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Distribution, detection and genetic background of herbicide-resistant *Alopecurus myosuroides* (Huds.) in Germany

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Index of abbreviations

***	Highest significant, $p \leq 0.001$
**	Highly significant, $p \leq 0.01$
*	Significant, $p \leq 0.05$
®	Registered trademark
Ø	Average
µ	Micro
a.i.	Active ingredient
a. m.	Ante meridiem
ACCase	Acetyl-coenzyme-A-carboxylase
AIC	Akaike information criterion
ALS	Acetolactate synthase
ATP	Adenosintriphosphat
ANOVA	Analysis of variance
BBCH-scale	Biologische Bundesanstalt, Bundessortenamt und Chemische Industrie, Growth stages of mono- and dicotyledonous plants
BC	Backcross
bp	Base-pair
BVL	Bundesamt für Verbraucherschutz und Lebensmittelsicherheit
dat	Day after treatment
DEN	Phenylpyrazoline
df	Degree of freedom
DIM	Cyclohexanediones
DNA	Deoxyribonucleic acid
e.g.	Example given
ED ₅₀	Herbicide dose at which plant response is reduced by 50%
F0	Parental generation
F1	Filial generation 1
FOP	Aryloxyphenoxy-propionate
GST	Glutathione S-transferase
HRAC	Herbicide Resistance Action Committee
HWE	Hardy-Weinberg equilibrium
KNO ₃	Potassium nitrate
M	Mol

MoA	Mode of action
n	Number
NaOH	Sodium hydroxide
NTSR	Non-target-site resistance
OECD	Organisation for economic co-operation and development
OSR	Oilseed rape
p	Probability of error
P450	Cytochrome P450 monooxygenase
PCR	Polymerase chain reaction
pH	Potentia hydrogenii
p. m.	Post meridiem
PRE	Pre emergence herbicide
PS II	Photosystem two
R	Resistant
r	Coefficient of correlation
r ²	Coefficient of determination
rep	Replication
RF	Resistance factor
RR	Homozygote resistant
RS	Heterozygote resistant
S	Susceptible
SNP	Single nucleotide polymorphism
SS	Homozygote susceptible
TSR	Target-site resistance
UK	United Kingdom
USA	United States of America
VLCFA	Very long chain fatty acid
WB	Winter barley
WW	Winter wheat

1 General introduction

In comparison to animal pests and pathogens (losses of 18% and 16%, respectively) weeds caused the highest potential yield loss (34%) on average (Oerke 2006). Before the invention of chemical weed control treatments, weed management largely depended on hand or mechanical weeding. The introduction of herbicides has dramatically changed the production of many crops. The first chemicals used for weed control were the inorganic copper salts in the early 1900s, and sulphuric acid some years later (Hamill et al. 2004). Herbicides increasingly replaced the labour-intensive mechanical weed control measures. The hormone-type herbicides with 2,4-dichlorophenoxyacetic acid introduced in 1942 were the first class of selective herbicides which revolutionised weed control in cereals. Today, in many countries weed control is dependent on herbicide use. However, the number of reported cases of herbicide-resistant weeds is increasing. These resistant weeds endanger the productivity of modern cropping systems in many regions of the world. Herbicide resistance is defined as “the 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” (WSSA 1998). The first case of an herbicide-resistant weed was found in 1968 (Heap 2014). Common groundsel (*Senecio vulgaris*) in a Washington tree nursery was shown to be resistant to herbicides in the triazine chemical class. Since that time, biotypes of 238 species (138 dicots and 100 monocots) expressing resistance to 22 different herbicide classes and 155 different herbicides have been found globally (Heap 2014). Herbicide-resistant weeds have been reported in 84 crop species in 65 countries (Heap 2014). The highest numbers of resistant weeds were found in the USA, Central Europa, Australia, and Canada (Heap 2014). These weeds occur especially in major crops (wheat, corn, rice, and soybean) in areas with high productivity where farmers rely primarily on chemical weed control measures (Heap & LeBaron 2001; Chauvel et al. 2009; Heap 2014). The Poaceae plant family is the family with the highest number of resistant weed species ($n = 75$) worldwide. One of Europe’s most problematic weeds *Alopecurus myosuroides* (Huds.) (black-grass) belongs to the Poaceae family. This species is well adapted to winter wheat. It is an allogamous, wind- and cross-pollinating grass with high genetic variation both within and between populations (Naylor 1972; Oleksiak et al. 2002; Menchari et al. 2007). The species can rapidly build up infestations which dramatically reduce yields and cause severe economic damage (Moss 1980; Orson 1999).

1.1 History of herbicide-resistant black-grass in Europe

The first cases of herbicide-resistant black-grass which targeted photosystem two (PS II) and acetyl-Co A carboxylase (ACCase) inhibitors were found in 1982 in the United Kingdom. Since then, resistance has been reported in different parts of Europe to triazines (Yaacoby et al. 1986; Mikulka 1987), substituted ureas (Niemann & Pestemer 1984; Moss & Cussans 1985), acetolactate synthase (ALS) inhibitors (Tranel & Wright 2002), and ACCase inhibitors (Eelen et al. 1996; Moss et al. 1999). All in all, 30 black-grass biotypes with different resistances were documented in Europe (Heap 2014). The reasons for the increasing number of herbicide resistances are various. The domination of cereals in narrow crop rotations, as well as early sowing, reduced tillage, and the intensive use of selective herbicides without changing the modes of action have increased the resistance evolution in weeds (Warwick 1991; Moss & Clark 1994; Chauvel et al. 2001; Beckie 2006; Chauvel et al. 2009). In particular, herbicides with only a single site of action were especially affected by herbicide resistance (e.g. ACCase and ALS inhibitors) (Heap 2014).

1.2 Resistance mechanisms

Two primary mechanisms are known to confer resistance in weeds. They differ according to their genetic basis, their distribution, and the background knowledge which is available. Both mechanisms have been found in the grass weed *A. myosuroides*. One mechanism is monogenetic target-site resistance (TSR) which is dominantly inherited (Délye et al. 2005a). It is the result of an altered target enzyme in which single point mutations change the amino acid structure, and exclude herbicides from effectively binding to the target enzyme. Herbicides which contain single site of action are particularly affected by this resistance mechanism. Seven alleles of the ACCase gene confer resistance (Délye et al. 2002; 2003; 2005b), and two alleles of the ALS gene are known to confer TSR in *A. myosuroides* (Tranel & Wright 2002; Délye & Boucansaud 2008). Plants with TSR are characterised by high resistance levels to a specific herbicide or herbicide group. Target-site gene mutations which endow herbicide resistance can be identified precisely by genetic analysis (Kaudun & Windass 2006; Délye & Boucansaud 2008). The frequencies of ACCase TSR in black-grass in some European countries was investigated by Délye et al. (2010), and proved to play a minor part in the herbicide resistance which was observed in Europe (25% in France, 14% in UK, 2% in Belgium, and 4% in Germany). However, this powerful selective pressure, targeting a single gene or a few genes, is consequently expected to rapidly increase the frequency of mutations conferring adapted phenotypes (Délye et al. 2004; Menchari et al. 2006; Moss et al. 2007; Délye et al. 2010).

The second mechanism is the so called “non-target-site resistance” (NTSR), which is a set of mechanisms governing the weed response to the abiotic stress exerted by herbicide application, which cause a reduction in the amount of herbicide molecules reaching their target site. NTSR plays a major role in resistance to the grass-specific herbicides inhibiting ACCase and ALS as well as other herbicides (Délye et al. 2010; Délye et al. 2013). To date, the genetic basis of NTSR is poorly understood (Preston 2003; Petit et al. 2010). It is known to be a polygenic, quantitative trait that can confer resistance to multiple modes of action (Petit et al. 2010). Biochemical studies have shown the involvement of different enzyme activities in NTSR, which can reduce the rate of herbicide penetration or translocation in the plant, or enhance the degradation of herbicide molecules by enzymes contributing to the general plant metabolism. In contrast to herbicide target-site mutations, fewer non-target mechanisms have been elucidated at the molecular level because of the inherently complicated biochemical processes and the limited genomic information available for weedy species (Yuan et al. 2007). Gene families involved in NTSR are cytochrome P450 mono-oxygenase, glutathione S-transferase (GST), and glycosyltransferase (Yuan et al. 2007).

1.3 Evolution of herbicide resistance

The described mechanisms lead to a broad range of resistances. More and more black-grass biotypes with resistance to more than one single site of action have evolved (Heap 2014). The study conducted by Letouzé and Gasquez (2001) detected that both mechanisms, TSR and NTSR, can coexist in the same plant. There are many investigations which show that herbicide resistance is nuclear inherited, which allows the resistance alleles to be moved through both pollen and seed (Letouzé & Gasquez 2001; Busi et al. 2011; Busi et al. 2012). The only known exception was found for triazine resistance, which is inherited cytoplasmically (Darmency & Gasquez 1981). The outcrossing nature of black-grass enables the combination and accumulation of a number of different resistance genes in one plant (Christopher et al. 1992; Preston 2003; Zhang & Powles 2006). This might explain why herbicide-resistant plants were found before certain herbicides had been introduced to the market (Petit et al. 2010). The plants accumulate the ability to withstand different herbicides with different sites of action. Due to the ongoing selection by herbicides, the number of resistant plants is increasing, which can result in higher costs for herbicide control and lower crop yields. In some cases herbicide resistance can reduce the profitability of agriculture dramatically.

1.4 Objective of the thesis and aim of the study

The problem of herbicide resistance is a major challenge to agriculture (Powles & Yu 2010). Therefore, the present study focused on different aspects addressing herbicide-resistant black-grass in Germany.

The first paper is focused on ACCase and ALS-resistant black-grass samples in Germany. It pertains to the distribution and development of TSR in Germany over a time scale of nine years. The research questions were: How widespread is TSR in Germany? Has the amount of TSR changed over the years? Are there “TSR-hot spots” in Germany? Answers to these questions will provide deeper knowledge about the distribution and development of TSR in Germany, and may contribute to highlighting the cropping systems which are problematic from the point of view of herbicide resistance.

As herbicide resistance is mainly conferred by NTSR (Délye et al. 2010), the second paper investigated the inheritance of NTSR in six different black-grass plants. How many genes are involved in NTSR? Are there differences between the plants, and are there typical resistance patterns which are repeated in different plants? Knowledge about this is important for estimations of further spread and occurrence of herbicide resistance.

The third paper deals with a more practical question. It is known that diverse sites of action can be affected by NTSR. First populations with resistance to pre-emergence herbicides belonging to the HRAC groups C₂, N, and K₃ were detected (Heap 2014). Considering this, a monitoring programme for the early detection of decreasing efficacies of pre-emergence herbicides is needed. Each case of detected resistance or decreasing herbicide efficacy can avoid erroneous applications. Therefore, the spread of those resistances might be delayed by field specific measures. Hence, the third paper was aimed at finding a reliable test system.

In summary, the studies conducted in the present thesis dealt with the spread and background of herbicide-resistant black-grass in Germany, in order to complement the existing knowledge about herbicide-resistant black-grass. This knowledge will support the management of herbicide resistant weeds in the future.

2 Publications

The present cumulative thesis consists of three articles which have been published in pre-reviewed, international journals.

Publication I:

Development of target-site resistance (TSR) in *Alopecurus myosuroides* (Huds.) in Germany between 2004 and 2012

Publication II:

Segregation of non-target-site based resistance to herbicides in multiple resistant *Alopecurus myosuroides* plants

Publication III:

Bioassay development for the identification of pre-emergence herbicide resistance in *Alopecurus myosuroides* (Huds.) populations

2.1 Publication I

ROSENHAUER M, JASER B, FELSENSTEIN F and PETERSEN J (2013) **Development of target-site resistance (TSR) in *Alopecurus myosuroides* (Huds.) in Germany between 2004 and 2012.** *Journal of Plant Diseases and Protection*, **120** (4), 179-187.

This article is online available at:

<http://www.jpdp-online.com/Development-of-target-site-resistance-TSR-in-iAlopecurus-myosuroidesi-in-Germany-between-2004-and-2012,QUIEPTQwNDI5NzgmTUIEPTUxODAyJkFST09UPTE1MjE1MCZURU1QX01BSU49U2NpZW50aWZpY3NfUG9ydHJhaXQuaHRt.html?UID=36F54DB2942486FA9653A2A3F1AF176F79E7BFE46D6F84E6>

Abstract

Alopecurus myosuroides (black-grass) is one of the most important grass weeds on arable land in Germany. For many years, it has developed herbicide-resistant populations. ACCase- and ALS-inhibiting herbicides, in particular, have lost significant efficacy. Two different mechanisms are commonly known to confer resistance to herbicides: target-site resistance (TSR) and non-target-site resistance (NTSR). In Germany, only approximately 5 to 10% of the herbicide-resistant black-grass populations have been reported to show TSR so far. To quantify the status and the distribution of TSR in Germany, 653 black-grass samples were collected between 2004 and 2012 all over Germany. The samples were tested by greenhouse bioassays and genetic analysis. Averaged over all tested samples, ACCase-TSR increased from 5.0% in 2004 to 54.3% in 2011. In 2012, the proportion of ACCase-TSR was lower than the previous years (38.5%). Similarly, ALS-TSR rose from 0.8% in 2007 to 13.9% in 2012. Regions with a high density of herbicide resistant black-grass samples were identified in the north and south of Germany. The data suggest a rapidly working selection of herbicide-resistant populations which may be due to current agricultural practices and the increasing use of herbicides acting on a single site.

Key words: ACCase inhibitors, ALS inhibitors, black-grass, herbicide resistance, non-random screening

2.2 Publication II

ROSENHAUER M, FELSENSTEIN F, PIEPHO H-P, HÖFER M and PETERSEN J (2015) **Segregation of non-target-site based resistance to herbicides in multiple resistant *Alopecurus myosuroides* plants.** *Weed Research*, 55 (3), 298-308.

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Abstract

Non-target-site resistance (NTSR) comprises a set of mechanisms conferring resistance to multiple modes of action. Investigation of the number of loci involved in NTSR will aid in the understanding of these resistance mechanisms. Therefore, six different multiple herbicide-resistant *Alopecurus myosuroides* (Huds.) plants with different herbicide histories were crossed in two generations with a susceptible wild-type. Seeds from the backcrossing generation were studied for their segregation rate for resistance to five different herbicides with four different modes of action belonging to the HRAC-groups C₂, A, B, and K₃. Taking into account that NTSR is a set of quantitative traits, the numbers of loci controlling NTSR were estimated using a normal mixture model fitted by the NLMIXED procedure of SAS. Each resistance to a different herbicide was controlled by a different number of loci comparing the six plants. In most of the cases, chlorotoluron resistance was controlled by one locus, whereas resistance to fenoxaprop-P-ethyl needed one or two loci. Resistance to pinoxaden was in all plants conferred by two loci. Cross-resistance of fenoxaprop-P-ethyl and pinoxaden was found in all backcrossings, indicating that at least one of the two loci is responsible for both resistances. Resistance to mesosulfuron + iodosulfuron was conferred by a minimum of two loci. Results indicated that a minimum of five different loci can be involved in a multiple NTSR plant. Furthermore, the plant-specific accumulation of NTSR loci was demonstrated. This is important for estimations of further spread and occurrence of herbicide resistance in the future.

Keywords: Black-grass, gene accumulation, herbicide resistance, inheritance of NTSR, mode of action, resistance linkage

2.3 Publication III

ROSENHAUER M and PETERSEN J (2015) **Bioassay development for the identification of pre-emergence herbicide resistance in *Alopecurus myosuroides* (Huds.) populations.** *Gesunde Pflanze*, DOI: 10.1007/s10343-015-0346-0.

This article is online available at:

<http://link.springer.com/article/10.1007/s10343-015-0346-0>

Abstract

Herbicide-resistant *Alopecurus myosuroides* (Huds.) is an increasing problem in winter wheat in Western Europe. An increasing number of farmers need sequential herbicide applications to control biotypes with non-target-site resistance (NTSR). Pre-emergence herbicides play an increasing and important role in resistance management today. However, cases of pre-emergence herbicide resistance caused by non-target-site mechanisms occur. This paper presents the results of bioassays to determine a reliable standard protocol for the diagnosis of resistance to pre-emergence herbicides in *A. myosuroides*. Testing was conducted to determine the influence on herbicide efficacy of two different soil types, three kinds of seed coverage and an analyses of the optimal assessment date. Additionally, dose-response experiments in soil-based pot bioassays under two growth conditions with pre-germinated seeds and a confirmatory field trial were carried out. Four different herbicides (HRAC-groups N, K₃, and C₂) were analysed (prosulfocarb, flufenacet, chlorotoluron, and metazachlor). The data showed no statistical influence of sandy loam or a mixture of sandy loam with sand as soil to the differentiation between susceptible and resistant biotypes. The seed coverage influenced the herbicide efficacy. Reliable results for all herbicides within the soil-based pot bioassay under greenhouse conditions at specific assessment dates were obtained. Suggested discriminating rates are 2000 g a.i. ha⁻¹ for prosulfocarb (dat 14), 75 g a.i. ha⁻¹ for metazachlor (dat 14), 2100 g a.i. ha⁻¹ for chlorotoluron (dat 28), and 125 g a.i. ha⁻¹ for flufenacet (dat 14). The pot assay was shown to be an appropriate procedure for the identification of pre-emergence herbicide resistance to prosulfocarb, metazachlor, chlorotoluron, and flufenacet in *A. myosuroides*. Consequently, a reliable, quick, and easy to handle bioassay which is required for high throughput screening was detected in the present study.

Keywords: black-grass, bioassay, discriminating rate, non-target-site resistance, pre-emergence herbicide

5 General discussion

The economic losses because of decreasing herbicide efficacy became more due to an increase in the number of resistant black-grass populations. To date, two main resistance mechanisms are known to confer herbicide resistance in black-grass: TSR and NTSR. TSR is monogenic and the spread of this dominantly inherited gene can be quite fast due to selection. An increasing frequency of TSR in black-grass can be assumed. Therefore, it is important to know how widespread TSR in Germany is, to avoid further selection of this trait. NTSR is a set of mechanisms and a quantitative trait. The number of loci determines the speed of distribution. The more loci are involved in specific resistances, the slower the evolution of NTSR, because it is more unlikely that many different loci will accumulate in one plant if the loci are independently inherited. However, if many different loci can confer NTSR, the likelihood increases to select any of those loci conferring new resistance patterns in plants. Thus, it is important to know how many loci are involved in NTSR and if they are connected. Farmers will need to be diligent in order to prevent resistance, because prevention costs significantly less than dealing with resistance once it is fully developed (Orson 1999). The question of the predictability of resistance development is therefore of importance. A first step for prediction is the collection of information about the occurrence and distribution of resistance. Monitoring programmes are widespread, and test systems were established for the identification of several herbicide resistances (Beckie et al. 2000). Due to the first cases of resistant black-grass against pre-emergence herbicides (Heap 2014), a test system for those herbicides is needed to avoid further selection and economic losses.

5.1 TSR

The frequent occurrence of weed populations resistant to ACCase- and ALS inhibitors can be attributed to the widespread usage of these herbicides. In several cropping systems, ACCase- and ALS-inhibiting herbicides were used repeatedly as the primary mechanisms of weed control. Overreliance on a single herbicide (or a group of herbicides with the same site of action) is likely to result in weed populations which are resistant to that particular herbicide (or group of herbicides) (Menchari et al. 2006; Délye et al. 2007; Preston 2013). In particular, ALS- and ACCase-inhibitors have been affected by the development of herbicide-resistant weeds (Heap 2014). A total of seven point mutations in the gene encoding ACCase, causing a total of five amino-acid replacements, have been demonstrated to confer resistance to ACCase-inhibiting herbicides in black-grass (Délye 2005). Five conserved amino acids have been identified in the ALS gene, which are known to confer resistance to ALS inhibitors in black-grass (Tranel &

Wright 2002). Target-site resistance is conferred by a single dominant, nuclear-encoded gene. A detailed molecular and biochemical understanding in resistance-endowing mutations in the genes coding for ALS and ACCase targets is available, and TSR has been extensively reported (reviewed in Tranel & Wright 2002; Délye 2005; Powles & Yu 2010). The actual mutation rate of ALS and ACCase is not known, and there is no evidence that ALS and ACCase mutate at an unusually high rate (Tranel & Wright 2002). Mutation rates are typically estimated to be in the range of 10^8 to 10^{10} per nucleotide base-pair per generation (Gardner & Snustad 1984). Délye et al. (2007) assumed that 25% of the resistance to ACCase-inhibiting herbicides is based on TSR in French black-grass populations. In the UK, the frequency of TSR was estimated at around 50% (Moss et al. 2007). The percentage of TSR black-grass populations in Germany is expected to be between 5 and 10% (Délye et al. 2010). Due to the powerful selective pressure of targeting a single or a few genes, a rapid increase in the frequency of mutations conferring adapted phenotypes is expected (Menchari et al. 2006). Insights about the occurrence and distribution of TSR black-grass in Germany were achieved with the first publication. Resistance to two major herbicides used in the cultivation of winter wheat in Germany, pinoxaden and mesosulfuron + iodosulfuron, were assessed. A total of 653 black-grass samples which were non-randomly collected between 2004 and 2012 were tested using bioassay and genotyping techniques. The frequency of samples with pinoxaden-resistant plants increased from 40.8% in 2004 to 71.6% in 2012. The frequency of ACCase-TSR increased from 4.0% in 2004 to 38.5% in 2012. Resistance to mesosulfuron + iodosulfuron increased from 1.5% in 2006 to 34.2% in 2012. The frequency of ALS-TSR rose from 0.8% in 2007 to 13.9% in 2012. These findings show that TSR develops very rapidly as soon as a mutant (resistant ACCase or ALS allele) has been selected (Délye et al. 2004; Beckie & Gill 2006; Menchari et al. 2006; Moss et al. 2007). TSR has been shown to be more widespread than estimated by Délye et al. (2010). Its frequency in Germany ranges between the frequencies of France and the United Kingdom. The detected TSR samples were distributed in hot-spots. Accumulations of TSR were found at the Baltic Sea coast, in North Baden-Württemberg and Central Franconia, in Westphalia, and at the Black Forest. These regions can be characterised by comparable agricultural practices. Short rotation cropping systems with winter cereals and oilseed rape combined with the repeated use of ACCase and ALS inhibitors might result in an increase in the number of incidences of TSR (Moss 1980; Moss et al. 2007). Consequently, this study indicates that those farming systems are threatened by the evolution of TSR. The widespread occurrence of ACCase-TSR in nearly 40% of the non-randomly collected samples indicates an ongoing selection progress for TSR in Germany. Changes in agricultural practices such as integrating mechanical weed control

measures are necessary to avoid or at least to delay the selection of TSR in black-grass in the field.

5.2 NTSR

NTSR has been frequently reported as a resistance mechanism in plants, especially in grasses (Beckie & Tardif 2012). NTSR is a set of quantitative traits which can coexist with TSR in the same plant (Letouzé & Gasquez 2001). The basic function of NTSR mechanisms is to minimise the amount of herbicide that reaches the herbicide site of action, so that plants can maintain fitness under herbicide selection (Busi et al. 2013). NTSR is the most common mechanism in *A. myosuroides* conferring partial resistance to a wide range of herbicides (Moss et al. 2007). The number of genes involved in NTSR is not known. The current study which focused on NTSR (Paper no. 2) found different numbers of loci conferring resistance in six different black-grass plants. The number of loci differed between the herbicide treatments and the plants. Resistance to chlorotoluron and fenoxaprop-P-ethyl were mostly conferred by at least one locus. Resistances to pinoxaden and mesosulfuron + iodosulfuron were mostly conferred by at least two loci, and resistance to flufenacet was mainly conferred by a minimum of three loci. A minimum of five different loci were found to confer multiple resistances in the plants studied. Resistance linkages differ between the plants. Only fenoxaprop-P-ethyl and pinoxaden resistances were linked in all six populations. This linkage was previously described by Petit et al. (2010). The other linkages were plant-specific, and no general resistance linkages except for the two ACCase inhibitors could be identified. Powles & Yu (2010) highlighted NTSR as a very problematic mechanism because of unpredictable cross-resistances between different modes of action which can appear (Powles & Yu 2010). Our results confirm that the accumulation of different NTSR loci in different plants is possible (Christopher et al. 1992; Letouzé & Gasquez 2003; Preston 2003; Zhang & Powles 2006; Petit et al. 2010; Busi et al. 2012). The gene pool in populations will be enriched in resistance loci because of herbicide selection (Beckie & Gill 2006; Petit et al. 2010). Surviving weed plants will subsequently reproduce via cross-pollination, which will recombine resistance loci in individual plants. Novel genotypes with various levels of resistance and a variety of cross- and multiple resistances to different herbicides may occur (Menchari et al. 2007). The six F₀-populations (“601”, “710”, “Elbe”, “9509”, “ST26”, and “ST44”) represented those multiple resistant genotypes. Any mechanism which allows a plant to survive the herbicide application can be selected. Some mechanisms will occur more commonly than others as a result of increased fitness in the presence or absence of herbicide use, but a wide variety of mechanisms are possible (Preston 2013). In plants, NTSR mechanisms are mediated by stress-response enzymes including complex constitutive

and/or induced interactions of cytochrome P450 mono-oxygenase, glutathione S-transferase (GST), glycosyltransferase, and/or the ATP-binding cassette transporter polygene families (Yuan et al. 2007; Powles & Yu 2010; Délye 2013). P450 enzymes can simultaneously metabolise herbicides which have different modes of action (Powles & Yu 2010). Some of the P450s involved in herbicide resistance might be specific for one herbicide group (Letouzé & Gasquez 2003; Busi et al. 2012). Studies on resistance to diclofop-methyl, chlorsulfuron, and chlorotoluron show the involvement of P450s (Busi et al. 2011; 2012). Thus, some of the loci conferring NTSR in the present study may belong to the P450s. Glutathione-S-transferase enzymes can play a role in herbicide conjugation and in stress response within evolved herbicide resistances (Read et al. 2004). The involvement of GST in fenoxaprop-P-ethyl resistance was demonstrated in the English black-grass population “Peldon”, in which GST activity was significantly increased in the presence of the herbicide (Cummis et al. 1997). Furthermore, Letouzé and Gasquez (2003) suggested that the increased activity of GST may be responsible for fenoxaprop-P-ethyl degradation in another black-grass population. Our study predominantly assumed that one locus conferring resistance to fenoxaprop-P-ethyl might be involved in GST activity. A few studies report current progress concerning the identification and functional analysis of candidate NTSR genes in *Alopecurus myosuroides*. Petit et al. (2012) detected the herbicide-induced up-regulation of *GSTL* in resistant plants compared to sensitive plants. Cummis et al. (2013) identified high expression levels of *GSTF1* orthologs in two species of multiple herbicide-resistant weeds. They concluded an important role for these proteins in herbicide resistance to several graminicides, such as chlorotoluron and fenoxaprop-P-ethyl. To date, details about the specific genes involved in NTSR remain unknown. In order to provide better insights, “omic”-based approaches might be a promising method. Stewart et al. (2009) and Délye (2013) reviewed “omics” for the rapid identification of genes governing NTSR through the increasing accessibility of genomics and transcriptomics supported by next-generation sequencing technologies. These studies promise a deeper understanding of the role of NTSR genes in complex detoxification pathways associated with herbicide resistance. A better understanding of the genetic basis of NTSR is the necessary first step towards the development of management solutions (Shaner & Beckie 2014). PCR-based DNA markers for NTSR screening and detection such as those that exist for TSR could be developed based on this knowledge (Burgos et al. 2012). The segregation analysis of NTSR in black-grass is a contribution to the investigation of the genetic background of NTSR.

5.3 Pre-emergence herbicides

The third paper aimed at finding a test system to identify resistance against pre-emergence herbicides in black-grass populations. Due to increasing problems with herbicide-resistant black-grass populations, a change in the modes of action of the herbicides is recommended (Bailly et al. 2012). Furthermore, sequential herbicide applications are increasingly required to control resistant biotypes in the field. Pre-emergence herbicides play an important and increasing role in resistance management (Moss & Hull 2009; Bailly et al. 2012). The first cases of black-grass biotypes with resistance to different pre-emergence herbicides have been reported (Heap 2014). It is known that NTSR can also affect the efficacy of pre-emergence herbicides (Moss & Hull 2009; Heap 2014). In Germany, a multiple resistant black-grass biotype with resistance against flufenacet was reported in 2007 (Heap 2014). Investigations in the UK gave clear evidence of partial resistance to flufenacet in the UK populations. The repeated use of flufenacet resulted in a progressive decrease of 5 - 6% efficacy per year (Hull & Moss 2014). This increasing tendency of resistance emphasises the importance of a reliable test system for pre-emergence herbicides. Specific discriminating rates for each herbicide for further bioassays need to be found to distinguish between susceptible and resistant biotypes. Different impacts on herbicide efficacy were tested with standard susceptible and two multiple resistant black-grass biotypes “Peldon” and “Elbe”. Resistances to four different herbicides (pro sulfocarb, metazachlor, chlorotoluron, and flufenacet) were detected using different test systems. The tests were conducted in agar, soil, and in field conditions. Dose-response curves showed clear differentiation between susceptible and resistant biotypes when tested in soil. The most reliable test system to distinguish between susceptible and resistant biotypes was a soil-based greenhouse assay. It was possible to identify discriminating rates for the tested herbicides. These rates were: 2000 g a.i. ha⁻¹ pro sulfocarb, 75 g a.i. ha⁻¹ metazachlor, 2100 g a.i. ha⁻¹ chlorotoluron, and 125 g a.i. ha⁻¹ flufenacet. This provides a helpful strategy for monitoring programmes for herbicide-resistant black-grass in the future.

Successful test systems need to be economically feasible according to the time-, space-, and materials consumed (Moss 1995). Working with one single discriminating rate per herbicide reduces the effort of the system. Altogether, 21 to 35 days were needed to detect resistances from ripe seeds of populations, including the time for pre-germination. The outcomes of the third paper can be used for future monitoring programmes. Additional selection pressure can be avoided due to the detection of the decreasing efficacy of relevant pre-emergence herbicides, and the choice of herbicides can be adjusted to the field-specific resistance situation.

5.4 Outlook

The practical relevance for both resistance mechanisms, TSR and NTSR, is increasing. These resistance mechanisms are widespread and can broadly affect many different herbicides with distinct modes of action. In Germany, the increasing importance of NTSR and TSR can be assumed with unknown consequences for the efficacy of herbicides (Yuan et al. 2007). Looking at the future of weed control, the knowledge generated regarding TSR and NTSR is essential to emphasise the urgency of this topic. The wide-spread distribution and the increasing number of cases reported concerning both resistance mechanisms in black-grass show that effective measures to counteract resistance evolution are required as soon as possible.

The history of weed control in the industrialised countries over the past half-century has shown that costs, simplicity, and convenience are the top three criteria utilised by growers to make weed management decisions (Shaner & Beckie 2014). Managing herbicide resistance solely with herbicides is doomed to failure. Integrated weed management strategies have to be incorporated into the farming practices, and the efficacy of the existing modes of action must be preserved. No predominant new mode of action has been introduced to the market place for over 20 years (Duke 2012), and it will remain difficult for the industry to develop new chemicals for weed control in the near future (Ruegg et al. 2007). Délye et al. (2013) assume that the local evolution of TSR and/or NTSR is possible in each population. The repeated use of the same mode of action will result in the adoption and selection of resistant individuals (Powles & Preston 2006). Field-specific decisions are necessary to address the different resistance situations in the field. Therefore, easy and cheap detection methods have to be established and introduced into practice. Sustainability will only be achieved if there is diversity in the agroecosystem, the herbicide, and non-herbicide tools employed for weed control (Délye 2013). The results of further “omic” studies can be used for the development of “resistance-breakers” counteracting the herbicide-degrading enzymes (e.g. cytochrome P450s, GSTs etc.) (Heap 2014; Shaner & Beckie 2014). These synergists might improve the efficacy of herbicides. However, the problem of selection and the adoption of individuals cannot be resolved by new chemical technologies alone. Consequently, it remains essential to combine mechanical weed control and integrated measures such as later sowing date, tillage, and crop rotation with chemical weed control.

In order to maintain herbicide efficacy in the future, the results of the present study have to be taken into account. We indicated that TSR is more widespread in Germany than expected, and that an increasing frequency can be assumed. Although the initial frequencies might be low, the rapid distribution of this trait is likely occurring due to continuous selection by herbicides.

Furthermore, NTSR has been shown to be a quantitative trait with a minimum of five different loci conferring specific resistances. The resistance patterns conferred by these different loci were various without any detectable rules of connection. The accumulation of NTSR loci can result in unpredictable resistance patterns, and even herbicides with new modes of action may lose their efficacy (Petit et al. 2010). With each herbicide application there is a chance to select multiple resistant biotypes. Therefore, it is quite difficult to find the most effective herbicide for a certain situation because no general recommendations can be given due to the complex structure of NTSR. Reacting to specific resistance situations on a field scale might improve the choice of herbicides. Field-specific knowledge about the resistance mechanisms requires the option of avoiding ineffective herbicide applications which may promote NTSR. In order to generate field-specific knowledge, quick and easy resistance tests would be a part of the decision support systems which might be useful for the purposeful choice of herbicides. Consequently, it is important that people advance those test systems for all types of herbicides and quickly respond to the ongoing problematic of herbicide-resistant weeds.

6 Summary

Weed control is an important part in agricultural practice. Since selective herbicides were introduced, the labour-intensive mechanical weed control was replaced by chemicals. The use of chemicals for weed control has become increasingly problematic due to the evolution of herbicide-resistant weeds. Herbicide resistance is defined as “the inherited ability of a plant to survive and reproduce following exposure to a dose of herbicide normally lethal to the wild-type” (WSSA 1998). The first resistant weed was found in 1968. Since that time, biotypes of 238 species (138 dicots and 100 monocots) have expressed resistance to 22 different herbicide classes globally. In Germany, *Alopecurus myosuroides* (Huds.) is one of the most problematic weeds concerning herbicide resistance. It is an allogamous, wind- and cross-pollinating grass with high genetic variation both within and between populations which is well adapted to winter wheat. The first resistant black-grass biotype in Germany was found in 1982.

After more than 30 years, there are still numerous unsolved questions and challenges concerning the problem of herbicide resistance. Further knowledge about the distribution, the detection, and the genetic background of different resistance mechanisms is needed to find comprehensive solutions for the future. Knowledge about the occurrence and distribution of herbicide-resistant black-grass in Germany, and the herbicides primarily affected may provide more detailed information for farmers to quickly react on upcoming resistances. Moreover, if the genetic background of resistance is better understood, practical conclusions regarding the choice of herbicides and management tools can be drawn. Furthermore, a reliable and easy-to-handle test system for the detection of resistances would enable farmers to react faster and in a more targeted manner.

The aim of the present study was to investigate these aspects addressing herbicide-resistant black-grass in Germany.

How widespread is TSR in Germany? Did the amount of TSR change over the years? Are there “TSR-hot spots” in Germany?

The first paper addressed ACCase and ALS-resistant black-grass samples in Germany. It dealt with the distribution and development of TSR in Germany over a period of nine years. It could be demonstrated that TSR was more widespread than expected. The frequency of ACCase-TSR increased from 4.0% in 2004 to 38.5% in 2012. ALS-TSR rose from 0.8% in 2007 to 13.9% in 2012. Both TSRs significantly increased over time within a standing number of seed samples over the years. The study detected TSR hot-spots (Baltic Sea coast, North Baden-Württemberg/Central Franconia, Westphalia, and the Black Forrest) which can be character-

ised by comparable agricultural practices. The repeated use of ACCase and ALS inhibitors in short rotation cropping systems with winter cereals and oilseed rape can rapidly lead to an increase in the incidences of TSR which are observed.

How many genes are involved in NTSR? Are there differences between the plants and can we detect cross-resistances?

The second paper dealt with the inheritance of NTSR conferring resistance to chlorotoluron, fenoxaprop-P-ethyl, pinoxaden, mesosulfuron + iodosulfuron, and flufenacet in six different black-grass plants. Segregation analyses of the quantitative trait showed a minimum of five loci conferring specific resistances. The resistances against chlorotoluron and fenoxaprop-P-ethyl were mostly conferred by one locus, whereas resistances against pinoxaden and mesosulfuron + iodosulfuron were mostly conferred by a minimum of two loci. A minimum of one to three loci explained resistance to flufenacet. The accumulation of resistance loci in individual plants could be achieved by the study. Furthermore, the number of loci was shown to be herbicide- and plant-specific which further confirms the complexity of NTSR. Resistance linkage patterns were found to vary between the plants. The only linkage detected in all six plants was fenoxaprop-P-ethyl and pinoxaden resistance. This knowledge is important for estimations of further spread and occurrence of NTSR in black-grass.

How is it possible to test pre-emergence herbicides in black-grass? Which test system is the most reliable?

The aim of the third paper was to find a reliable test system to monitor pre-emergence herbicide resistance in black-grass. It is widely known that diverse sites of action can be affected by NTSR. Moreover, resistance against pre-emergence herbicides belonging to the HRAC groups N, K₃, and C₂ occur. The outcomes of the study indicate that a soil-based greenhouse test with pre-germinated seeds is most suitable for resistance detection. Discriminating herbicide rates which were able to distinguish between the resistant and susceptible black-grass biotypes were found for all of the herbicides tested. This enables a reliable, quick, and easy way to identify pre-emergence resistance. Detecting resistance provides the option to reduce the selection pressure in fields where the decreasing herbicide efficacy of pre-emergence herbicides has been found.

In conclusion, herbicide-resistant black-grass has become an increasing problem in Germany. The high frequency of nearly 40% ACCase TSR on resistance suspected sites highlights the importance of changes in agricultural practices. The aim should be to avoid the repeated use of single site of action herbicides in short term crop-rotations with large quantities of winter cereals. The accumulation of NTSR loci in single plants increases the risk of biotypes with broad

resistances against many different modes of action. Resistance linkages were found to be plant-specific which may result in unpredictable resistance situations in the field. Even pre-emergence herbicides can be affected by NTSR. An option to detect these resistances is provided by a soil-based greenhouse bioassay with pre-germinated seeds.

In regard to herbicide resistance, field-specific decisions on herbicide strategy and the integration of agronomical practices are necessary to avoid a rapid increase in TSR and NTSR in black-grass populations in Germany.

7 Zusammenfassung

Unkrautregulierung ist einer der wichtigsten Aspekte im Ackerbau. Seit der Einführung selektiver Herbizide wurde die intensive Handarbeit durch chemische Unkrautbekämpfung ersetzt. Mittlerweile ist der verstärkte Herbizideinsatz zunehmend problematisch, da sich herbizidresistente Unkräuter entwickeln. Herbizidresistenz wird als die „vererbte Fähigkeit einer Pflanze eine normalerweise für den Wildtyp tödliche Herbiziddosis zu überleben und sich zu reproduzieren“ definiert (WSSA 1998). Das erste herbizid-resistente Unkraut wurde 1968 entdeckt. Seitdem zeigten weltweit Biotypen aus 238 Arten (138 Zweikeimblättrige und 100 Einkeimblättrige) Resistenzen gegenüber 22 verschiedenen Herbizidklassen. In Deutschland gilt der Ackerfuchsschwanz (*Alopecurus myosuroides* Huds.) als eines der problematischsten Unkräuter hinsichtlich der Herbizidresistenz. Es ist ein einjähriges, wind- und fremdbestäubendes Gras, das vornehmlich im Winterweizen vorkommt. Es weist eine hohe genetische Variabilität innerhalb und zwischen Populationen auf. Der erste Resistenzfund in Deutschland stammt aus dem Jahr 1982.

Obwohl seitdem mehr als 30 Jahre vergangen sind, bestehen in der Wissenschaft noch viele ungelöste Fragen bezüglich Herbizidresistenz. Zusätzliches Wissen über die Verbreitung, die Erkennung und den genetischen Hintergrund verschiedener Resistenzmechanismen ist nötig, um umfassende Lösungsansätze für die Zukunft zu entwickeln. Die Kenntnis über das Auftreten und die Verbreitung von herbizid-resistentem Ackerfuchsschwanz in Deutschland und darüber, welche Herbizide am häufigsten betroffen sind, kann Landwirten zielgerichtet Informationen geben, um schnell auf aufkommende Resistenzen zu reagieren. Weiterhin kann ein besseres Verständnis des genetischen Hintergrunds von Resistenz zu praktischen Schlussfolgerungen für die Herbizidwahl benutzt werden. Außerdem kann ein zuverlässiges und einfach zu handhabendes Testsystem zur Resistenzerkennung den Landwirten eine schnelle und zielgerichtete Reaktion ermöglichen.

Das Ziel der vorliegenden Arbeit war es, diese verschiedenen Aspekte bezüglich herbizid-resistenten Ackerfuchsschwanz in Deutschland näher zu beleuchten.

Wie verbreitet ist Zielortresistenz in Deutschland? Veränderte sich der Anteil von Zielortresistenz in den letzten Jahren? Existieren Orte mit besonders hohem Zielortresistenzanteil?

Die erste Publikation untersuchte ACCase und ALS-resistenten Ackerfuchsschwanzproben in Deutschland und betrachtete die Verbreitung und Entwicklung von TSR über neun Jahre in Deutschland. Zielortresistenz zeigte sich als weiter verbreitet als vermutet. Die Häufigkeiten

von ACCase-TSR stieg von 4,0% im Jahr 2004 auf 38,5% im Jahr 2012. ALS-TSR stieg von 0,8% im Jahr 2007 auf 13,9% im Jahr 2012. Beide TSR Anteile stiegen über die Jahre signifikant an. Die Untersuchung ergab TSR-Brennpunkte (Ostseeküste, Nord Baden-Württemberg/Mittelfranken, Westphalen und Schwarzwald) die durch eine ähnliche ackerbauliche Praxis charakterisiert werden können. Der wiederholte Einsatz von ACCase- und ALS-Hemmern in engen Fruchtfolgen mit Winterweizen und Winterraps kann zu einem schnellen Anstieg von TSR auf den entsprechenden Feldern führen.

Wie viele Genorte sind an Nicht-Zielortresistenz (NTSR) beteiligt? Bestehen Unterschiede zwischen Pflanzen und gibt es Kreuz-Resistenzen?

Die weite Publikation handelte von der Vererbung von NTSR gegen Chlortoluron, Fenoxaprop-P-Ethyl, Pinoxaden, Mesosulfuron + Iodosulfuron und Flufenacet in sechs verschiedenen Ackerfuchsschwanzpflanzen. Die Aufspaltungsverhältnisse des quantitativen Merkmals zeigten ein Minimum von fünf Loci, die herbizid-spezifische Resistenzen bewirken. Resistenzen gegenüber Chlortoluron und Fenoxaprop-P-ethyl wurden meistens durch einen Genort bedingt, während an den Resistenzen gegenüber Pinoxaden und Mesosulfuron + Iodosulfuron meistens mindestens zwei Genorte beteiligt waren. Mindestens drei Genorte zeigten sich an der Resistenz gegenüber Flufenacet bei den meisten der untersuchten Pflanzen beteiligt. Die Anreicherung von Resistenz-Genorten in einzelnen Pflanzen konnte in der Studie gezeigt werden. Weiter zeigte sich die Anzahl Genorte als pflanzen-spezifisch, was die Komplexität von NTSR bekräftigt. Resistenzverknüpfungen zeigten sich ebenfalls als herbizid- und pflanzenspezifisch. Nur die Verknüpfung von Fenoxaprop-P-Ethyl und Pinoxaden konnte in allen sechs Pflanzen gefunden werden. Dieses Wissen ist für die Einschätzung des weiteren Auftretens und der zukünftigen Verbreitung von NTSR im Ackerfuchsschwanz bedeutsam.

Wie kann Resistenz gegen Voraufdauerherbizide bei Ackerfuchsschwanz getestet werden? Welches ist das geeignetste Testsystem?

Das Ziel der dritten Veröffentlichung war es, ein verlässliches Testsystem für Resistenz-Monitorings von Voraufdauerherbiziden bei Ackerfuchsschwanz zu finden. Es ist bekannt, dass NTSR verschiedene Wirkmechanismen betreffen kann. Auch Resistenzen gegenüber Voraufdauerherbiziden, die zu den HRAC Gruppen C₂, N und K₃ gehören, treten auf. Die Ergebnisse der dritten Untersuchung zeigen, dass ein Gewächshaustest mit Boden und vorgekeimten Samen eine geeignete Methode ist, um diese Resistenzen nachzuweisen. Kritische Dosierungen für die Unterscheidung zwischen resistenten und sensitiven Ackerfuchsschwanzbiotypen konnten für alle getesteten Herbizide gefunden werden. Dies ermöglicht eine verlässliche, schnelle und praktikable Durchführung von Resistenztests. Die Erkennung von Resistenz ermöglicht es,

den Selektionsdruck zu reduzieren, indem Felder mit abnehmender Wirkung von Vorauf-herbiziden erkannt werden und die Wahl des Herbizides verändert wird. Zusammenfassend ergibt sich, dass herbizid-resistenter Ackerfuchsschwanz in Deutschland ein zunehmendes Problem darstellt. Der hohe Anteil von fast 40% ACCase-TSR bei Resistenzverdachtsfällen hebt die Wichtigkeit von Veränderungen in der ackerbaulichen Praxis deutlich hervor. Der wiederholte Gebrauch von Herbiziden mit einem einzelnen Wirkort sollte in engen Fruchtfolgen mit hohem Winterweizenanteil vermieden werden. Die Anreicherung von NTSR Loci in Einzelpflanzen erhöht das Risiko für Biotypen mit breiten Resistenzen gegen viele verschiedene Wirkstoffe. Bis zu drei verschiedene NTSR Loci können zu herbizid spezifischen Resistenzen führen. Pflanzenspezifische Resistenzverknüpfungen wurden gefunden, wodurch nicht vorhersagbare Resistenzsituationen auf den Feldern möglich sind. Sogar Voraufherbizide können durch NTSR unwirksam werden. Eine Möglichkeit, diese Resistenzen zu überprüfen ist durch einen Gewächshaustest mit Boden und vorgekeimten Samen für die geprüften Wirkstoffe gegeben.

Berücksichtigt man Herbizidresistenz, sind feldspezifische Entscheidungen bezüglich der Herbizidstrategie und die Integration ackerbaulicher Maßnahmen notwendig, um einen schnellen Anstieg von TSR und NTSR innerhalb der Ackerfuchsschwanzpopulationen in Deutschland zu vermeiden.

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Affirmation

I, Maria Rosenhauer, declare:

The present thesis: ‘Distribution, detection and genetic background of herbicide-resistant *Alopecurus myosuroides* (Huds.) in Germany’ was prepared autonomously and exclusive of using the mentioned additives. Literally or contextually adopted test passages are marked correspondingly. The work has not been submitted to any examination authority in similar or exact profile.

Mainz, 12.01.2015

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(Signature)