selectivity. *Oxford University Press, Oxford, New York, Tokyo.*

He M, Nie YF & Xu P (2003). A T42M substitution in bacterial 5-Enolpyruvylshikimate-3-phosphate synthase (EPSPS) generates enzymes with increased resistance to glyphosate. *Bioscience, Biotechnology, and Biochemistry* **67**, 1405-1409.

Heap I (2011). International survey of herbicide resistant weeds. http://www.weedscience.org, last accessed 31.12.2012.

Hemingway J & Ranson H (2000). Insecticide Resistance in Insect Vectors of Human Disease. *Annual Review of Entomology* **45**, 371-391.

Herrmann KM & Weaver LM (1999). The shikimate pathway. *Annual Review of Plant Physiology and Plant Molecular Biology* **50**, 473-503.

Heslop-Harrison JS & Schwarzacher T (2011). The plant genome: An evolutionary view on structure and function. Organisation of the plant genome in chromosomes. *The Plant Journal* **66**, 18-33.

Hess M, Barralis G, Bleiholder H, Buhr L, Eggers TH, Hack H & Stauss R (1997). Use of the extended BBCH-scale - general for the description of the growth stages of mono- and dicotyledonous weed species. *Weed Research* (**37**), 433-441.

Holm LG, Plucknett DL, Pancho JV & Herberger JP (1991). The World's Worst Weeds: Distribution and biology. 2nd edn. East West Center, University Press of Hawaii, Honululu, Hawaii, USA, 609; ISBN: 0 894 644 157.

Horak MJ & Loughin TM (2000). Growth analysis of four *Amarantus* species. *Weed Science* **48**, 347-355.

Horowitz M (1972). Early development of Johnsongrass. *Weed Science* **20**, 271-273.

HRAC (2011). Herbicide Resistance Action Committee. http://www.hracglobal.com, last accessed: 31.12.2011.

Huangfu CH, Song XL, Qiang S & Zhang HJ (2007). Response of wild *Brassica juncea* populations to glyphosate. *Pest Management Science* **63**, 1133-1140.

Huff DR, Peakall R & Smouse PE (1993). RAPD variation within and among natural populations of outcrossing buffalograss [*Buchloë dactyloides* (Nutt.) Engelm.]. *Theoretical and Applied Genetics* **86**, 927-934.

Hull RJ (1970). Germination control of Johnsongrass rhizome buds. *Weed Science* **18**, 118-121.

Jasieniuk M, Brûlè Babel AL & Morrison IN (1996). The evolution and genetics of herbicide resistance in weeds. *Weed Science* **44**, 176-193.

Jasieniuk M & Maxwell BD (2001). Plant diversity: New insights from molecular biology and genomics technologies. *Weed Science* **49**, 257-265.

Jasieniuk M, Ahmad R, Sherwood AM, Firestone JL, Perez-Jones A, Lanini WT, Mallory-Smith C & Zachary Stednick (2008). Glyphosate-resistant Italian Ryegrass (*Lolium multiflorum*) in California: distribution, response to glyphosate, and molecular evidence for an altered target enzyme. *Weed Science* **56**, 496-502.

Jaworski EG (1972). Mode of action of N-Phosphonomethylglycine: Inhibition of aromatic amino acid biosynthesis.*Journal of Agricultural and Food Chemistry* **20**, 1195-1198.

Jha P, Norsworthy JK, Riley MB, Bielenberg DG & Bridges W Jr. (2008). Acclimation of Palmer Amaranth (*Amaranthus palmeri*) to shading. *Weed Science* **56**, 729-734.

Johal GS & Huber DM (2009). Glyphosate effects on diseases of plant. *European Journal of Agronomy* **31**, 144-152.

Jones CJ, Edwards KJ, Castaglione S, Winfield MO, Sala F, van de Wiel C, Bredemeijer G, Vosman B, Matthes M, Daly A, Brettschneider R, Bettini P, Buiatti M, Maestri E, Malcevschi A, Marmiroli N, Aert R, Volckaert G, Rueda J, Linacero R, Vazquez A & Karp A (1997). Reproducibility testing of RAPD, AFLP and SSR markers in plants by a network of European laboratories. *Molecular Breeding* **3**, 381-390.

Kahrizi D, Salmanian AH, Afshari A, Moieni A & Mousavi A (2007). Simultaneous substitution of Gly96 to Ala and Ala183 to Thr in 5-enolpyruvylshikimate-3-phosphate synthase gene of *E. coli* (k12) and transformation of rapeseed (*Brassica napus* L.) in order to make tolerance to glyphosate. *Plant Cell Reports* **26** 95-104.

Kaloumenos NS & Eleftherohorinos IG (2009). Identification of a Johnsongrass (*Sorghum halepense*) biotype resistant to ACCase-inhibiting herbicides in northern Greece. *Weed Technology* **23**, 470-476.

Kapitonov VV & Jurka J (2001). Rolling-circle transposons in eukaryotes. *Proceedings of the National Academy of Science* **98**, 8714-8719.

Kaundun SS & Park Y-G (2002). Genetic structure of six Korean tea populations as revealed by RAPD-PCR markers. *Crop Science* **42**, 594-601.

Kaundun SS (2010). An aspartate glycine change in the carboxyl transferase domain of acetyl CoA carboxylase and non-target-site mechanism(s) confer resistance to ACCase inhibitor herbicides in a *Lolium multiflorum* population. *Pest Management Science* **66**, 1249-1256.

Keeley PE, Carter CH & Thullen RJ (1987). Influence of planting date on growth of Palmer Amaranth (*Amarantus palmeri*). *Weed Science* **35**, 199-204.

King SR (2007). Effect of mesosulfuron rate and formulation on wild oat (*Avena fatua*) control and malt barley tolerance. *Weed Technology* **21**, 771-776.

Kishore GM, Brundage L, Kolk K, Padgette SR, Rochester D, Huynh QK & Della Cioppa G (1986). Isolation, purification and characterization of a glyphosate-resistant mutant *E. coli* EPSP Synthase. *Federation of American Societies for Experimental Biology* **45**, 1506.

Kivlin PT & Doll JD (1988). Quackgrass (*Agropyron repens*) control with SC-0224 and glyphosate. *Weed Technology* **2**, 147-152.

Klingaman TE & Oliver LR (1994). Palmer Amaranth (*Amarantus palmeri*) interference in Soybeans (*Glycine max*). *Weed Science* **42**, 523-527.

Kluge AG & Farris JS (1969). Quantitative phyletics and the evolution of Anurans. *Systematic Biology* **18**, 1-32.

Knezevic SZ, Streibig JC & Ritz C (2007). Utilizing R software package for dose-response studies: The concept and data analysis. *Weed Technology* **21**, 840-848.

Koger CH & Reddy KN (2005). Role of absorption and translocation in the mechanism of glyphosate resistance in horseweed (*Conyza canadensis*). *Weed Science* **53**, 84-89.

Koger CH, Shaner DL, Henry WB, Nadler-Hassar T, Thomas WE & Wilcut JW (2005). Assessment of two nondestructive assays for detecting glyphosate resistance in horseweed (Conyza canadensis). *Weed Science* **53**, 438-445.

Larionov A, Krause A & Miller W (2005). A standard curve based method for relative real time PCR data processing. *BMC Bioinformatics* **6**, doi:10.1186/1471-2105-6-62.

Larney FJ & Blackshaw RE (2003). Weed seed viability in composted beef cattle feedlot manure. *Journal of Environmental Quality*, **32** 1105-1113.

LaRossa R & Schloss JV (1984). The sulfonurea herbicide sulfometuron Methyl is an extremly potent and selective inhibitor of acetolactate Synthase in *Salmonella typhimurium*. *The Journal of Biological Chemistry* **14**, 8753-8757.

Lebrun M, Leroux B & Sailland A (1997). Chimeric gene for the transformation of plants. *United States Patent* Patent number 5633448.

Legleiter TR & Bradley KW (2008). Glyphosate and multiple herbicide resistance in Common Waterhemp (*Amaranthus rudis* populations from Missouri. *Weed Science* **56**, 582-587.

Levitt G (1978). Herbicidal sulfonamides. U.S. Patent 4127405.

Li J, Smeda RJ, Sellers BA & Johnson WG (2005). Influence of formulation and glyphosate salt on absorption and translocation in three annual weeds. *Weed Science* **53**, 153-159.

Li X, Schuler MA & Berenbaum MR (2007). Molecular mechanisms of metabolic resistance to synthetic and natural xenobiotics. *Annual Review of Entomology* **52**, 231-53.

Lineweaver H & Burk D (1934). The determination of enzyme dissociation constants. *Journal of the American Chemical Society* **56**, 658-666.

Liu W, Harrison DK, Chalupska D, Gornicki P, O'Donnell CC, Adkins SW, Haselkorn R & Williams RR (2007). Single-site mutations in the carboxyltransferase domain of plastid acetyl-CoA carboxylase confer resistance to grass-specific herbicides. *Proceedings of the National Academy of Science* **104**, 3627-3632.

Livak KJ & Schmittgen TD (2001). Analysis of relative gene expression data using real time quantitative PCR and the $2(-^{\Delta\Delta}C(t))$ method. *Methods* **25**, 402-408.

Lorentz L, Beffa R & Kraehmer H (2011). Recovery of plants and histological observations on advanced weed stages after glyphosate treatment. *Weed Research* **51**, 333-343.

Lorraine-Colwill DF, Powles SB, Hawkes TR, Hollinshead PH, Warner SAJ & Preston C (2002). Investigations into the mechanism of glyposate resistance in *Lolium rigidum*. *Pesticide biochemistry and physiology* **74**, 62-72.

Lynch M & Milligan BG (1994). Analysis of population genetic structure with RAPD markers. *Molecular Ecology* **3**, 91-99.

Manalil S, Busi R, Renton M & Powles SB (2011). Rapid evolution of herbicide resistance by low herbicide dosage. *Weed Science* **59**, 210-217.

McCue KF & Conn EE (1990). Induction of shikimic acid pathway enzymes by light in suspension cultured cells of parsley (*Petroselinum crispum*). *Plant Physiology* **94**, 507-510.

McWorter CG, Jordan TN & Wills GD (1980). Translocation of ^{14}C-glyphosate in Soybeans (Glycine max) and Johnsongrass (Sorghum halepense). *Weed Science* **28**, 113-118.

Menchari Y, Camilleri C, Michel S, Brunel D, Dessaint F, Le Corre V & Delye C (2006). Weed response to herbicides: regional-scale distribution of herbicide resistance alleles in the grass weed *Alopecurus myosuroides*. *New Phytologist* **171**, 861-874.

Menchari Y, Delye C & Le Corre V (2007). Genetic variation and population structure in black-grass (*Alopecurus myosuroides* Huds.), a successful, herbicide resistant, annual grass weed of winter cereal fields. *Molecular Ecology* **16**, 3161-3172.

Menchari Y, Chauvel B, Darmency H & Délye C (2008). Fitness costs associated with three mutant acetylcoenzyme A carboxylase alleles endowing herbicide resistance in black-grass *Alopecurus myosuroides*. *Journal of Applied Ecology* **45**, 939-947.

Metraux JP (2002). Recent breakthroughs in the study of salicylic acid biosynthesis. *Trends in Plant Science* **7**, 332-334.

Michel A, Peterson J, Dogan MN & Ernst V (1999). Anleitung zur Anlage und Auswertung von Versuchen zur Erstellung quantitativer Dosis-Wirkungsbeziehungen am Beispiel der Wirksamkeit von Herbiziden. *Gesunde Pflanzen* **51**, 10-19.

Michitte P, De Prado R, Espinoza N, Ruiz-Santaella JP & Gauvrit C (2007). Mechanisms of resistance to glyphosate in a ryegrass (*Lolium multiflorum*) biotype from Chile. *Weed Science* **55**, 435-440.

Mikula BC (1995). Environmental programming of heritable epigenetic changes in paramutant *r*-gene expression using temperature and light at a specific stage of early development in maize seedlings. *Genetics* **140**, 1379-1387.

Monaghan N (1979). The biology of Johnson grass (*Sorghum halepense*). *Weed Research* **19**, 261-267.

Mortimer AM (1997). Phenological adaptation in weeds - an evolutionary response to the use of herbicides? *Pesticide Science* **51**, 299-304.

Mueller TC, Massey JH, Hayes RM, Main CL & Stewart CN (2003). Shikimate accumulates in both glyphosate-sensitive and glyphosate-resistant Horseweed (*Conyza canadensis* L. Cronq.). *Journal of Agricultural and Food Chemistry* **51**, 680-684.

Muers M (2011). Transcriptome to proteome and back to genome. *Nature Reviews Genetics* **12**, 518.

Muzik TJ & Mauldin WG (1964). Influence of environment on the response of plants to herbicides. *Weeds* **12**, 142-145.

Nafziger ED, Widholm JM, Steinrucken HC & Killmer JL (1984). Selection and characterization of a carrot line tolerant to glyphosate. *Plant Physiology* **76**, 571-574.

Naito K, Zhang F, Tsukiyama T, Saito H, Hancock CN, Richardson AO, Okumoto Y, Tanisaka T & Wessler SR (2009). Unexpected consequences of a sudden and massive transposon amplification on rice gene expression *Nature* **461**, 1130-1134.

Nei M (1973). Analysis of gene diversity in subdivided populations. *Proceedings of the National Academy of Science* **70**, 3321-3323.

Nei M (1978). Estimation of average heterozygosity and genetic distance from a small number of individuals. *Genetics* **89**, 583-590.

Nei M & Li W (1979). Mathematical model for studying genetic variation in terms of restriction endonucleases. *Proceedings of the National Academy of Science* **76**, 5269-5273.

Nei M & Chesser RK (1983). Estimation of fixation indices and gene diversities. *Annals of Human Genetics* **47**, 253-259.

Neve P, Vila-Aiub M & Roux F (2009). Evolutionary-thinking in agricultural weed management. *New Phytologist* **18**, 783-793.

Ng CH, Wickneswary R, Salmijah S & Ismail BS (2004a). Inheritance of glyphosate resistance in Goosegrass (*Eleusine indica*). *Weed Science* **52**, 564-570

Ng CH, Wickneswary R, Salmijah S, Teng YT & Ismail BS (2004b). Glyphosate resistance in *Eleusine indica* (L.) Gaertn. from different origins and polymerase chain reaction amplification of specific alleles. *Australian Journal of Agricultural Research* **55**, 407-414

Norsworthy JK (2008). Effect of tillage intensity and herbicide programs on changes in weed species density and composition in the southeastern coastal plains of the United States. *Crop Protection* **27**, 151-160.

Norsworthy JK, Smith KL, Steckel LE & Koger CH (2009). Weed seed contamination of Cotton gin trash. *Weed Technology* **23**, 574-580.

Nybom H & Bartish IV (2000). Effects of life histroy traits and sampling strategies on genetic diversity estimates obtained with RAPD markers in plants. *Perspectives in Plant Ecology, Evolution and Systematics* **3**, 93-114.

Nybom H (2004). Comparison of different nuclear DNA markers for estimating intraspecific genetic diversity in plants. *Molecular Ecology* **13**, 1143-1155.

Oerke EC (2006). Crop losses to pests. *The Journal of Agricultural Science* **144**, 31-43.

Oliveira JS, Mendes MA, Palma MS, Basso LA & Santos DS (2003). One-step purification of 5-enolpyruvylshikimate-3-phosphate synthase enzyme from Mycobacterium tuberculosis. *Protein Expression and Purification* **28**, 287-292.

Onofri A, Carbonell EA, Piepho HP, Mortimer AM & Cousens RD (2009). Current statistical issues in weed research. *Weed Reseach* **50**, 5-24.

O'Sullivan J, Zandstra J & Sikkema P (2002). Sweet corn (*Zea mays*)cultivar sensitivity to mesotrione. *Weed Technology* **16**, 412 - 425.

Owen MDK (2008). Weed species shifts in glyphosate-resistant crops. *Pest Management Science* **64**, 377-387.

Owen MDK (2011). Weed resistance development and management in herbicide-tolerant crops: experiences from the USA. *Journal of Consumer Protection and Food Safety* **6**, 85-89.

Padgette SR, Re DB, Gasser CS, Eichholtz DA, Frazier RB, Hironaka CM ,Levine EB, Shah DM, Fraley RT & Kishore GM (1991). Site-directed mutagenesis of a conserved region of the 5-enolpyruvylshikimate-3-phosphate synthase active site. *Journal of Biological Chemistry* **266** 22364-22369.

Patzoldt WL, Tranel PJ & Hager AG (2002). Variable herbicide responses among Illinois waterhemp (*Amaranthus rudis* and *A. tuberculatus*) populations. *Crop Protection* **21**, 707-712.

Papanikou E, Brotherton JE & Widholm JM (2004). Length of time in tissue culture can affect the selected glyphosate resistance mechanism. *Planta* **218**, 589-598.

Perez A & Kogan M (2003). Glyphosate-resistant *Lolium multiflorum* in Chilean orchards. *Weed Research* **43**, 12-19.

Perez A, Alister C & Kogan M (2004). Absorption, translocation and allocation of glyphosate in resistant and susceptible Chilean biotypes of *Lolium multiflorum*. *Weed Biology and Management* **4**, 1445-6664.

Perez-Jones A, Park KW, Polge N, Colquhoun J & Mallory-Smith CA (2007). Investigating the mechanisms of glyphosate resistance in *Lolium multiflorum*. *Planta* **226** 395-404.

Peterson J, Dresbach-Runkel M & Wagner J (2010). A method to determine the pollen-mediated spread of target-site resistance to acetyl-coenzyme A carboxylase inhibitors in black grass (*Alopecurus myosuroides* Huds.). *Journal of Plant Diseases and Protection* **117**, 122-128.

Pielberg G, Day AE, Plastow GS & Andersson L (2003). A sensitive method for detecting variation in copy numbers of duplicated genes. *Genome Research* **13**, 2171-2177.

Piffanelli P, Ramsay L, Waugh R, Benabdelmouna A, D'Hont A, Hollricher K, Jorgensen JH, Schulze-Lefert & Panstruga R (2004). A barley cultivation-associated polymorphism conveys resistance to powdery mildew. *Nature* **430**, 887-891.

Pihakaski S & Pihakaski K (1980). Effects of glyphosate on ultrastructure and photosynthesis of Pellia epiphylla. *Annals of Botany* **46**, 133-141.

Pline WA, Wilcut JW, Duke SO, Edmisten KL & Wells R (2002). Tolerance and application of shikimic acid in response to glyphosate applications in glyphosate-resistant and nonglyphosate-resistant Cotton (*Gossypium hirsutum* L.). *Journal of Agricultural and Food Chemistry* **50**, 506-512.

Pline-Srnic W (2005). Technical performance of some commercial glyphosate-resistant drops. *Pest Management Science* **61**, 225-234.

Pline-Srnic W (2006). Physiological mechanisms of glyphosate resistance. *Weed Technology* **20**, 290-300.

Porebski S, Bailey LG & Baum BR (1997). Modification of a CTAB DNA extraction protocol for plants containing high polysaccharide and polyphenol components. *Plant Molecular Biology Reporter* **15**, 8-15.

Powell W, Morgante M, Andre C, Hanafey M, Vogel J, Tingey S & Rafalski A (1996). The comparison of RFLP, RAPD, AFLP and SSR (microsatellite) markers for germplasm analysis. *Molecular Breeding* **2**, 225-238.

Powles SB, Lorraine-Colwill DF, Dellow JJ & Preston C (1998). Evolved resistance to glyphosate in rigid ryegrass (*Lolium rigidum*) in Australia. *Weed science* **46**, 604-607

Powles SB (2008). Evolved glyphosate-resistant weeds around the world: lessons to be learnt. *Pest Management Science* **64**, 360-365.

Powles SB (2010). Gene amplification delivers glyphosate-resistant weed evolution. *Proceedings of the National Academy of Science* **107**, 955-956.

Powles SB & Yu Q (2010). Evolution in Action: Plants resistant to herbicides. *Annual Review of Plant Biology* **61**, 317-347.

Pratley J, Urwin N, Stanton R, Baines P, Broster J, Cullis K, Schafer D, Bohn J & Krueger R (1999). Resistance to glyphosate in *Lolium rigidum*. I Bioevaluation. *Weed Science* **47**, 405-411.

Pratt DB & Clark LG (2001). *Amarantus rudis* and *A. tuberculatus* - one species or two? *Journal of the Torrey Botanical Society* **128**, 282-296.

Preston C & Wakelin AM (2008). Resistance to glyphosate from altered herbicide translocation patterns. *Pest Management Science* **64**, 372-376.

Price HJ, Dillon SL, Hodnett G, Rooney WL, Ross L & Johnston JS (2005). Genome evolution in the genus *Sorghum* (Poaceae). *Annals of Botany* **95**, 219-227.

Proctor VW (1968). Long-distance dispersal of seeds by retention in digestive tract of birds. *Science* **160**, 321-322.

R Development Core Team (2011). R: A language and environment for statistical computing. *R Foundation for Statistical Computing*, Vienna, Austria. ISBN 3-900051-07-0, URL http://www.R-project.org/.

Ream JE, Steinrucken HC, Porter CA & Sikorski JA (1988). Purification and properties of 5-Enolpyruvylshikimate-3-phosphate synthase from dark-grown seedlings of *Sorghum bicolor*. *Plant Physiology* **87**, 232-238.

Reinbothe S, Nelles A & Parthier B (1991). N-(Phosphonomethyl)glycine (glyphosate) tolerance in Euglena gracilis acquired by either overproduced or resistant 5-enolpyruvylshikimate-3-phosphate synthase. *European Journal of Biochemistry* **198**, 1432-1033.

Riar DS, Norsworthy JK, Johnson DB, Scott RC & Bagavathiannan M (2011). Glyphosate resistance in Johnsongrass (*Sorghum halepense*) biotype from Arkansas. *Weed Science* **59**, 299-304.

Ritz C & Streibig JC (2005). Bioassay Analysis using R. *Journal of Statistical Software* **12**, 1-22.

Ronaghi M (2001). Pyrosequencing sheds light on DNA sequencing. *Genome Research* **11**, 3-11

Rosset P (2009). Agrofuels, Food sovereignty, and the contemporary food crisis. *Bulletin of Science, Technology & Society* **29**, 189 - 193.

Rowe ML, Lee DJ, Nissen SJ, Bowditch BM & Masters RA (1997). Genetic variation in North American Leafy Spurge (*Euphorbia esula*) determined by DNA-markers. *Weed Science* **45**, 446-454.

Sachs L (2003). Angewandte Statistik, Anwendung statistischer Methoden. *Springer-Verlag Berlin Heidelberg New York* 11. Auflage, ISBN 3-540-40555-0.

Saitou N & Nei M (1987). The Neighbor-joining Method: A New Method for Reconstructing Phylogenetic Trees. *Molecular Biology and Evolution* **4**, 406-425.

Sammons RD, Ivashuta SI, Liu H, Wang D, Feng PC, Kouranov AY & Andersen SE (2011). Polynucleotide molecules for gene regulation in plants. Monsanto Technology LLC, *World patent* WO2011/112570 A1.

Sanchez-Rodriguez E, Moreno DA, Ferreres F, del Mar Rubio-Wilhelmi M & Ruiz JM (2011). Differential responses of five cherry tomato varieties to water stress: Changes on phenolic metabolites and related enzymes. *Phytochemistry* **72**, 723-729.

Sandegren L & Andersson DI (2009). Bacterial gene amplification: implications for the evolution of antibiotic resistance. *Nature* **7**, 578-588.

Sauer JD (1967). The grain Amaranths and their relatives: A revised taxonomic and geographic survey. *Missouri Botanical Garden Press* **54**, 103-137.

Sauer JD (1972). The dioecious Amaranths: a new species name and major range extensions. *Madrono* **21**, 426-434.

Schönbrunn E, Eschenburg S, Shuttleworth WA, Schloss JV, Amrhein N, Evans JNS & Kabsch W (2001). Interaction of the herbicide glyphosate with its target enzyme 5-enolpyruvylshikimate 3-phosphate synthase in atomic detail. *Proceedings of the National Academy of Science* **98**, 1376-1380.

Schwanhäusser B, Busse D, Li N, Dittmar G, Schuchhardt J, Wolf J, Chen W & Selbach M (2011). Global quantification of mammalian gene expression control. *Nature* **473**, 337-342.

Seefeldt SS, Jensen JE & Fuerst EP (1995). Log-logistic analysis of Herbicide dose-response relationships. *Weed Technology* **9**, 218-227.

Sellers BA, Smeda RJ, Johnson WG, Kendig JA & Ellersieck MR (2003). Comparative growth of six *Amaranthus* species in Missouri. *Weed Science* **51**, 329-333.

Shah J (2003). The salicylic acid loop in plant defense. *Current Opinion in Plant Biology* **6**, 365-371.

Shah DM, Horsch RB, Klee HJ, Kishore GM, Winter JA, Turner NE, Hironaka CM, Sanders PR, Gasser CS, Aykent S, Siegel NR, Rogers SG & Fraley RT (1986). Engineering herbicide tolerance in transgenic plants. *Science* **233**, 478-481.

Shaner D, Feist DA & Retzinger EJ (1997). SAMOA®: One Company's approach to herbicide-resistant weed management. *Pesticide Science* **51**, 367-370.

Shaner D, Nadler-Hassar T, Brien Henry W & Koger CH (2005). A rapid in vivo shikimate accumulation assay with excised leaf discs. *Weed Science* **53**, 769-774.

Shaner DL (2009). Role of translocation as a mechanism of resistance to glyphosate. *Weed Science* **57**, 118-123.

Shields EJ, Dauer JT, VanGessel MJ & Neumann G (2006). Horseweed (*Conyza canadensis*) seed collected in the planetary boundary layer. *Weed Science* **54**, 1063-1067.

Singh BK & Shaner D (1998). Rapid determination of glyphosate injury to plants and identification of glyphosate-resistant plants. *Weed Technology* **12**, 527-530.

Sitthiwong K, Matsui T, Sukprakarn S, Okuda N & Kosugi Y (2005). Classification of Pepper (*Capsicum annuum* L.) accessions by RAPD analysis. *Biotechnology* **4**, 305-309.

Slack A, Thornton PC, Magner DB, Rosenberg SM & Hastings PJ (2006). On the mechanism of gene amplification induced under stress in Escherichia coli. *Public Library of Science Genetics* **2**, 385-398.

Slotta TA (2008). What we know about weeds: Insights from genetic markers. *Weed Science* **56**, 322-326.

Smart CC, Johänning D, Müller G & Amrhein N (1985). Selective Overproduction of 5-*enol*-Pyruvylshikimic Acid 3-Phosphate synthase in a plant cell culture which tolerates high doses of the herbicide glyphosate. *The Journal of Biological Chemistry* **260**, 16338-16346.

Smeda RJ, Snipes CE & Barrentine WL (1997). Identification of graminicide-resistant johnsongrass (*Sorghum halepense*). *Weed Science* **45**, 132-137.

Sprague CL, Stoller EW, Wax LM & Horak MJ (1997). Palmer amaranth (*Amaranthus palmeri*) and common waterhemp (*Amaranthus tuberculatus*) to selected ALS-inhibiting herbicides. *Weed Science* **45**, 192-197.

Staub JE, Danin-Poleg Y, Fazio G, Horejsi T, Reis N & Katzir N (2000). Comparative analysis of cultivated melon groups (*Cucumis melo* L.) using random amplified polymorphic DNA and simple sequence repeat markers. *Euphytica* **115**, 225-241.

Steckel LE & Sprague CL (2004). Common Waterhemp (*Amaranthus rudis*) interference in corn. *Weed Science* **52**, 359-364.

Steckel LE, Main CL, Ellis AT & Mueller TC (2008). Palmer Amaranth (*Amarantus palmeri*) in Tennessee has low level glyphosate resistance. *Weed Technology* **22**, 119-123.

Steinau AN, Skinner DZ & Steinau M (2003). Mechanism of extreme genetic recombination in weedy *Amaranthus* hybrids. *Weed Science* **51**, 696-701.

Steinrücken HC & Amrhein N (1980). The herbicide glyphosate is a potent inhibitor of 5-enolpyruvylshikimic acid-3-phosphate synthase. *Biochemical and Biophysical Research Communications* **94**, 1207-1212.

Stewart CN, Tranel PJ, Horvath DP, Anderson JV, Rieseberg LH, Westwood JH, Mallory-Smith CA, Zapiola ML & Dlugosch KM (2009). Evolution of Weediness and Invasiveness: Charting the Course for Weed Genomics. *Weed Science* **57**, 451-462.

Strek HJ, Hess M & Kaiser J (2012). Glyphosat-Resistenz in amerikanische Reihenkulturen und Lehren für die Unkrautbekämpfung in Europa. *25th German Conference on Weed Biology and Weed Control*, 171-176.

Sun S, Berg OG, Roth JR & Andersson DI (2009). Contribution of gene amplification to evolution of increased antibiotic resistance in *Salmonella typhimurium*. *Genetics* **182**, 1183-1195.

Sost D, Schulz A & Amrhein N (1984). Characterization of a glyphosate-insensitive 5-enolpyruvylshikimic acid-3-phosphate synthase. *Federation of European Biochemical Societies. Letters* **173**, 238-242.

Southern EM (1975). Detection of specific sequences among DNA fragments separated by gel electrophoresis. *Journal of Molecular Biology* **98** 503-517.

Tamagnone L, Merida A, Stacey N, Plaskitt K, Parr A, Chang CF, Lynn D, Dow JM, Roberts K & Martin C (1998). Inhibition of phenolic acid metabolism results in precocious cell death and altered cell morphology in leaves of transgenetic tobacco plants. *The Plant Cell* **10**, 1801-1816.

Thormann CE, Ferreira ME, Camargo LEA, Tivang JG & Osborn TC (1994). Comparison of RFLP and RAPD markers to estimating genetic relationships within and among cruciferous species. *Theoretical and Applied Genetics* **88**, 973-980.

Timmons FL (1970). A history of weed control in the United States and Canada. *Weed Science* **18**, 294-307.

Tranel PJ, Riggins CW, Bell MS & Hager AG (2010). Herbicide resistances in *Amaranthus tuberculatus*: A call for new options. *Journal of Agricultural and Food Chemistry* **59**, 5808-5812

Tomlin CDS (2000). The Pesticide Manual; fifteenth edition. *British Crop Protection Council*, ISBN 1901396185.

Tong XH, Daud MK, Sun YQ & Zhu SJ (2009). Physiological and molecular mechansims of glyphosate tolerance in an *in vitro* selected cotton mutant. *Pesticide Biochemistry and Physiology* **94** 100-106.

Tuesca D & Puricelli E (2007). Effect of tillage systems and herbicide treatments on weed abundance and diversity in a glyphosate resistant crop rotation. *Crop Protection* **26**, 1765-1770.

Vaithanomsat P & Brown KA (2007). Isolation and mutation of recombinant EPSP synthase from pathogenic bacteria *Pseudosomonas aeruginosa*. *Process Biochemistry* **42**, 592-598.

VanGessel MJ (2001). Rapid Publication: Glyphosate-resistant horseweed from Delaware. *Weed Science* **49**, 703-705.

Velini ED, Alves E, Godoy MC, Meschede DK, Souza RT & Duke SO (2008). Glyphosate applied at low doses can stimulate plant growth. *Pest Management Science* **64**, 489-496.

Vereecken H (2005). Mobility and leaching of glyphosate: a review. *Pest Management Science* **61**, 1139-1151.

Vila-Aiub MM, Neve P & Powles SB (2005). Resistance cost of a cytochrome P450 herbicide metabolism mechanism but not an ACCase target site mutation in a multiple resistant *Lolium rigidum* population. *New Phytologist* **167**, 787-796.

Vila-Aiub MM, Neve P & Powles SB (2009). Fitness costs associated with evolved herbicide resistance alleles in plants. *New Phytologist* **184**, 751-767.

Wakelin, AM, Lorraine-Colwill, DF & Preston, C (2004). Glyphosate resistance in four different populations of Lolium rigidum is associated with reduced translocation of glyphosate to meristematic zones. *Weed Research* **44**, 453-459.

Wakelin AM & Preston C (2006). A target site mutation is present in a glyphosate-resistant *Lolium rigidum* population. *Weed Research* **46**, 432-440.

Warwick SI (1991). Herbicide resistance in weedy plants: Physiology and population biology. *Annual Review of Ecology and Systematics* **22**, 95-114.

Warwick SI & Black LD (1983). The biology of Canadian weeds. 61. *Sorghum halepense* (L.) Pers.. *Canadian Journal of Plant Science* **63**, 997-1014.

Warwick SI, Legere A, Simard MJ & James T (2008). Do escaped transgenes persist in nature? The case of an herbicide resistance transgene in a weedy *Brassica rapa* population. *Molecular Ecology* **17**, 1387Ũ1395.

Watanabe T, Tanabe H & Horiuchi T (2011). Gene amplification system based on double rolling-circle replication as a model for oncogene-type amplification. *Nucleic Acids Research* **39**, e106.

Watrud LS, Lee EH, Fairbrother A, Burdick C, Reichman JR, Bollman M, Storm M, King G & Van de Water PK (2004). Evidence for landscape-level, pollen-mediated gene flow from genetically modified creeping bentgrass with CP4 EPSPS as a marker. *Proceedings of the National Academy of Sciences* **101**, 14533-14538.

Weaver LM & Hermann KM (1997). Dynamics of the shikimate pathway in plants. *Trends in Plant Science* **2**, 346-351.

Webster TM (2005). Weed survey-southern states: broadleaf crops subsection. *Proceedings of the Southern Weed Science Society* **58**, 291-304.

Webster TM & Sosnoskie LM (2010). Loss of glyphosate efficacy: A changing weed spectrum in georgia cotton. *Weed Science* **58**, 73-79.

Welsh J & McClelland M (1990). Fingerprinting genomes using PCR with arbitrary primers. *Nucleic Acids Research* **18**, 7213-7218.

Widholm JM, Chinnala AR, Ryu JH, Song HS, Eggett T & Brotherton JE (2001). Glyphosate selection of gene amplification in suspension cultures of 3 plant species. *Physiologia Plantarum* **112**, 540-545.

Williams CS & Hayes RM (1984). Johnsongrass (*Sorghum halepense*) competition in soybeans (*Glycine max*). *Weed Science* **32**, 498-501.

Williams JG, Kubelik AR, Livak KJ, Rafalski JA & Tingey SV (1990). DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. *Nucleic Acids Research* **18**, 6531-6535.

Williams GM, Kroes R & Munro IC (2000). Safty evaluation and risk assessment of the herbicide roundup and its active ingradient, glyphosate, for humans. *Regulatory Toxicology and Pharmacology* **31**, 117-165.

Wright SR, Coble HD, Raper CD Jr. & Rufty TW Jr. (1999). Comparative responses of Soybean (*Glycine max*), sicklepod (*Senna obtusifolia*), and Palmer amaranth (*Amarantus palmeri*) to root zone and aerial temperatures. *Weed Science* **47**, 167-174.

Yeh FC & Boyle TJB (1997). Population genetic analysis of co-dominant and dominant markers and quantitative traits. *Belgian Journal of Botany* **129**, 157. Software free available at: http://www.ualberta.ca/~fyeh/popgene_download.html

Yu Q, Cairns A & Powles S (2007). Glyphosate, paraquat and ACCase multiple herbicide resistance evolved in a *Lolium rigidum* biotype. *Planta* **225**, 499-513.

Yu Q, Abdallah I, Han H, Owen M & Powles S (2009). Distinct non-target site mechanisms endow resistance to glyphosate, ACCase and ALS-inhibiting herbicides in multiple herbicide-resistant *Lolium rigidum*. *Planta* **230**, 713-723.

Yuan CI, Hsieh YC, Lin LC & Chiang MY (2006). Study on resistance of broadleaf fleabane *Conyza sumatrensis* to glyphosate and variation of 5-enolpyruvylshikimate-3-phosphate synthase gene. *Zhiwu Baohu Xuehui Huikan* **48**, 229-241.

Zelaya IA, Owen MDK & VanGessel MJ (2004). Inheritance of evolved glyphosate resistance in *Conyza canadensis* (L.) Cronq. *Theoretical and Applied Genetics* **110**, 58-70

Zelaya IA & Owen MDK (2005). Differential response of *Amaranthus tuberculatus* (Moq ex DC) JD Sauer to glyphosate. *Pest Management Science* **61**, 936-950.

Zhu J, Patzold WL, Shealy RT, Vodkin LO, Clough SJ & Tranel PJ (2008). Transcriptome Response to Glyphosate in Sensitive and Resistant Soybean. *Journal of Agricultural and Food Chemistry* **56**, 6355-6363.

9 Supplements

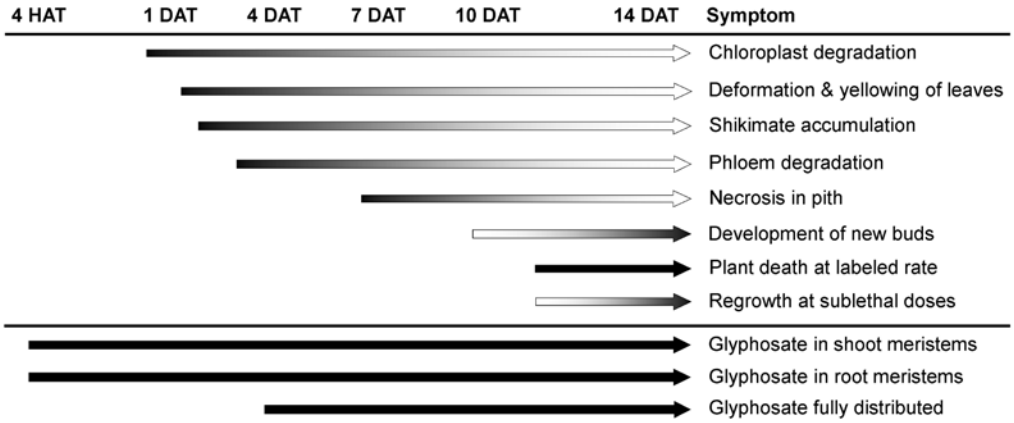

Fig. 45: General symptoms of *A. palmeri* after glyphosate application at labeled rate; changes in plant tissues and herbicide distribution in plant after application of glyphosate in a time frame of 14 days, according to Lorentz et al. (2011).

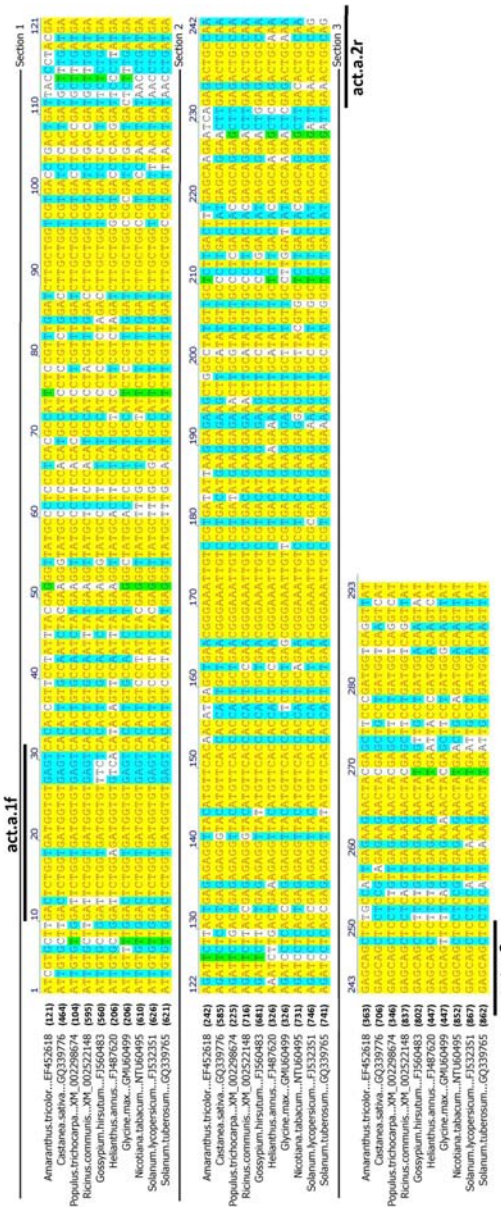

Fig. 46: Localization of the oligonucleotide sequences act.a.1f & act.a.2r, Tab. 5 used to determine the EPSPS gene expression of *A. palmeri* & *A. tuberculatus* in the alignment of *Actin* sequences of *Amaranthus tricolor* (NCBI accession number: EF452618); *Helianthus annus* (NCBI accession number: FJ487620); *Solanum lycopersicum* (NCBI accession number: FJ532351); *Gossypium hirsutum* (NCBI accession number: FJ560483); *Glycine max* (NCBI accession number: GMU60499); *Solanum tuberosum* (NCBI accession number: GQ339765); *Castanea sativa* (NCBI accession number: GQ339776); *Nicotiana tabacum* (NCBI accession number: NTU60495); *Populaus trichocarpa* (NCBI accession number: XM_002298674); *Ricinus communis* (NCBI accession number: XM_002522148).

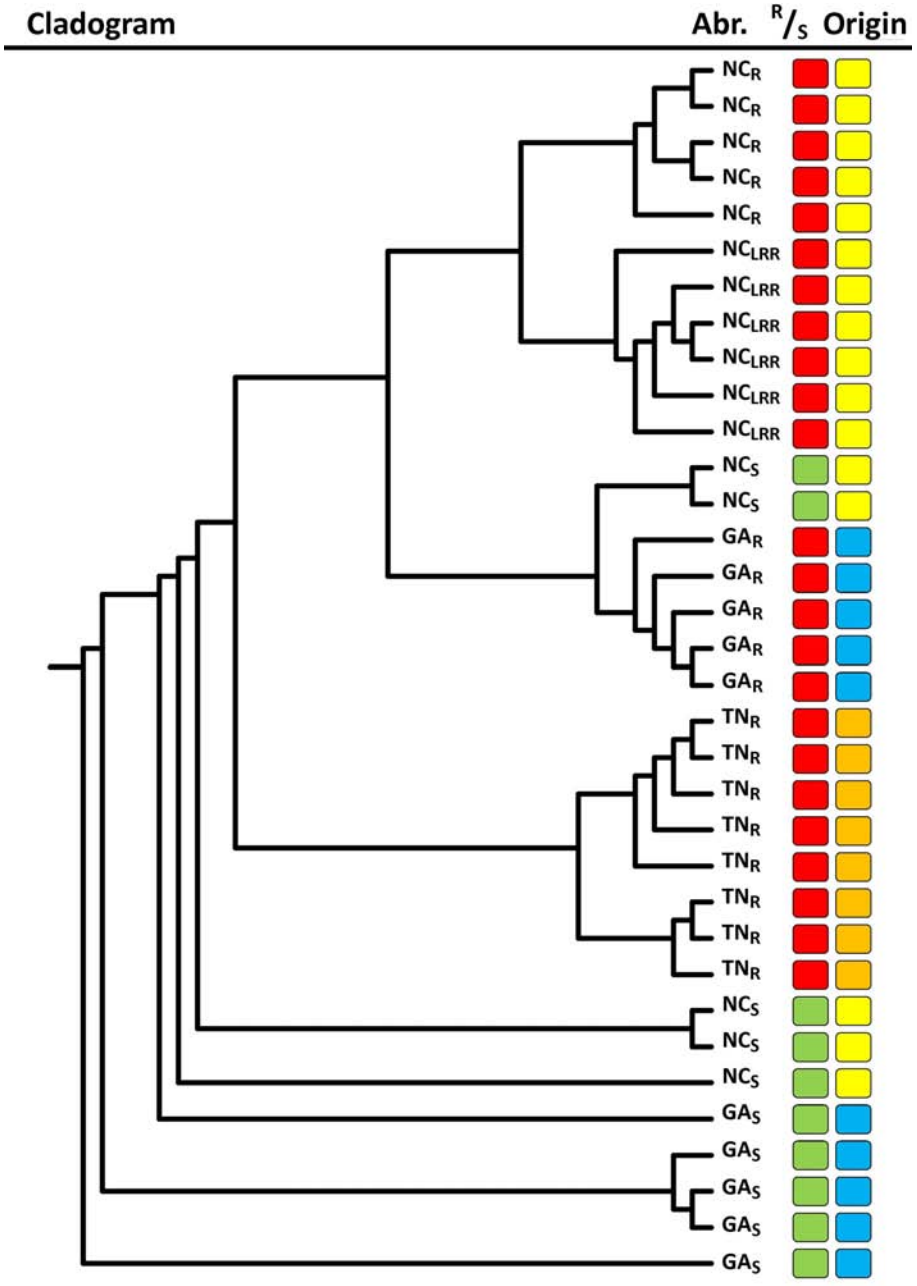

Fig. 47: Cladogram of *A. palmeri* populations; relationship of the populations GA_S, NC_S, NC_{LRR}, NC_R, TN_R and GA_R based on 243 RAPD-marker; calculation of the unrooted phylogenetic tree based on the most parsimonious method according to Felsenstein (1985) & Felsenstein (1989), the color code on the graphic is related to the glyphosate response (left side) and to the geographical origin (right side, according to Tab. 2) of the populations, green color on the left side indicates glyphosate sensitive populations, red color indicates glyphosate resistant populations.

Fig. 48: RAPD-markers of the relationship analyses (1) in Fig. 24 & 47 among *A. palmeri* populations GA_S, NC_S, NC_{LRR}, NC_R, TN_R and GA_R based on 243 RAPD-marker; naming of the populations according to Tab. 2.

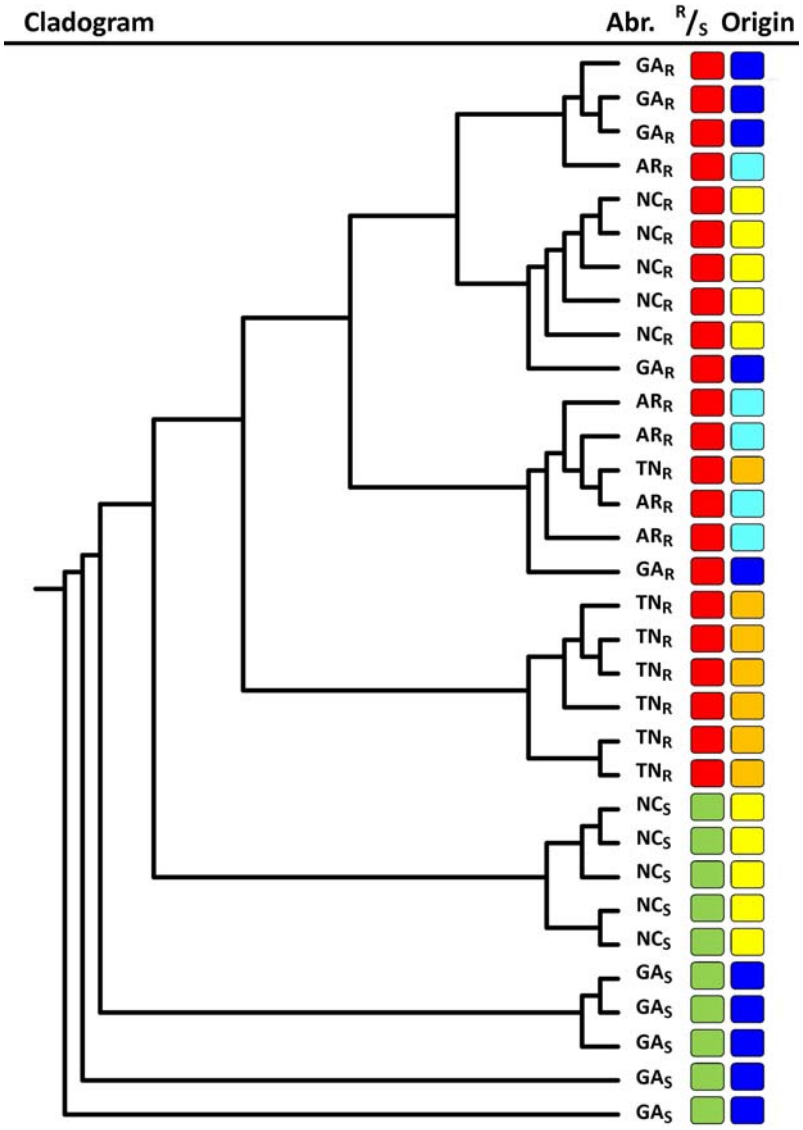

Fig. 49: Cladogram of *A. palmeri* populations; relationship of the populations GA_S, NC_S, NC_R, GA_R, AR_R and TN_R based on 202 RAPD-marker; calculation of the unrooted phylogenetic tree based on the most parsimonious method according to Felsenstein (1985) & Felsenstein (1989), the color code on the graphic is related to the glyphosate response (left side) and to the geographical origin (right side, according to Tab. 2) of the populations, green color on the left side indicates glyphosate sensitive populations, red color indicates glyphosate resistant populations.

Fig. 50: RAPD-markers of the relationship analyses (2) in Fig. 25 & 49 among the *A. palmeri* populations GA_S, NC_S, NC_R, GA_R, AR_R and TN_R based on 202 RAPD-marker; naming of the populations according to Tab. 2.

Fig. 51: RAPD-markers of the relationship analyses in Fig. 34 among the *A. tuberculatus* populations IL_1, Mo_{13} and Mo_{18} based on 117 RAPD-marker; naming of the populations according to Tab. 3.

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