University of Hohenheim

Institute of Phytomedicine

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Investigations on herbicide resistant grass weeds

Dissertation

submitted in fulfilment of the requirements for the degree "Doktor der Agrarwissenschaften"
(Dr.sc.agr./ Ph.D. in Agricultural Sciences)

to the

Faculty Agricultural Sciences

presented by

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from

Hannover

2009

This thesis was accepted as a doctoral dissertation in fulfilment of the requirements for the degree “Doktor der Agrarwissenschaften” by the Faculty Agricultural Sciences at the University of Hohenheim on September 15, 2009.

Date of oral Examination:	November 17, 2009
Examination Committee:	
Supervisor and Review	Prof. Dr. R. Gerhards
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Die Natur hat sich so viel Freihalt vorbehalten, dass wir mit Wissen und Wissenschaft ihr nicht durchgängig beikommen oder sie in die Enge treiben können.

Johann Wolfgang von Goethe (1749 -1832)

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CHAPTER I

General Introduction

Natalie Balgheim

1 General Introduction

Weeds are the most important pest complex that threatens world fibre and food production while herbicides represent the most prevalent pesticide used (Hock et al. 1995; Heap and LeBaron 2001). From all pests' threats, weeds produced the highest potential crop losses (34 %), with insect pests (18 %) and pathogens (16 %) being much less important (Oerke, 2006). They compete with crops for environmental resources (available in limited supply) like nutrients, water and light (Wilson and Wright 1990; Froud-Williams 2002), hinder harvest, decrease food quality, might be toxic for animals and humans (Hock et al. 1995), and serve as hosts for pathogens and insect pests (Ross and Lembi 2009). Because of that processing costs and human health problems are increasing (Naylor and Lutman 2002). Currently herbicides are used on the majority of the crop acres and provide economically acceptable control of weed pests. But despite their benefits, strong concerns have been developed since they have been used intensively. However, herbicides can lead to residues and are associated with food safety issues. They have an adverse impact on the environment and are responsible for the widespread occurrence of herbicide resistant weeds (Heap and LeBaron 2001). These rapidly increasing herbicide resistant weeds are the challenge for the agricultural production today.

1.1 Whys and wherefores of herbicide resistance

The evolution of herbicide resistance is mainly governed by the biology of weedy plant species and by herbicide characteristics and their use patterns (Neve and Powles 2005a). It occurs as the result of heritable changes to biochemical processes that enable plant survival when treated with herbicides (Preston and Mallory-Smith 2001). Herbicide resistance is not a new topic. First reported cases are out of the late 1960s, and came along with the broad use of chemical weed control (Heap and LeBaron 2001). Today 330 resistant biotypes of 189 species with herbicide resistance to one or more modes of action are known: 113 dicot and 76 monocot weeds (Heap 2009).

1.1.1 Herbicide resistance - what does is mean?

However, to understand the whole problematic of herbicide resistance it is quite essential to comment on this term in the context of this thesis. According to Heap and LeBaron (2001) the overall definition of herbicide resistance is the evolved capacity of a previously herbicide-susceptible weed population to withstand a herbicide and complete its life cycle, if the herbicide is used at its normal rate in an agricultural situation.

With few exceptions, one or more of three general mechanisms cause herbicide resistance: an altered herbicide target enzyme, enhanced herbicide metabolism, or reduced herbicide translocation (Hall et al. 1997).

Whereas target-site resistance is the result of a modification of the herbicide binding site, usually the target enzyme, mostly by a single nucleotide polymorphisms (SNP) which precludes herbicides from effectively binding on the corresponding enzyme (Devine and Shukla 2000), non-target-site resistance is due to all other mechanisms than target-site modifications, as enhanced metabolism, reduced uptake or translocation of herbicides that reduce the amount of herbicide active ingredient reaching the herbicide binding site (Preston and Mallory-Smith 2001).

The plant detoxification mechanism causing non-target site resistance are processing different detoxifications steps within the plant. Four gene families are involved in these processes: cytochrome P 450 monooxygenases, glutathione S-transferases, glycosyltransferases, and ABC transporters (Yuan et al. 2006).

If a single resistance mechanism provides resistance to two or more herbicides acting at the same target, cross resistance occurs (Heap and LeBaron 2001). If two or more resistance mechanisms are involved in resistance against herbicides acting at different target sites, it is a question of multiple resistance.

Meanwhile target-site resistance is the best understood resistance mechanism and is suggested to be the predominant resistance mechanism in weeds.

1.1.2 Evolution of herbicide resistant weeds

The development of herbicide resistance in weeds is an evolutionary process as a consequence of environmental changes brought about by man (Maxwell and Mortimer 1994). It is mainly the evolutionary response to the continuous use of selective agents as herbicides with the same or similar modes of action (Gressel 2002; Cousens and Mortimer 1995; Heap and LeBaron 2001). Weed populations change in genetic composition in a way

that the frequency of resistance alleles and resistant individuals increases (Jasienuok et al. 1996). Susceptible phenotypes were removed from the population, leaving more tolerant phenotypes in greater proportions in the field which survive herbicide applications (Cousens and Mortimer 1995). This process arises because genetic variations are almost always present within wild populations at high rates; so evolutionary responses are inevitable according to intensity of selection (Beckie and Gill 2006).

In the late 1960s a biotype of *Senecio vulgaris* was found to be the first herbicide resistant weed (Ryan 1970). A few years later the occurrence of the first target-site based resistance in *Senecio vulgaris*, again associated with resistance to triazine herbicides was reported. Since then reported cases of herbicide resistance are rapidly increasing.

Out of the today known 189 species which evolved herbicide resistance, the most important ones are: *Lolium rigidum*, *Avena fatua*, *Amaranthus retroflexus*, *Chenopodium album*, *Setaria viridis*, *Echinochloa crus-galli*, *Eleusine indica*, *Kochia scoparia*, *Conyza canadensis*, and *Amaranthus hybridis* (Heap 2009).

Most of these resistances rose up in the developed world, in countries like the USA, Australia, Canada, and in Central Europe (Heap 2009). An analysis of the resistance phenomenon in the developed nations in contrast to the developing world showed that the prevalence of herbicide resistant weeds in developed countries, occurs especially in major crops and in the most productive and fertile areas where there is a heavy reliance on herbicides is predominating (Heap and LeBaron 2001). Fewer weed problems associated with herbicide resistance exist in the developing world, because these countries depend due to economic limitations and the availability of cheap labour not as much on herbicides as the developed nations. But if developing countries industrialize, the evolution of herbicide resistant weeds will increase.

The reasons for the different situation of developed and developing countries make plain that the evolutionary process depends on the selection pressure exerted to the weed, often due to an increase on the reliance on herbicides, in combination with a decrease of the importance of all other agronomic factors (Cousens and Mortimer 1995; Beckie and Gill 2006). In many areas the situation becomes even more problematic, because multiple cultivation for weed control was changed to reduced tillage to prevent soil erosion which led to a greater dependence on herbicides (Thill and Lemerle 2001). Moreover different herbicides exert different selection pressures on weeds. Nonpersistent herbicides generally exert less selection pressure than persistent ones. This persistence depends on timing of the

herbicide application and the germination characteristics of the target species (Beckie and Gill 2006). However, single-site-of-action herbicides are supposed to exert a high selection pressure on target weeds and enhance the risk of resistance evolution, multi-site-of-action herbicides on the other hand have a minor risk to select herbicide resistant weeds (Coupland 1994). Herbicides that have only a single site of action, are i.e. acetyl-coenzyme A (ACCase) and acetolactate synthase (ALS) inhibiting herbicides, whereas low resistance risk herbicides, targeting multiple sites of action, are i.e. ureas and dinitroanilines (Beckie and Gill, 2006). Therefore and because of the rapid evolution of species being resistant to ACCase and ALS inhibiting herbicides, those are classified as high risk and most resistance prone herbicides. Today, ALS inhibiting herbicides count for 101 detected and ACCase for 36 proved cases of herbicide resistance (Figure 1.1) (Heap 2009).

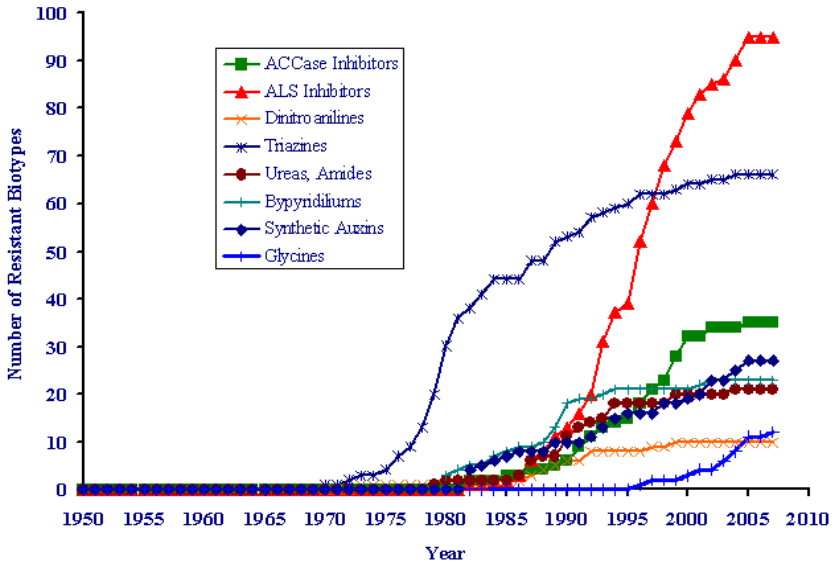

Figure 1.1: Development of herbicide resistance weeds divided into the mode of action, to which weeds developed herbicide resistance.

Source: Heap (2009)

1.1.3 Grass weed resistance to ACCase and ALS inhibiting herbicides

Nowadays, ACCase and ALS inhibitors are the most resistance prone herbicides. These modes of action are mainly used in cereals and, in case of the ACCase inhibitors, in dicot crops as well, to control annual grass weeds.

Herbicides targeting ACCase are inhibiting the first committed step of fatty acid biosynthesis which is catalysed by Acetyl-CoA carboxylase, an enzyme which catalyzes the ATP dependent carboxylation of acetyl-CoA to malonyl-CoA (Harwood 1988). However, their selectivity is expressed at the level of the plastid localized ACCase, where fatty acids are synthesized (Sasaki et al. 1995; Sasaki and Nagano 2004). Three catalytic domains are contained on the two different types of plastidics: the biotin carboxyl-carrier (BCCP), the biotin carboxylase (BC), and the carboxyl transferase (CT) domain. Kinetic analysis showed that herbicides inhibiting ACCase interfere with the CT domain (Sasaki and Nagano 2004). Thus, it is suggested that changes within the CT domain entail resistance to ACCase inhibiting herbicides.

These herbicides are selective against the plastidic form of ACCase on grasses and do not affect significantly the enzyme of other monocotyledons, dicotyledons or from other species such as bacteria and animals (Price et al. 2003). Three different herbicidal groups interfere with the ACCase: Aryloxyphenoxypropionate (APPs) and Phenylpyrazoline (DENs) which were used in cereals and Cyclohexanedione (CHDs) used in dicot crops as oilseed rape and sugar beet to control grass weeds.

Another herbicide group used to control grass and dicot weeds in cereal crops are herbicides which are inhibiting the Acetolactate-synthase (ALS), a nuclear-encoded, chloroplast-localized enzyme in higher plants (Duggleby and Pang 2000) which catalysis the first common step of the synthesis of the branched chained amino acids leucine, isoleucine and valine (Ray 1982b). These amino acids are synthesised from pyruvate, with 2-ketobutyrate additionally required for the biosynthesis of isoleucine. Two molecules pyruvate are condensed to form 2-acetolactate with elimination of CO_2 for the biosynthesis of valine and leucine, while a molecule of pyruvate is condensed with 2-ketobutyrate in a similar reaction for the biosynthesis of isoleucine (Ball et al. 2007). At least five chemical groups are known inhibiting ALS: Sulfonylureas (SUs), Imidazolinones (IMIs), Pyrimidinylthiobenzoates (PTBs), Sulfonylaminocarbonyltriazolinone (SCTs), and Triazolopyrimidines (TPs). Their unique mode of action coupled with the low mammalian toxicity and high efficacy set new standards in herbicide technology (Shaner and Singh, 1997).

Both, ACCase and ALS inhibiting herbicides have a high activity and result in high levels of weed control, and were therefore used in high production systems, especially in cereal production. According to Heap and LeBaron (2001) grass weeds with resistance to

ACCase and ALS inhibiting herbicides account for the majority of the cereal production area worldwide. France, Germany, and Great Britain are the major wheat producing countries of the European Union. Within these countries *Alopecurus myosuroides* Huds., *Apera spica-venti* L. Beauv., and *Lolium* ssp. are the most troublesome grass weeds associated with herbicide resistance (Naylor and Lutman 2002).

1.1.4 Current situation in Germany

The most affected herbicides in correlation with resistance in Germany are ACCase and ALS inhibitors (Heap 2009).

In 2002 Niemann et al. confirmed the first occurrence of an ALS inhibitor resistance *A. myosuroides* biotype. Two years later monitoring results of 50 *A. myosuroides* biotypes from Northwest Germany proved ACCase inhibitor resistance in 84 % and ALS inhibitor resistance in 68 % of the investigated biotypes (Bünte und Niemann 2004). Conservative estimations assume a resistance infestation level of 5 to 10 % on the German arable land with naturally occurring *A. myosuroides* populations (Petersen and Wagner 2009). Therefore *A. myosuroides* is the most problematic weed linked with herbicide resistance in Germany. Nevertheless reports about ALS inhibitor resistance in *A. spica-venti* accumulate as well (Niemann and Zwerger 2006). Especially in the intensive wheat monocultures in Northwest Germany herbicide resistant grass weeds are known to cause difficulties in allying appropriate management strategies.

Although it is assumed that the most occurring herbicide resistance cases in Germany are due to enhanced metabolism (Menne et al. 2008) and alternative modes of action to control grass weeds in cereals are missing, multiple resistant biotypes are still rare (Heap 2009).

1.2 Detection of herbicide resistance

In literature, several methods are known for detecting herbicide resistance: from simple seedling bioassays to costly molecular methods. But not all of them differentiate between different resistance mechanisms and aim clear results.

Seedling bioassays

The most common method for detecting resistance is the glasshouse bioassay, where seedlings where proved against several active ingredients applied with different doses

(Corbett and Tardif 2006). These seedlings originated from fields, where resistance was assumed. Therefore seeds of the surviving plants were collected after ripening from the field and prepared for the following assays. To compare the collected samples, seeds of already known resistant and susceptible populations are commercially available. After herbicide response to tested plants is completed, efficacy will be assessed by different methods, but each in comparison to an untreated control.

This method is the most conventional one and is practiced with variations, using agar or soil, growing chambers or greenhouses, special spraying chambers or manually driven application vehicles. Simultaneously it is one of the most time consuming methods, and results can be obtained not until the growing season has been finished, because weeds have to produce seeds. Nevertheless the most relevant disadvantage of this method is that the molecular reason for the detected resistance can not be clarified exactly. Anyhow, with the information of the resistance pattern, presumptions can be made about the level of resistance, which active ingredients are affected, and if it is a matter of target- or non target-site resistance, or cross resistance. But no information can be given about the molecular background and possible mutations. To obtain this information DNA analysing techniques have to be used.

Enzyme assays

To detect the activity of the affected enzyme assays were developed. With the results of these assays conclusions can be drawn on the resistance mechanism. De Prado et al. (2004) described assays for ACCase and ALS enzymes as well. The principles of such assays are independent of the affected enzyme. Isolated target enzymes were tested against several herbicides. Plants with target site-based resistance have an enzyme that is less affected by the inhibiting herbicide than enzyme from wild-type populations (Corbett and Tardif, 2006). But with this method too, no answer can be given about the molecular substitutions on the corresponding gene.

DNA based detection of herbicide resistance

PCR amplification of specific alleles (PASA) (Délye et al. 2002), cleaved amplified polymorphic sequences (CAPS) (Kaundun and Windass 2006), real time polymerase chain reaction (RT-PCR) (Kaundun et al. 2006), and Pyrosequencing™ (Wagner 2008 unpublished) are the main used techniques in weed science. To develop such marker

techniques, a basic knowledge about the affected gene sequence is required. In favour, a classic DNA sequencing method is therefore essential. But these techniques generate information about the underlying resistance mechanism.

1.3 Grass weed dynamics

A. myosuroides and *A. spica-venti* are the most trouble causing grass weeds in Germany. They are showing different population dynamic parameters and different characteristics in the evolution of herbicide resistance. Whereas *A. myosuroides* prefers heavy, loamy, and waterlogged soils, the occurrence of *A. spica-venti* is associated with light and sandy soils. *A. myosuroides* has a seed production of about 200 seeds per plant with a lifetime of up to 10 years (Moss 1985). However, *A. spica-venti* has a higher seed potential of 2000 seeds per plant, but with a seed viability of 2 years on average (Chomans and Kells 2001).
Seedlings of both species germinated in fall and are highly adapted to cereals (Warwick et al. 1985). In general, the spatial and temporal distribution of weeds within arable fields is known to be heterogeneous (Marshall 1988). They often occur in patches of varying sizes, which were persistent over years (Wilson and Brain 1991).
This evolution and occurrence of weeds and the stability of weed patches depend on several factors which are well described in literature, i.e. cultivated crop, crop rotation, drilling date, applied herbicides and tillage system. However, it is widely known that infestation levels of *A. spica-venti* and *A. myosuroides* tend to increase when the proportion of winter cereals, particularly wheat, in the crop rotation is increased (Melander 1995). Later drilling dates are associated with increased sowing densities of wheat seeds which are correlated to a reduced competitive ability of *A. myosuroides* and *A. spica-venti* seedlings (Balgheim 2006). Streit et al. (2000) proved that a change to reduced tillage systems leads to a shift among weed populations. Higher amounts of weeds contributing to the soil seed bank are known to be the consequences of reduced tillage systems (Melander et al. 2008). Along with this development, reduced tillage systems often have a greater reliance on herbicides, which can result in weed populations dominated only by a few species, often grass weeds (Melander et al. 2008). Under non-inversion tillage systems, herbicide resistant *A. myosuroides* evolves much quicker than under continuous ploughing (Clarke et al. 2000).

1.4 Thesis objectives

The overall purpose of this thesis is to understand the molecular patterns and the spatial distribution of herbicide resistance. Lab and agricultural field experiments might be connected, to clear up the evolution and to fight herbicide resistance. Therefore the interest on this work is based on different topics, concerning *A. myosuroides* and *A. spica-venti* biotypes exhibiting resistance to acetyl-CoA-carboxylase and acetolactate-synthase, respectively. The objectives of this work are herein:

- To characterise the resistance of the two species against several modes of action.
- To identify the resistance mechanisms and their molecular background.
- To develop mechanisms for fast, easy, and cheap molecular detection of the alleles of interest.
- To get an idea of the spatial and temporal distribution of herbicide resistance in fields.

The first paper deals with the characterisation of two different *A. myosuroides* biotypes with evolving resistance to ACCase inhibiting herbicides. The aim was to analyse resistance patterns and responses to different modes of action and therefore to gain information about the underlying resistance mechanisms. Sequencing results might complete the analysis of the underlying resistance mechanisms.
The second paper deals with the development of molecular markers to detect known target-site mutations in ACCase inhibitor resistant *A. myosuroides*. An easy to handle tool for detecting known changes on the molecular structure of the ACCase coding gene will be provided within this project.
The intention of the third paper was to ascertain information about the resistance mechanism in ALS inhibitor resistant *A. spica-venti*. Dose response assays on seedling and enzyme level reveal knowledge of the resistance patterns of the first proved target site resistance in *A. spica-venti* in Germany. Developed molecular markers serve as tool for detecting proved target-site resistance.
Data collected during studies for the fourth paper demonstrate the spatial and temporal distribution of herbicide resistant *A. myosuroides* within arable fields over two growing

seasons. Results shall contribute to an expanded knowledge about the dynamics of herbicide resistant populations within arable field.

The evolution of herbicide resistant weeds and their distribution in arable fields is discussed. Likewise the use of molecular marker technologies and their appliance in weed science.

CHAPTER II

Biotypes of *Alopecurus myosuroides* Huds. with target-site resistance to ACCase inhibiting herbicides in Germany

Natalie Balgheim, Jean Wagner and Roland Gerhards

2 Biotypes of *Alopecurus myosuroides* Huds. with target-site resistance to ACCase inhibiting herbicides in Germany

Abstract - Reports about herbicide resistant weeds are increasing steadily, also in Germany it is no longer a curiosity. Resistance against ACCase inhibiting herbicides is a challenge for today's weed control.

Investigations on two different German biotypes of *A. myosuroides* with resistance against ACCase inhibitors were carried out. Seeds collected from infested fields were analysed to determine the reason for loss of effectiveness of the used herbicides and to detect the molecular background of herbicide resistance. Greenhouse dose-response assays were conducted to determine the degree of resistance to different herbicides. Results showed resistance of the biotype $BR_{(R)}$ against different ACCase inhibitors from both, APPs and CHDs. However the biotype $BL_{(R)}$ showed resistance only against APPs but not to CHDs. No cross resistance was detected against other modes of action than ACCase inhibiting herbicides. Thus, the requirements for controlling these biotypes are also fulfilled.

To elucidate the reason for the resistance phenomenon, DNA sequencing of the ACCase CT domain revealed a change of isoleucine to leucine at amino acid position 1781 of the biotype $BR_{(R)}$ and a change from glycine to alanine at position 2096 in the resistant biotype $BL_{(R)}$, respectively.

Keywords: APP, blackgrass, CHD, fenoxaprop-p-ethyl, herbicide resistance, SNP

2.1 Introduction

Analogue to the demand of agricultural commodities for food and energy as well, the world wheat production has to be increased. For the growing season 2008/09 the International Grain Council forecasted a world grain production of 688 million tons (IGC 2009). Thus, more cereals have to be produced on a constant arable area. Therefore plant protection and plant cultivation measurements have to be intensified. Wheat monocultures or rotations with a high proportion of wheat, combined with reduced soil cultivation and the use of pesticides with the same or similar mode of action are the consequences. These

cropping systems are known to enhance the risk for herbicide resistance evolution because of the increasing selection pressure on the weed populations (Maxwell and Mortimer 1994; Heap and LeBaron 2001; Moss 2002).

Within the European Union, France, Germany, and Great Britain are the major wheat producing countries. In these countries the most problematic weed associated with a dramatic increase of herbicide resistance is *Alopecurus myosuroides* Huds. (Thill and Lemerle 2001; Heap 2009). Meanwhile field populations of *A. myosuroides* have been reported, being resistant to acetyl coenzyme A (ACCase), Acetolactate synthase (ALS), photosystem II (ureas, amides), and microtubule assembly (dinitroanilines) inhibiting herbicides (Heap 2009).

Resistance can be due to two different mechanisms, target-site and non-target site resistance (Preston and Mallory-Smith 2001). A modification of the herbicide-binding-site which precludes the herbicides from binding the target results in a so called target-site resistance (Gressel 2002). Mechanisms others than target-site modifications can be summarised as non target-site resistance and can be endowed by several mechanisms such as enhanced metabolism (Cocker et al. 1999).

Because of a lack of alternative modes of action, ACCase inhibiting herbicides are intensively used during the cultivation of wheat, and resistance evolved therefore in ten major grass weed species (Gressel 2002). In Germany monitoring results show an increasing proportion of ACCase inhibitor resistant biotypes of *A. myosuroides* (Balgheim 2006; Drobny at al. 2006; Heap 2009). According to the herbicide resistance action committee (HRAC) the herbicidal group A, containing ACCase inhibitors, summarised the active ingredients of APPs (Aryloxyphenoxypropionates), CHDs (Cyclohexanediones), and DENs (Phenylpyrazoline). These herbicides are inhibiting the first committed step of fatty acid biosynthesis which is catalysed by Acetyl-CoA carboxylase an enzyme which catalyzes the ATP dependent carboxylation of acetyl-CoA to malonyl-CoA (Harwood 1988). This enzyme is located in both, cytosol and chloroplasts, and is responsible for the carboxylation of acetyl-CoA to malonyl-CoA. However, selectivity of ACCase inhibiting herbicides is expressed at the level of the plastid localized ACCase, where fatty acids are synthesised (Sasaki et al. 1995; Sasaki and Nagano 2004). Three catalytic domains are contained on the two different types of plastidic: the biotin carboxyl-carrier (BCCP), the biotin carboxylase (BC), and the carboxyl transferase (CT) domains (Sasaki and Nagano 2004).

Mutations within the CT domain might reveal target-site resistance (Nikolskaya et al. 1999). For *A. myosuroides* six amino acid substitutions within the CT domain are known to be responsible for target-site resistance: changes on the amino acids isoleucine (Ile) at position 1781 (position numbered according to the *A. myosuroides* plastid ACCase [EMBL accession no. AJ310767]), tryptophane (Trp) at position 1999, Trp at position 2027, Ile at position 2041, asparagine (Asp) at position 2078, and glycine (Gly) at position 2096 can be exchanged by one or in case of Ile_{2041} and Gly_{2096} at least two alternative amino acids, respectively (Zhang and Powles 2006; Liu et al. 2007). Substitutions on these positions generate different resistance patterns. According to Cocker et al. (2000) and Délye et al. (2003) non target-site resistant weeds expressed resistance only to APP, but not to CHD herbicides, whereas different substitutions on the CT domain exhibit different resistance patterns. Substitutions on Trp_{2027}, Ile_{2041}, and Gly_{2096} confer resistance to APPs, but not to CHDs, whereas mutations on Ile_{1781} and Asp_{2078} confer resistance to both.

Objectives

The challenge of herbicide resistance today is to understand the genetic background of herbicide resistance and to combine revealed results with weed management strategies. The purpose of this research is to generate knowledge about the molecular background of resistance to understand the whole complex, why changes appear, the influence of the interaction of agricultural and plant protection measurements and plant production systems, to change the weed management system in general to reduce the risk of the evolution of herbicide resistant weeds. Therefore the objectives of this paper were the following: (i) to confirm and quantify the specific resistance towards ACCase inhibitors and examine herbicides with alternative mode of action in *A. myosuroides*, and (ii) to sequence the CT domain to find responsible non-synonymous mutations in the resistant populations.

2.2 Materials and methods

2.2.1 Plant material

Two *A. myosuroides* biotypes of which farmers reported lower effects of the used active ingredients fluazifop-p-butyl and fenoxaprop-p-ethyl, respectively, were selected for the following investigations. Seeds of the biotype $BR_{(R)}$ were collected in autumn 2003 from a

sugar beet field near Stuttgart (Germany), whereas seeds of the biotype $BL_{(R)}$ were collected at a winter wheat field near Hanover (Germany) during summer 2006. At both sites control with ACCase inhibiting herbicides failed. A susceptible $BS_{(S)}$ biotype (commercial available from Herbiseed, Twyfort, UK) was used as a reference population.

2.2.2 Dose-response assays

Seeds of the resistant and sensitive biotypes of *A. myosuroides* were sown and germinated directly on flooded vermiculite. Seedlings were planted in 8cm * 8cm jiffy pots (two plants per pot) or 6cm * 6cm jiffy pots (one plant per pot), respectively, filled with compost soil and placed in the greenhouse (24/20 ºC day/night and 14 h additional lighting of 300 µmol photosynthetic photon-flux density m^{-2} s^{-1}).

Seedling were sprayed with six ACCase inhibitors and three herbicides of other mode of action using a laboratory track sprayer equipped with a single nozzle "Teejet 8004EVS" applying 400 liters ha^{-1} at 3 bar at plant leaf stage 11 – 12 (BBCH). Foliage fresh or dry weight was taken 21 days after treatment (DAT), after herbicide response was completed.

Herbicide active ingredients of clethodim, clodinafop-propagyl, cycloxydim, fenoxaprop-p-ethyl, fluazifop-p-butyl, haloxyfop-p-methyl, quizalofop-p-ethyl, pinoxaden, isoproturon, flupyrsulfuron-methyl-sodium and glyphosate were used to determine herbicidal response to the resistant and sensitive biotypes described above.

2.2.3 Statistical analysis

Non linear regression according to Seefeldt et al. (1995) was used to calculate dose response relationships in consideration of the log-logistic model of Streibig (1988):

$$y = C + \frac{D - C}{1 + \exp\{b[\log(x) - \log(ED_{50})]\}}$$

Whereas y is the shoot dry and fresh weight respectively, C and D the upper and lower limit, x the herbicides doses, b the slope of the curve, and ED_{50} the herbicide doses, which causes 50 % weight reduction. With PASW Statistics17 (Release 17.0.2.; SPSS Inc., 2009) statistical analysis were calculated.

2.2.4 DNA analyses

Genomic DNA was extracted from leaves of resistant and sensitive biotypes of *A. myosuroides* according to the manufacturers´ recommendations (DNeasy® Plant Mini Kit; Qiagen GmbH, Hilden, Germany). PCR primers were designed using the genetic information of the well known chloroplastic ACCase sequence of *A. myosuroides* (EMBL accession no. AJ310767). The primer pair For/Rev ACCase-n600 yielded a 600 bp fragment (Table 2.1) encompassing the triplet for Leu_{1781}. The primer pair For/Rev ACCase-n591 yielded a 591 bp fragment (Table 2.1) encompassing the information of the amino acid positions 1999, 2027, 2041, 2078 and 2096. A single PCR reaction consisted of approx. 30 ng DNA template in a final volume of 25 µl, containing 0.4 µM of each primer, 200 µM dNTPs (Fermentas GmbH; St. Leon-Rot, Germany), and 2 U of Taq DNA polymerase (Invitrogen GmbH; Karlsruhe, Germany) with the supplied buffer with 1 x concentration. The reactions were carried out on an Eppendorf Mastercycler Personal (Eppendorf AG; Hamburg, Germany), with following cycle steps: a 5 min initial denaturation step at 95 °C, followed by 35 cycles of 95 °C for 30 s, 57 °C for 30 s, and 72°C for 1 min, followed by a final extension step of 7 min at 72 ºC. PCR products were analysed by gel electrophoresis and fragment sizes were determined.

Amplified products of seven independent PCR reactions were purified using QIAquick Gel Extraction Kit (Qiagen GmbH; Hilden, Germany) and sequenced directly on both strands using the CycleReader™ Auto DNA Sequencing Kit (Fermentas GmbH; St. Leon-Rot, Germany). Sequencing was carried out on ALFexpress®II (Amersham Pharmacia Biotech Europe GmbH; Nümbrecht, Germany). Results were analysed via MegAlign 5.03 (DNASTAR Inc.; 1990).

Table 2.1: Primers used to amplify fragments encompassing the genetic information of the variable amino acids within the ACCase CT-domain. The primers positions are referred to EMBL/GenBank Accession Number AJ310767.

Primer	Sequence (5'-3')
For ACCase-n600	GCGTGC TGC TGG GCT GAA T
Rev ACCase-n600	CCG GTC AAA ATA ATG GGC TGG TC
For ACCase-n591	AAG GAT GGG CGA AGA CAG TAG TTA
Rev ACCase-n591	CTC CAT CAG ATA GGC TTC CAT TT

2.3 Results and discussion

2.3.1 Dose-response assays

Dose response experiments were conducted to verify resistance und to describe the resistance patterns, thus, conclusions can be drawn to the underlying resistance mechanism. The investigated susceptible reference population was sensitive (100 % reaction) to all tested herbicides. Results for all investigated ACCase inhibitors show, in case of the biotype $BR_{(R)}$, ED_{50} values which were significant higher than for the sensitive reference population (Table 2.2). In comparison ED_{50} values of the biotype $BL_{(R)}$ shows significant differences against all tested APPs, but not against CHDs and DENs. Significance was proved via F-test (α=0.05) and the derived resistance factors resulted in different degrees of resistance. In the biotype $BR_{(R)}$ resistance for the herbicides fenoxaprop-P-ethyl and cycloxydim was highly developed (RF = 51; RF = 130), whereas the herbicides haloxyfop-P-methyl, fluazifop-P-butyl, and quizalofop-P-ethyl showed a moderate resistance level with values ranging up from 10 to 19.

Table 2.2: Parameters of the log-logistic model used to calculate the herbicide dose (g a.i. ha^{-1}) required for 50 % reduction of fresh weight (ED_{50}) of R and S biotypes of *A. myosuroides*.

	ED_{50} (g a.i. ha^{-1})		**RF**	**ED_{50} (g a.i. ha^{-1})**		**RF**
Herbicide	$BR_{(R)}$	$BS_{(s)}$	$BR_{(R)}/BS_{(s)}$	$BL_{(R)}$	$BS_{(S)}$	$BL_{(R)}/BS_{(S)}$
Clodinafop-propagyl	-	-	-	12.9	0.38	34
Fenoxaprop-P-ethyl	1118	21.5	52	331.9	49.1	6.35
Fluazifop-P-butyl	162	9	18	-	-	-
Haloxyfop-P-methyl	107	10.7	10	-	-	-
Quizalofop-P-ethyl	45.8	2.5	19	-	-	-
Clethodim	57	6.5	8.8	23.9	23.9	1
Cycloxydim	2348	18	130	-	-	-
Pinoxaden	-	-	-	2.06	2.06	1
Flupyrsulfuron*	5.9	6	1	-	-	-
Glyphosate*	564	570	1	-	-	-
Isoproturon*	169	173	1	-	-	-

*Biotype $\boldsymbol{BL_{(R)}}$ was assessed only against single doses of labelled herbicides. Visual rating showed no cross resistance against tested herbicides.

Biotype $BL_{(R)}$ showed strong resistance against clodinafop-propagyl (RF = 34) and moderate resistance against fenoxaprop-P-ethyl (RF = 6.35) (Figure 2.1). No resistance was observed for clethodim and pinoxaden.

Furthermore no significant reaction shifts were observed in the response to glyphosate, isoproturon; and flupyrsulfuron for both biotypes. Therefore a cross-resistance to herbicides with other modes of action than inhibiting ACCase could not be proved within this study. But biotypes with metabolic or multiple resistance against ACCase inhibiting herbicides and cross-resistance against ALS inhibiting herbicides, such as flupyrsulfuron are already known (Letouze and Gasquez 2001; Moss et al. 2003; Yu et al. 2007).

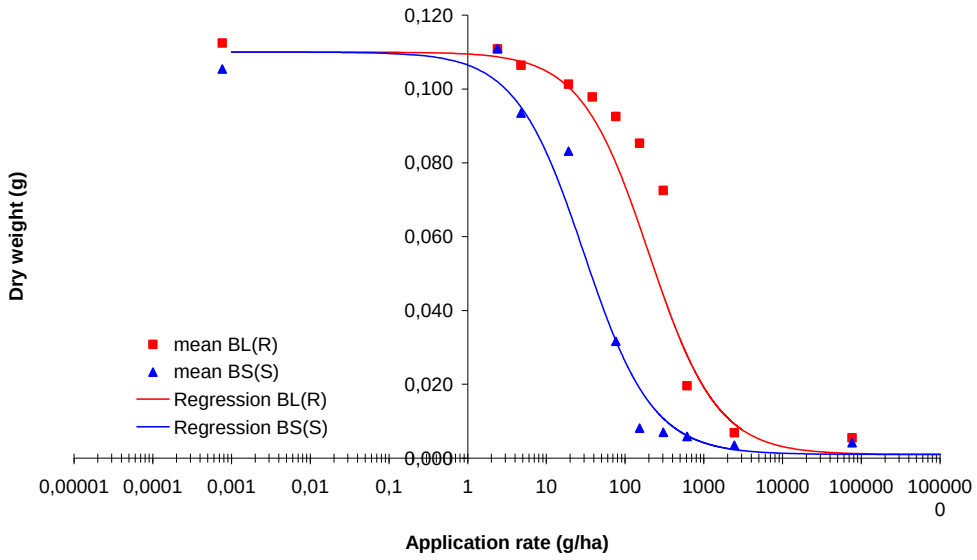

Figure 2.1: Dose response of the biotype $BL_{(R)}$ against fenoxaprop-p-ethyl.

While both biotypes show different resistance patterns, it can be assumed that their resistance is due to different mechanisms or different target-site mutations, respectively. It is known from literature that plants with a metabolic resistance to fenoxaprop-p-ethyl are susceptible to the CHD herbicides cycloxydim and sethoxydim, whereas a resistance against cycloxydim clearly indicates a target-site resistance mechanism (Cocker et al. 2000; Délye et al. 2003; Délye et al. 2008). According to results of Délye et al. (2008) the carried out dose-response assays lead to the presumption that the biotype $BR_{(R)}$ with evolved resistance to both, APPs and CHDs are of target-site resistance, most likely on Ile_{1781} or Asp_{2078}, as underlying resistance mechanisms. Whereas biotype $BL_{(R)}$ shows just

a resistance against APP herbicides: either a non-target site resistance or a target-site mutation on Trp_{2027}, Ile_{2041}, or Gly_{2096} might confer resistance just to APPs.

2.3.2 DNA analyses

To identify the responsible mutations for the suggested target-site resistances, DNA of the relevant parts of the CT domain was sequenced. PCR products of seven individual plants of the resistant and sensitive biotypes were sequenced and compared.

DNA analyses of the $BR_{(R)}$ biotype proved an exchange of a base at the first position within the triplet coding for the amino acid Ile (ATA) at position 1781. Thus, the derived amino acid Ile in the sensitive biotype is exchanged by Leu (CTA) in the resistant biotype. This mutation for target-site resistance against ACCase inhibitors seems to be the most wide spread in grass weeds. Sequencing of the biotype $BL_{(R)}$ revealed an exchange of the second position of the triplet coding for Gly (GGT) to Ala (GCT) on position 2096 within the CT domain. No other non-synonymous changes within the analysed parts of the CT domain of both resistant biotypes were identified.

Both mutations are already described in biotypes of *A. myosuroides* to be responsible for target-site resistance conferring cross-resistance to CHDs in case of the Ile_{1781} mutations and conferring no cross resistance in case of mutations on Gly_{2096} herbicides inhibiting plastidic ACCase (Moss et al. 2003). Zagnitko et al. (2001) showed that a Leu residue on the corresponding position change a formally sensitive ACCase into a resistant one. High levels of resistance against diclofop-P-methyl, fenoxaprop-P-ethyl, fluazifop-P-butyl, cycloxydim, sethoxydim and tralkoxydim, but not to haloxyfop, clodinafop-propagyl and clethodim for the Ile_{1781} mutation were reported elsewhere (Délye 2005). But however, in the conducted greenhouse dose-response experiments, the $BR_{(R)}$ biotype displayed a significant resistance to clethodim and haloxyfop at whole plant level (Table 2.2). Therefore it was concluded that the Ile_{1781}Leu mutation also confers lower resistance to these herbicides. This has to be taken into account if management strategies will be based on the use of one these herbicides in crop rotation.

Compared with literature resistance levels due to an Ile_{1781}Leu substitution within the CT domain of *A. myosuroides* seems to be comparable between biotypes of different origins. Cocker et al. (1999) found an ED_{50} value of 1588 g a.i ha^{-1} for fenoxaprop-P-ethyl in the biotype of *A. myosuroides* "Notts" A1 which is resistant due to the substitution. Similar results are obtained for the second investigated biotype $BL_{(R)}$ with the Gly_{2096}Ala

substitution. Délye et al. (2004) showed resistance ratios for the enzyme activity of *A. myosuroides* biotypes with substitutions on position 2096 of the ACCase ranging up from 6.5 for clethodim and 20.5 for fenoxaprop to 57.5 for clodinafop-propagyl in ACCase extracts. For both substitutions similar resistance levels were observed in this work.

Investigated biotypes were collected from fields were ACCase inhibiting herbicides were used over a period of 10 years. The proved target-site resistance of both biotypes might be due to the frequent use of ACCase inhibitors, combined with wheat monoculture ($BL_{(R)}$) or high proportions of winter wheat with the crop rotation ($BR_{(R)}$). Their management will be possible with other modes of action than inhibiting ACCase. But because of the restricted use of isoproturon, ALS inhibiting herbicides are the only available herbicides managing these biotypes.

Therefore general resistance management strategies have to be spotlighted. Neve (2007) demand from weed scientists to focus less on simply describing resistance and to drive more towards a deeper understanding of the evolutionary forces that underpin resistance evolution. For this, all factors concerning herbicide resistance and their evolutionary process have been taken into account.

Mismanagements in the past as reduced crop rotation combined with minimum tillage led to increasing densities of *A. myosuroides* populations (Moss and Clarke, 1994). But the most important factor affecting the rate of resistance evolution in weeds is the selection pressure exerted by the used herbicides. Thus evolution of target-site resistance is attributed to the frequent use of herbicides of the same mode of action and the ease of selection by these modes of action. Furthermore it is proved that lower herbicide use rates will increase herbicide resistance, because of a higher survival frequency of the target population (Neve and Powles 2005a; Neve and Powles 2005b).

Studies of Zwerger et al. (2002) proved that the application of herbicides with alternative modes of action can reduce the resistance problem; if the herbicides are used in that way that no multiple resistances will be developed. Thus, herbicide management strategies are just a part of the managing of herbicide resistant weeds. In long time view it is necessary to get resistant populations under control by a combination of different crop management strategies. Integrated weed management (IWM) becomes the overall slogan. Therefore Beckie and Gill (2006) describe the reduction of the selection pressure as the underlying principle of any management strategy, because this factor has the greatest impact on resistance evolution and can be controlled by the farmer himself. In this case the non

selective controlling methods, as mechanical weed control or the cultivation of competitive sorts, are of a higher importance (Zwerger at al. 2002).

Due to a consequent combination of different plant cultivation measurements only, herbicide resistant weeds can be controlled and their evolutionary process can be stopped.

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CHAPTER III

Designing molecular markers for detecting target-site based resistance in *Alopecurus myosuroides* Huds.

Natalie Balgheim, Jean Wagner and Roland Gerhards

3 Designing molecular markers for detecting target-site based resistance in *Alopecurus myosuroides* Huds.

Abstract – The detection of the underlying resistance mechanisms of weeds escaping herbicide applications is time and money consuming process. Often single dose assays with collected seeds were carried out, but they do not reveal clear results of the underlying resistance mechanism. As a consequence of the increasing widespread occurrence of herbicide resistance, today fast, easy to handle and less expensive marker technologies are required to confirm weed resistance. High-throughput methods have to be developed.

With the identification of the target-site resistance causing alleles, different molecular techniques found their way into weed science. Currently dCAPS markers are the method of choice. Rapid, easy, and cheap, they are the fitting technique for detecting such alleles. The genetic information of the target gene serves as a basis for the development of these markers. So primer pairs can be designed for creating a recognition site for specific restriction endonucleases during performed PCRs.

With the help of these techniques results can be obtained faster and farmers can be informed earlier, so that appropriate measurements can be implemented earlier within the growing season.

Key words: Acetyl-CoA-carboxylase, black grass, dCAPS, PCR, SNP

3.1 Introduction

Weeds are often more in discussions, since they developed resistance against several herbicides. Since then, weed control practices were second-guessed and integrated weed management (IWM) becomes more important. Even in cereals herbicides with alternative modes of action for controlling grass weeds except the very effective Acetyl CoA (ACCase) inhibitors were missing over a long period (Gressel 2002). All three groups of ACCase inhibitors, the APP, CHD and DEN herbicides are targeting the fatty acid biosynthesis which takes place within plastids and is catalysed by two enzymes, ACCase and fatty acid synthase (Post-Beittenmiller et al. 1992). However, ACCase catalyses the

first committed step of *de novo* fatty acid biosynthesis the carboxylation of acetyl-CoA to malonyl-CoA. This enzyme consists of a carboxyl carrier protein (BCCP), a biotin carboxylase (BC), and a carboxyltransferase (CT) (Sasaki and Nagano 2004). ACCase inhibiting herbicides are interacting with the CT domain of the ACCase, which is more sensitive for inhibition (Nikolskaya et al. 1999). Therefore it is suggested, that all mutations correlated with herbicide resistance are localised inside the CT domain.

As a consequence of the widespread use of ACCase inhibiting herbicides resistance of *A. myosuroides* against ACCase inhibiting herbicides occurs in Europe. Conservative estimations emanate from about 26000 ha infested with herbicide resistant *A. myosuroides* in Europe (De Prado and Franco 2004). In Germany about 5 to 10 % of the arable land with an occurrence of *A. myosuroides* is infested with herbicide resistant populations (Petersen and Wagner 2009).

Two principle biochemical mechanisms are associated with herbicide resistance: an alteration in the target enzyme, often due to a change in the molecular structure, caused by a single nucleotide polymorphism (SNP) on the target enzyme, that reduces sensitivity to the herbicide, the so called target-site resistance, or an increased herbicide detoxification rate, a so called non-target site resistance (Cocker et al. 1999; Heap and LeBaron 2001).

Six amino acid changes on the gene coding for the ACCase enzyme are responsible for different resistance patterns in several grass weed species against ACCase inhibiting herbicides: $Ile_{1781}Leu$ and $Asp_{2078}Gly$ confer resistance to APP and CHD herbicides, whereas $Trp_{1999}Cys$, $Trp_{2027}Cys$, $Ile_{2041}Asn$, and $Gly_{2096}Ala$ [positions numbered according to EMBL accession no. AJ310767] confer resistance to APPs only (Zhang and Powles 2006; Liu et al. 2007; Délye et al. 2008). Such target-site resistance causing SNPs can be used for developing several molecular marker technologies (Gupta et al. 2001).

Herbicide resistance in weeds is usually detected using seedling bioassays (Corbett and Tardif 2006). Seeds from surviving populations have to be collected in fields, prepared and then planted in glasshouse environment. Herbicidal applications with different modes of action and doses are necessary to discriminate between resistant and sensitive plants. The different resistance patterns exhibited by different mutations and mechanisms, allows just a rough differentiation between target-site resistance and non-target site resistance. Clear statements have to be given by DNA sequencing methods. Therefore the development kept off these methods and tends to molecular marker techniques, because they are less time and money consuming and labour intensive and allow a strong discrimination between

target-site and non-target site resistance. The first marker technology used widespread in weed science was the allele-specific PCR published by Délye et al. (2003). However, currently the dCAPS technology is the marker technique of choice. Kaundun and Windass (2006) established this method in weed science for the Ile_{1781} mutation in different grass weed species. Two years later Délye and Boucansaud (2008) presented dCAPS markers for ALS inhibitor resistant *A. myosuroides*. Both research studies based on the CAPS technique which uses gene-specific primers to amplify template DNA to detected polymorphic nucleotides by the loss or gain of a restriction enzyme recognition site (Neff at al. 1998). This technique was modified by Neff et al. (1998) to eliminate the need for the "investigated" SNP to fall within a recognition site for an available restriction enzyme. A restriction enzyme recognition site which includes the SNP is introduced into the PCR product by a primer containing one or more mismatches to the template DNA. The modified PCR product is then digested via an appropriate restriction enzyme, and the presence or absence of the SNP will be identified by the resulting resistance patterns. For designing such primers Neff et al. (2002) initiate a web based system.

Objectives

The widespread occurrence of resistance required fast and easy to handle marker technologies to confirm herbicide resistance. Therefore this paper pursues the object to provide an appropriate tool for detecting target-site based resistance in ACCase inhibitor resistant populations of *A. myosuroides*, based on dCAPS technology.

3.2 Designing and testing dCAPS marker

Plant material and seed source

Seeds of the investigated biotypes were collected from different fields where control with ACCase inhibiting herbicides failed. Preliminary herbicide assays and conducted DNA sequences revealed target-site resistance. dCAPS marker were only developed for resistance alleles which has already been detected in our lab: biotypes with substitutions on Ile_{2041}, Asp_{2078}, and Gly_{2096}.

DNA was extracted with the DNeasy Plant Mini Kit (Qiagen GmbH; Hilden, Germany) following the manufactures recommendations, whereas sequencing was carried out using the CycleReader™ Auto DNA Sequencing Kit (Fermentas GmbH; St. Leon-Rot, Germany).

dCAPS Marker (derived Cleaved Amplified Polymorphic Sequence)

dCAPS primer and their corresponding restriction enzymes were developed using dCAPS Finder 2.0 (Neff et al. 2002), PrimerSelect 5.03 and MapDraw 5.03 (DNASTAR Inc.; 1990). EMBL accession no. AJ310767 and own sequencing results serves as basic sequences. Rules, state by (Délye and Boucansaud 2008) for designing primers to get optimal results were considered. All designed primers were purchased from biomers.net (biomers.net GmbH; Ulm, Germany).

PCRs were performed containing 0.4 µM of each primer, 200 µM dNTPs, 1.25 U Taq polymerase with the appropriate amount of the supplied puffer, and 10-100 mg genomic DNA in a total volume of 25 µl. Reaction was performed on an Eppendorf Mastercycler Personal (Eppendorf) with 35 cycles of 95 °C for 30 s, 57 °C for 30 s, and 72 °C for 60 s, followed by a final extension step of 10 min at 72 ºC. PCR products were analysed by gel electrophoresis. Digestion of amplified PCR products was carried out according to the manufactures recommendations and analysed via gel electrophoresis after reaction has been completed.

Table 3.1: dCAPS primers and their corresponding restriction enzymes.

Target codon (Position; Allele)	Primer	Sequence (5´-3´)	Tm (PCR)	Restriction enzyme; recognition site	Expected dCAPS patterns (length in bp)	
					S	R
2027 (TGC; R)	For ACCase 591 Rev ACCase 591	AAG GAT GGG CGA AGA CAG TAG TTA CTC CAT CAG ATA GGC TTC CAT TT	57 °C	Pst I (CTGCAG)	292 + 73 + 226	246 + 46 + 73 + 226
2041 (ATT; S)*	For ACCase 591 Rev ACCase 591	AAG GAT GGG CGA AGA CAG TAG TTA CTC CAT CAG ATA GGC TTC CAT TT	57 °C	EcoRI (GAATTC)	283 + 308	591
2078 (GAT; S)	For dCAPs-2078 Rev dCAPs-2078	CAG CGC AGG CGA TGT TGG ACT TC ATA GCA CTC GAT GCG ATC TGG GTT TAT CTT GAT A	61 °C	EcoRV (GATATC)	33 + 215	248
2096 (GCT; R)	For dCAPs-2096 Rev dCAPs-2096	CAG ATC GCA TCG AGT GCT ATG CTG AGA GGA CTG CAA AAG CAA CTG TTT CTT CCG AGC TTC TAT GC	59 °C	AluI (AGCT)	204 + 18	39 + 165 + 18

* modified according to Zhang and Powles, 2006

Positions referred to EMBL accession no. AJ 310767

3.3 Results and discussion

As well known in literature, six amino acid substitutions within the CT domain are conferring resistance against ACCase inhibiting herbicides: Ile_{1781}Leu (ATA to CTA or TTA), Trp_{1999}Cys (TGG to TGT), Trp_{2027}Cys (TGG to TGT or TGC), Ile_{2041}Val-Asn (ATT to GTT or AAT), Asp_{2078}Gly (GAT to GGT), and Gly_{2096}Ala (GGT to GCT) (Zhang and Powles 2006; Liu et al. 2007; Délye et al. 2008).

The dCAPS marker which has been developed for the sensitive Ile_{1781} allele by (Kaundun and Windass 2006) serves as basis for designing further dCAPS marker. They are providing dCAPS marker which uses gene-specific primers to amplify and to introduce a restriction enzyme recognition site into the template DNA to detect SNPs by the loss or gain of this recognition site via gel electrophoresis. Via dCAPS Finder 2.0 and DNAStar Primer Select primers were designed which create restriction enzyme recognition sites in the resistant or sensitive alleles, respectively. The primer pairs and their corresponding restriction enzymes were selected according technical and monetary rules (Délye and Boucansaud 2008).

On Trp_{2027} (TGG) two known alleles (TGT and TGC) can cause an amino acid change from Trp to Cys and are responsible for resistance against APP herbicides.

The designed primer pair ACCase-n591 and Rev ACCase-n591 amplifies a 591 bp long fragment which is encompassing the naturally occurring recognition for restriction enzyme PstI in case of resistant allele TGC. During digestion reaction of the PCR generated fragment the sensitive allele was cut into three fragments, whereas the resistant one revealed four (Table 3.1). The 591 bp sized fragment was digested by the restriction enzyme PstI into three fragments in case of the sensitive and in four fragments in case of the resistant allele.

On position 2078 on the CT domain only the SNP on the second position on the triplet coding for Asp (GAT) confers a change to Gly (GGT) and thus resistance against ACCase inhibitors. The 248 bp long fragment amplified via the two primers For CAPS-2078 and Rev CAPS-2078 contains a recognition site for EcoRV in case of the sensitive allele and digested fragment sizes are 33 bp and 215 bp, whereas in the resistant biotype there was no recognition site created will be created during PCR. Fragments stay undigested with a total length of 247 bp (Table 3.1).

The change from Gly to Ala on position 2096 is due to a change on the second position on the triplet of GGT (coding for Gly) to GCT (Ala). In the fragment of the resistant allele a recognition site for AluI was introduced and the resulted fragment lengths after digestion were 39 bp, 165 bp, and 18 bp (Table 3.1).

In case of the SNP on Ile_{2041} the natural occurrence of the recognition site for EcoRI on the sensitive allele has been used. This was described by Zhang and Powles (2006) before. The primers For ACCase-n591 and Rev ACCase-n591 were used to amplify a 591 bp long DNA fragment. After digestion fragments containing the resistant allele are still undigested, whereas fragments containing the sensitive allele were digested into two different sized fragments of 283 bp and 308 bp lengths.

All these above mentioned markers can be used to discriminate not only resistant and sensitive alleles; they discriminate between heterozygous alleles as well. Heterozygous resistant biotypes owe both, the resistant and sensitive alleles, and therefore after digestion, fragments of both alleles were revealed.

Since the early beginnings, herbicide resistance was usually detected using simple seedling bioassays. Although the genetic background of herbicide resistance is elucidated today, these bioassays are used further on. These assays are very simple, but they are very time and space consuming; seeds have to be collected in the fields, prepared for germination, planted and cultivated in greenhouse, etc. (Corbett and Tardif 2006). Clear results were obtained late in the growing season, when herbicide application is completed. Indeed, with the obtained resistance patterns conclusions can be drawn, but they can not readily differentiate between different resistance mechanisms (Kaundun and Windass 2006).

However, clear statements have to be given by molecular methods. Thus, sequencing was the only method which allows a clear prediction if target-site resistance is the revealed resistance mechanism or not. But distinction between homo- and heterozygous resistant alleles is not possible. For detecting new mutations, Sanger sequencing is an indispensable method. But there are some technical disadvantages of DNA sequencing, because both strands of the DNA are sequenced the likelihood of mismatches is very high and the likelihood of misidentifications increases as well (Corbett and Tardif 2006). Therefore easier ways to discriminate between resistant and sensitive alleles have to be developed.

As SNPs are the reasons for changes in the molecular structure of the DNA, they have already been used in large number for the human genome (Gupta at al. 2001). They are easy to detect and therefore several detection techniques moved on into in weed science.

Nowadays dCAPS markers are the technique of choice for identification of known SNPs to confirm the revealed resistance mechanism. They are easy to handle and once developed they can be used for high throughput processes. Mainly the availability of a recognition site for a fitting restriction enzyme may limit the application of this method (Neff et al. 1998). It depends on the availability of enzymes with the appropriate recognition site on the resistance conferring position, if a marker for the sensitive or the resistant allele can be created. Indeed special designed primers can be used to introduce base changes (additionally SNPs) into or nearby the corresponding alleles during PCR, but these possibilities are limited to the same factors. Therefore it is not possible to create dCAPS marker for every resistance conferring allele and other marker technologies have to be used.

The advantages of marker techniques are well described in literature and in the last years several markers have been developed and successfully applied in weed science (Neff et al. 1998; Délye et al. 2002; Corbett and Tardif 2006; Kaundun and Windass 2006). All developed markers allow a clear distinction between sensitive and resistant plants and furthermore dCAPS technology can be used to discriminate between homozygous and heterozygous individuals. The developed markers are the basis for an identification of the underlying resistance mechanism of suspected weed populations and serves as another tool in the management of herbicide resistant weeds.

CHAPTER IV

ALS inhibitor resistant *Apera spica-venti* Beauv. in Germany

Natalie Balgheim, Jean Wagner and Roland Gerhards

4 ALS inhibitor resistant *Apera spica-venti* Beauv. in Germany

Abstract - Reports on control failure of grass weeds due to herbicide resistance accumulate. Since production systems have changed to a higher proportion of winter cereals, *Apera spica-venti* L. Beauv. is one of the dominating grass weeds in European winter wheat fields. Especially in Germany, France, Denmark, Belgium, Switzerland and the Czech Republic *A. spica-venti* it is one of the most troublesome weeds in winter annual grains evolving resistance to ALS-inhibitors. In this study a biotype of *A. spica-venti* from a winter wheat field in Germany which showed low effect to ALS inhibitors was investigated. To prove resistance single dose assays were performed in greenhouse. Detailed dose-response experiments were used to characterize the reaction to sulfosulfuron, propoxycarbazone and isoproturon. A statistical significant resistance was observed to sulfosulfuron (RF=83.9) and propoxycarbazone (RF=10.9), while the absence of resistance against isoproturon (RF=1) led to the assumption of target-site resistance as the only responsible resistance mechanism. To confirm the role of reduced target susceptibility, target assays were performed. A statistical significant different ALS susceptibility of the resistant biotype against sulfosulfuron (RF=158) and propoxycarbazone (RF=31.5) proved again target-site resistance. To identify the responsible mutation of the ALS and to manifest the previous results, the relevant parts of ALS gene from resistant and susceptible biotypes were sequenced. Results were aligned and compared with ALS sequences of *A. myosuroides*. A SNP (Single Nucleotide Polymorphism) is responsible for the exchange of proline by threonine at the respective amino acid position 197. This mutation of the ALS protein is well known to cause target-site resistance in different grass weed species. In order to detect this mutation in future samples a molecular marker based on CAPS technology was developed using a naturally occurring enzyme recognition site. This technology is reasonable to detect resistance in field-collected leaf samples.

Key words: acetolactate-synthase, CAPS, dose-response relationship, herbicide resistance, molecular marker, silky bent-grass, SNP, target-site resistance.

4.1 Introduction

Since the proportion of winter cereals in crop rotation increases, infestation levels of grass weeds increase as well (Melander 1995). Today *Apera spica-venti* L. Beauv. counts for one of Europe's worst weeds associated with problems in winter cereals (Naylor and Lutman 2002). It has a high propagation potential of an average of 2000 seeds per plant (Warwick et al. 1985). Seeds are not long-lived in soil, with an exceed viability of 2 years and exhibit little primary dormancy (Melander et al. 2008). These characteristics and its preference for autumn germination, make *A. spica-venti* a problematic weed in autumn-sown crops, notably winter cereals (Chomans and Kells 2001).

But control measures for *A. spica-venti* have been limited; so there is a heavy reliance on acetolactate synthase (ALS) inhibiting herbicide. These herbicides are inhibiting the synthesis of the branched chained amino acids leucine, isoleucine and valine, of which ALS catalysis the first common step (Ray 1982b). The branched chain amino acids are synthesised from pyruvate, with 2-ketobutyrate additionally required for the biosynthesis of isoleucine. Five chemical groups are known inhibiting ALS: Sulfonylureas (SUs), Imidazolinones (IMIs), Pyrimidinylthiobenzoates (PTBs), Sulfonylaminocarbonyltriazolinone (SCTs), and Triazolopyrimidines (TPs).

The biological activity of the sulfonylurea herbicides is extremely high with typical field application rates of 10 to 100 g per hectare (Ray 1982a). The combination of the high potency and the minor toxicity to non target organisms, including mammalians, makes them very effective and safe herbicides (Shaner and Sigh 1997; Duggleby and Pang 2000).

Indeed, ALS inhibitors are one of the most important herbicide classes used in many cropping systems because of their broad spectrum of weed control activity and wide crop selectivity (Park and Mallory-Smith 2004), but, however, they are the most resistance-prone herbicide group (Délye and Boucansaud 2008; Heap 2009). Weeds evolved resistance to ALS inhibiting herbicides faster than for any other mechanism of action (Gressel 2002). Ironically, the high efficacy of ALS inhibiting herbicides that enables them to be used at very low rates is the reason of this quick evolution (Saari et al. 1994).

Five years after their commercial launch in 1982 resistant weed populations have already been detected (Mallory-Smith et al. 1990). Today 101 species are reported to be resistant against ALS inhibiting herbicides (Heap 2009). Herbicide resistance can be due to target-site and/or non-target site mechanisms.

The resistance to ALS inhibitors is often a consequence of amino acid substitutions in the ALS enzyme which prevent herbicide binding, commonly known as target-site resistance (Marshall and Moss 2008). However, target site resistance to ALS inhibiting herbicides can be conferred by a number of different point mutations: six mutation sites are known to confer target-site resistance to ALS inhibiting herbicides: Ala_{122}, Pro_{197}, Ala_{205}, Asp_{376}, Trp_{574}, and Ser_{653} (Tranel and Wright 2002; Whaley et al. 2007). All these mutations are of different resistance characteristics: substitutions on position Ala_{122} and Ser_{653} result in resistance to IMI herbicides but not to SU, Ser_{653} evolved additionally a resistance against PB herbicides; Trp_{574} conferred resistance to both, IMI and SU, and Pro_{197} results in SU and TP resistance, whereas the IMI resistance is depending on the substitute amino acid (Duggleby and Pang 2000; Tranel and Wright 2002). Substitutions on Asp_{376} revealed in resistance to all classes of ALS inhibiting herbicides (Whaley et al. 2007). Hitherto not all resistance patterns against most other herbicidal classes of the ALS inhibiting herbicides are investigated today.

Aims

The objectives of this study were (i) to investigate the reaction of a German biotype of *A. spica-venti* collected in a winter wheat field, where control with ALS inhibiting herbicides failed; (ii) to analyse the molecular background of this resistance to generate knowledge of the resistance mechanisms and the resistance patterns to different herbicides which is linked with the development of controlling strategies; (iii) to prove the resistance characteristics in greenhouse dose-response assays herein; and (iv) to verify target-site resistance as the underlying resistance mechanism due to ALS protein assays.

4.2 Materials and methods

4.2.1 Seed source

Seeds of the resistant biotype were collected in 2005 at a German winter wheat field. There were germinated on flooded vermiculite and emerged seedlings were planted into sandy-loam. Plants were cultivated under 25/20 °C day/night at a 12 h photoperiod. Progeny seeds were collected, cleaned and stored until dose-response experiments were carried out. Sensitive standard seeds are commercial available at Herbiseed (Herbiseed, Twyfort, United Kingdom).

4.2.2 Bioassays

Whole plant bioassays

To confirm resistance and to detect their degree and characteristics, greenhouse dose-response experiments were performed. Each with 10 – 20 replicates per dose, and one or two plants per pot, respectively. Herbicides were sprayed in a laboratory track sprayer applying 400 liters ha^{-1} with a single nozzle "Teejet 8004EVS" at 3 bar at plant leaf stage (BBCH) 12 – 13. Foliage fresh and dry weight was taken 21 DAT (days after treatment), if herbicide response was completed. Herbicide active ingredients of sulfosulfuron, isoproturon, and iodosulfuron in combination with mesosulfuron were used to determine herbicide response against the resistant and sensitive biotypes.

ALS protein assay

To confirm target-site resistance ALS enzyme assays according to Wagner (2004) were performed. ALS activity was measured in a crude protein extract obtained from fresh plant material. The inhibition of the ALS was quantified against formulated products of sulfosulfuron and propoxycarbazone.

Statistical analysis

Non linear regression according to Seefeldt et al. (1995) was used to calculate dose response relationships in consideration of the log-logistic model of Streibig (1988). Inhibiting of plant weight and enzyme activity was calculated in comparison to a sensitive reference population or enzyme of a sensitive population, respectively.

4.2.3 ALS sequencing

For identification of a possible target-site mutation PCR fragments were generated and sequenced. Plant DNA was extracted using DNeasy® Plant Mini Kit according to the manufactures recommendations (Qiagen GmbH, Hilden, Germany). Primer design based on the genetic information of *Alopecurus myosuroides*, *Bromus tectorum* and *Lolium multiflorum* ALS respectively (EMBL Accession no. AJ437300; AF488771; AF310684). Primer pair For ALS-n654 (5'- CGA GCC CCG CAA GGG CGC CGA CAT -3') and Rev ALS-n654 (5' -GCA GAG CAG CCA CCG CCA ACA TA -3') generates fragments of 654 bp long sizes, encompassing the genetic information of Ala_{122}, Pro_{197}, and Ala_{205}. PCR

reactions contain in a total volume of 25 µl, of approx. 30 ng template DNA, 0.4 µM of each primer, 200 µM dNTPs (Invitrogen GmbH; Karlsruhe, Germany), and 2 U of Taq DNA polymerase (Invitrogen GmbH; Karlsruhe, Germany) with the supplied buffer with 1 x concentration. Following program was performed on an Eppendorf Mastercycler Personal (Eppendorf AG; Hamburg, Germany): first a 5 min initial denaturation step at 95 °C, followed by 35 cycles of 95 °C for 30 s, 30 s at the special primer annealing temperature, and 72 °C for 1 min, followed by a final extension step of 10 min at 72 ºC. PCR products were analysed by gel electrophoresis and fragment sizes were determined.
For sequencing reactions, PCR products were purified using QIAquick Gel Extraction Kit (Qiagen GmbH; Hilden, Germany) and sequencing reactions were prepared using the CycleReader™ Auto DNA Sequencing Kit (Fermentas GmbH, St. Leon-Rot, Germany). Sequencing was carried out on ALFexpress®II (Amersham Pharmacia Biotech Europe GmbH; Nümbrecht, GermanyPharmacia Biotech). Results were analysed via DNASTAR MegAlign 5.03 (DNASTAR Inc.).

4.2.4 CAPS marker (Cleaved Amplified Polymorphic Sequence)

A CAPS marker was developed according to Konieczny and Ausubel (1993) using DNASTAR software. Sequencing results of sensitive and resistant *A. spica-venti* ALS serves as basis for developing CAPS marker for $Pro_{197}Thr$. The natural recognition site of the restriction enzyme BstEII (G↓GTNACC) (Fermentas GmbH, St. Leon-Rot, Germany) was used to distinguish resistant from sensitive plants after PCR and fragment digestion. PCR were performed as described above, using primer pair For ALS-n654 and Rev ALS-n375 (5'- GTG ATG GAG CGG GTG ACC TCT A -3').

4.3 Results and discussion

4.3.1 Bioassays

Whole plant bioassays

Reactions against the herbicides isoproturon, sulfosulfuron, and iodosulfuron in combination with mesosulfuron were quantified and compared against a susceptible reference population. Resulted dose-responses show a significant difference of the resistant biotype to the active ingredients sulfosulfuron (RF=83.9) (Figure 4.1) and

iodosulfuron/mesosulfuron (RF=10.9) in comparison to the sensitive reference (Table 4.1). Both herbicidal ingredients are out of the sulfonylurea group. However, no resistance was detected against isoproturon (RF=1) which is an urea class herbicide and inhibits the photosynthesis at photosystem II. Mutations on Ala_{122} and Ser_{653} are not conferring resistance against SUs, whereas Ala_{205} confers moderate resistance against SUs. However substitutions on Pro_{197}, Asp_{376}, and Trp_{574} confer high resistance against SUs. This suggests that detected resistance is conferred by a change on Pro_{197}, Asp_{376}, or Trp_{574}.

Table 4.1: Parameters of the log-logistic equation used to calculate the herbicide dose (g a.i. ha^{-1}) required for 50 % reduction of fresh weight (ED_{50}) of R and S biotypes of *A. spica-venti*.

	ED_{50} (g a.i. ha^{-1})		**RF**
Herbicide	**R**	**S**	**R/S**
Sulfosulfuron	161.95	1.93	83.9
Iodosulfuron+Mesosulfuron	24.66	2.26	10.9
Isoproturon	252.50	252.50	1.0

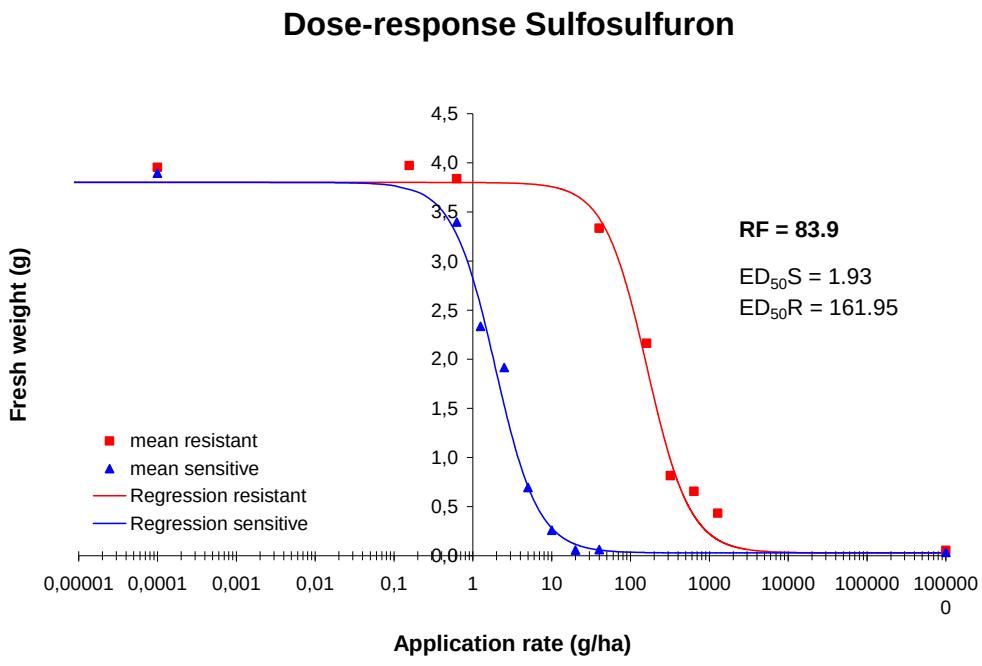

Figure 4.1: Dose-response of *A. spica-venti* tested against sulfosulfuron.

ALS protein assay

To confirm target-site resistance ALS enzyme assays were performed. The inhibition of the ALS protein was quantified against formulated products of sulfosulfuron and propoxycarbazone. Reaction of ALS protein extract from resistant and sensitive plants. I_{50} is representing the dose where 50 % reaction (inhibition of ALS activity) was measured. High resistant ratios were obtained for sulfosulfuron (RF = 158), lower for propoxycarbazone (31.5) (Table 4.2). The significant reaction shifts against both herbicides proved target-site resistance once more.

Table 4.2: Parameters of the log-logistic equation used to calculate the herbicide dose (g a.i. ha^{-1}) required for 50 % reduction of enzyme activity (I_{50}) of R and S biotypes of *A. spica-venti*.

	I_{50} (mg ml^{-1})		RF
Herbicide	**R**	**S**	**R/S**
Sulfosulfuron	0.0316	0.002	158
Propoxycarbazone	0. 0164	0.00052	31.5

4.3.2 ALS sequencing

For identification of resistance, PCR fragments encompassing the genetic information Ala_{122}, Pro_{197}, and Ala_{205} of ALS were sequenced. Results proved an exchange of CCC to ACC within the triplet coding Pro_{197} to be responsible for the predicted target-site resistance. Mutations on Pro_{197} are well known to confer target-site resistance in different grass weed species (Guttieri et al. 1995; Tranel and Wright 2002; Park and Mallory-Smith 2004). According to Guttieri et al. (1995) this allele confers resistance against SUs, but not against IMIs which was proven by the dose-response studies showed before once more. Resistance against TP herbicides described in literature could not be verified with the tested herbicides (Duggleby and Pang 2000). This Pro_{197} mutation was detected at the first time in a German *A. spica-venti* biotype.

For the identified mutation site nine different amino acid substitutions are known to confer herbicide resistance (Tranel et al. 2009). Thus, the herbicide binding site of the ALS is different from its active site, although the two sites are probably on close proximity, there is a large amount of flexibility in the herbicide binding site of the ALS, so substitutions at each of the several conserved amino acids with apparently minimal consequences to

normal catalytic function of the enzyme can be tolerated (Tranel and Wright 2002). Substitutions on Pro_{197} result in 100- to 1000-fold resistance to SUs and TPs (Duggleby and Pang 2000), but confer resistance to IMIs or PBs depending on the amino acid which is substituted (Gressel 2002). The Pro_{197}Trp exchange proved in this study is associated with resistance against the active ingredients of SUs and some IMIs. Guttieri et al. (1995) showed an RF value of 120 for chlorsulfuron and an RF value of 2 for imazethapyr. Resistance against SUs can be verified by our own results with similar RF values, whereas resistance against IMIs was not tested. Moreover Preston et al. (2006) showed that the Pro_{197}Trp substitution resulted in an enzyme that was highly resistant (>200-fold) to inhibition by SU herbicides and moderately resistant to TP and IMI herbicides. Similar results were obtained with the investigated biotype, which showed resistance to SU and SCT herbicides.

4.3.3 CAPS marker

The amino acid substitution on Pro_{197}Trp results in the predicted target-site resistance. The recognition site of the restriction enzyme BstEII (G↓GTNACC) which is naturally occurring in the resistant biotype with the resistance conferring and Trp coding allele ACC can be used to distinguish between resistant and sensitive plants

Figure 4.2). The 375 bp sized fragment generated during PCR have to be digested. Digestion reaction result for homozygous alleles in an undigested 375 bp long fragment for the sensitive (CCC) allele and in 304 bp and 71 bp long digested fragments for the resistant one. As CAPS marker can divided between target and non-target site herbicide resistant, three different fragments were obtained, if investigated samples are of heterozygous DNA: the undigested 375 bp and the digested 304 bp and 71 bp long fragments. This technique allows detection after one PCR and digestion step only. This makes such techniques simple, cheap and easy to handle for detecting target-site based resistance on a high throughput procedure, and allows detection early within the growing season for applying weed management strategies.

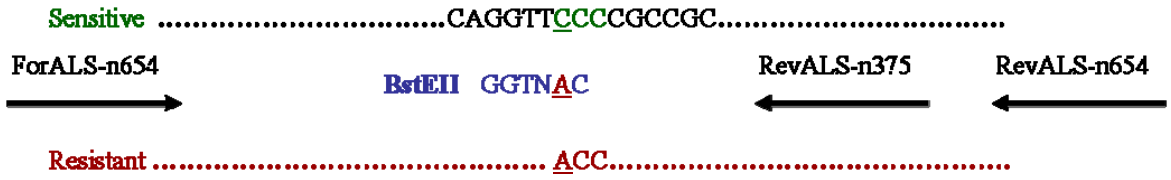

Figure 4.2: Design and results of the carried out molecular investigations.

The developed marker renders an important contribution to the diagnosis of target-site resistance against ALS inhibiting herbicides in *A. spica-venti*. Their use will lead to a clear prediction of the resistance mechanism and the underlying cross resistances. In contrast to the dCAPS marker, the herein presented marker does not need a modified primer to introduce a recognition site; it is rather using the natural occurring recognition site of resistant Trp allele.

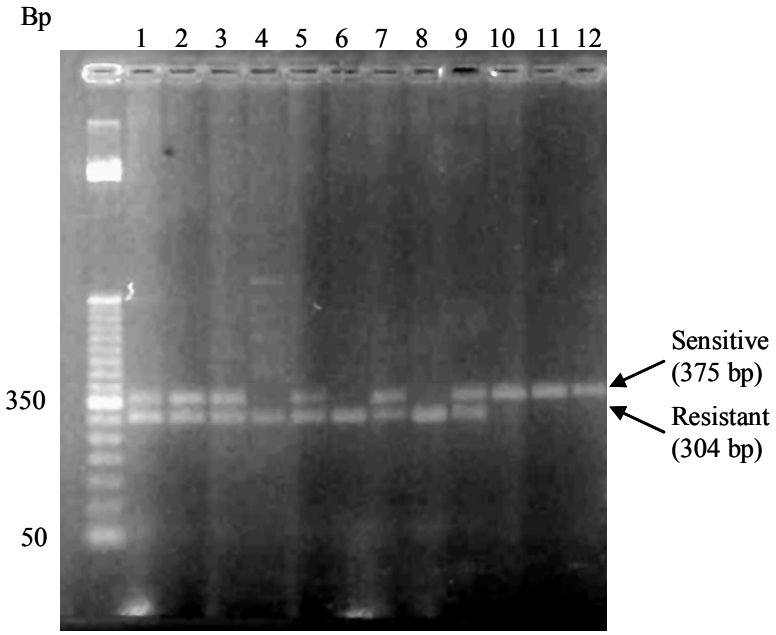

Figure 4.3: CAPS patterns of 12 different *A. spica-venti* individuals a. Samples in lanes 1-3, 5, 7, and 9 are heterozygous resistant; samples in lanes 4, 6, and 8 homozygous resistant and samples in lanes 10-12 homozygous sensitive.

4.4 Conclusions and management strategies

As a consequence of declining profit margins agricultural production systems changed. Today cereals and low tillage systems are characterising the crop rotations. However, without the weed management benefits of more tillage intensive practices, reduced tillage systems often have a greater reliance on herbicides, which can result in weed populations dominated by only a few species, often grass weed species (Melander et al. 2008).

Furthermore herbicide resistance is the result of the repeated use of herbicides with the same or similar mode of action (Thill and Lemerle 2001). As repeated use of a mode of action removes susceptible individuals from the population, leaving greater proportion of resistant individuals to reproduce and contribute to the soil seed bank (Corbett and Tardif, 2006). Moreover the application of low use rates leads to the selection of herbicide resistant individuals as well (Neve and Powles 2005a; Neve and Powles 2005b). Therefore agricultural production systems have to be reflected, to avoid and manage herbicide resistance.

Thereby the ecological consequences of resistance have to be taken into account. Whereas Saari et al. (1994) suggested that plants fitness may be unaffected by resistance resulting from changes in ALS sensitivity to ALS inhibitor herbicides. Eberlein et al. (1999) found higher branched chain amino acid concentrations in leafs and seeds of resistant biotypes which could be associated with earlier germination or a lack of thermodormancy, characteristics that could confer strategic competitive advantages. Nevertheless, recent studies of Park et al. (2004) showed a rapid and earlier germination of resistant biotypes which had reached 60% germination when the sensitive biotype initially germinated; produced seeds were larger, even if they were produced in a smaller amount, but no differences in competitive ability ware observed between resistant and sensitive biotypes on the basis of shoot dry weight, leaf area, or plant height.

However, Park et al. (2004) assumed if selection pressure of ALS inhibitors decreases, biotypes with resistance to ALS inhibiting herbicides are supposed to remain at a similar proportion in the field as sensitive ones will do. Therefore a consequent change of the herbicide and crop management strategies help managing herbicide resistant weeds and preserve the remaining modes of action.

CHAPTER V

Spatial distribution of herbicide resistant *Alopecurus myosuroides* Huds. on field-scale: A case study

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Journal of Plant Diseases and Protection
Special Issue XXI, 63-67, 2008

5 Spatial distribution of herbicide resistant *Alopecurus myosuroides* Huds. on field-scale: A case study

Abstract - The spatial distribution of target-site resistant *Alopecurus myosuroides* Huds. was assessed on field scale using Geographic Information System (GIS) in a field with crop rotation of two years winter wheat, followed by one year of sugar beet. After more than ten years with minimum tillage and application of ACCase inhibiting herbicides control of *A. myosuroides* was not possible due to a target-site resistance detected in 2003. This resistance is based on a mutation of Ile to Leu on position 1781 of the ACCase gene.
For weed mapping a 30m*30m grid was established in the field in spring 2006. Plant density was determined and plant leaf material (n = max. 10 plants) was collected on each intersection point, before and after herbicide application in 2006 and 2007. Individual plants were genotyped using the PyrosequencingTM technology. The distribution and frequencies of ACCase alleles was analysed and displayed.
Results show a heterogeneous distribution of *A. myosuroides* in the field and a correlation of weed density and the frequency of homozygous resistant plants, pointing out the role of plant densities in resistance evolution of allogamous species. The high frequency of homozygous plants reflected the intensity of resistance inbreeding in the population over the time.

Keywords: ACCase inhibitors, black grass, target-site resistance, weed map.

5.1 Introduction

Alopecurus myosuroides Huds. is the most important herbicide-resistant weed in Europe (Moss et al. 2007). Responsible for this evolution is the continuous use of herbicides with the same mode of action, which led to the selection of herbicide resistance (Park and Mallory-Smith 2004). This is particularly true for herbicides with a single target in weeds, such as herbicides inhibiting the acetyl-coenzyme A carboxylase (ACCase) in plant fatty acid biosynthesis (Délye 2005). In Germany ACCase inhibiting herbicides have been used repetitive for several years especially in areas, were cereals are cultivated in monoculture

(or almost in monoculture) and reduced tillage is performed. The evolved resistance mechanisms in *A. myosuroides* to ACCase-inhibitors are divided into two groups: target-site and non-target-site resistance. Target-site resistance is the result of a modification of the herbicide-binding site, which precludes a herbicide from effectively binding on its target. Non-target-site resistance is resistance due to a mechanism other than a target-site modification. So, enhanced metabolism is the most common mechanism in *A. myosuroides*, conferring partial resistance to a wide range of herbicides (Moss et al. 2007). The proportion of target-site resistance is lower than of non target-site mechanisms, especially in Germany (Drobny et al. 2006). All mechanisms are under genetic control. In case of target-site resistance in *A. myosuroides* five mutation sites on ACCase are known. Mutations of Ile_{1781} to Leu (amino acids are given in three letter code and numbers are referred to the amino acid position within ACCase [EMBL Accession No. AJ310767]), $Trp_{2027}Cys$, $Ile_{2041}Asn$, $Asp_{2078}Gly$, and $Gly_{2096}Ala$ confer resistance to fenoxaprop-P-ethyl at field rates (Délye 2005). In the UK the exchange of Ile to Leu at position 1781 appears to be the most widespread type of target-site resistance mutation (Moss et al. 2007), while the proportion of target-site resistance especially due to an Ile_{1781}-Leu mutation is much lower in Germany (Menne et al. 2008).

Two alleles of the ACCase gene are known to be responsible for $Ile_{1781}Leu$ mutations. An adenine-to-cytosine (A-to-C) and an adenine-to-thymine (A-to-T) transversion at the first position in amino acid codon 1781 (Table 5.2), which both cause an Ile to Leu substitution. These alleles and therewith target-site resistance can be diagnosed if mutations – generally referred to be single nucleotide polymorphisms (SNPs) – are detected with appropriate methods. One method for mutation detection used in weeds science is the derived cleaved amplified polymorphic sequence (dCAPS) technology (Kaundun and Windass 2006).

In the presented case target-site resistance in plants of *A. myosuroides* was selected in a field population over the last 15 years due to the continuous use of ACCase inhibiting herbicides like fenoxaprop-P-ethyl and clethodim. The resistance mechanisms and characteristics of this population are well known. This population has an $Ile_{1781}Leu$ mutation, which cause resistance to FOP and DIM herbicides (Balgheim et al. 2006). Furthermore analysed individuals of the investigated population showed a high proportion of homozygous Leu_{1781} genotypes (Leidinger 2007).

Different distribution and population dynamics studies about of *A. myosuroides* were carried out, especially in the course of site-specific weed control (Wilson and Brain 1991).

But however, today less is known about the spatial distribution of resistant weeds in arable fields and even less is known about the distribution of resistance alleles. It is important to understand the population dynamic characteristics of herbicides resistant weeds before herbicide measurements (reduction of the total herbicide amount used by site-specific weed control or management with different mode of action) are applied.
This study was performed to clarify how a resistant population is distributed in a single field and how the population is split up into homozygous and heterozygous resistant plants.

5.2 Materials and methods

Spatial distribution maps

Investigations were carried out at a 12.9 ha field near Stuttgart (Germany). Studies are containing the years 2006 and 2007 with sugar beet and winter wheat. The farmer practices a crop rotation with two years of winter wheat followed by one year of sugar beets. Furthermore non-tillage soil conservation with glyphosate to reduce the amount of black grass and self-sown grain were made. The practiced weed management measurements were carried out on the farmers own (Table 5.1).
For weed sampling and distribution analysis a regular 30 m * 30 m grid was established in the experimental field. On each grid intersection point weed density and leaf material of n ≤ 10 plants was collected before and after plant protection measurements and data were assessed by using a geographic information system (ArcGIS). Plant density was estimated at each grid intersection point with a 0.1 m² quadrant and data were projected on 1 m². Distribution maps of black grass for the homo- and heterozygous resistant and for sensitive plants (molecular analyses see below) were created, using the assessed results. Therefore plant density and grid intersection point locations were interpolated using inverse distant weighting (IDW). Thresholds were fixed on 0, 10, 20 and more than 30 *A. myosuroides* plants m^{-2} (Wahmhoff and Heitefuss 1984). In the year 2006 just the weed density was estimated, while in 2007 plants of sampling terms in March and July were genetically analysed.

Genotyping of individuals

In total 503 individuals were analysed using the Pyrosequencing™ technology according to Wagner (unpublished). Plants were screened for two alleles, the wild-type (sensitive, ATA for Ile) and the allele conferring target-site resistance (CTA for Leu) in *A.*

myosuroides (Table 5.2). Therefore DNA of the collected plant material was extracted according to Menchari et al. (2006) and genotyped at position 1781 using Pyrosequencing™.

Table 5.1: Plant protection measurements and sampling dates.

2006 Sugar beet		**2007 Winter wheat**	
29.03.	Data collection	17.11.06	Sowing
08.04.	3.0 l Glyphosate	13.03.	Data collection
22.04.	Sowing	07.04.	300 g Atlantis WG
			0.6 l FHS
			100 g Husar
			100 g Hoestar Super
			12 kg Bittersalz
09.05.	Data collection	03.05.	Data collection
10.05.	1.2 l Betanal Expert	12.07.	Data collection
	1.0 l Goltix SC		
22.05.	1.3 l Betanal Expert	09.10.	1.5 l Durano
	1.0 l Goltix SC		
29.05.	1.2 l Betanal Expert		
	1.7 l Goltix SC		
	0.4 l Clethodim		
	0.8 l Para Sommer		
03.07.	Data collection		

Table 5.2: Sensitive and resistant phenotype, DNA sequence of codon for Ile_{1787}, and resulting amino acid in ACCase.

Phenotype	**sensitive**	**resistant**	**resistant**
DNA-sequence	AAC**A**T**A**CAT	AAC**C**T**A**CAT	AAC**T**T**A**CAT
Amino acid at position 1781	Ile	Leu	Leu

5.3 Results and discussion

Results show a heterogeneous distribution of *A. myosuroides* in the observed field during all collection terms (Figure 5.1 and Figure 5.2) which is in confirmation to literature (Marshall 1988). The patches were persistent over the two investigated years. Wilson and Brain (1991) observed that patches of weeds had a 10-year long persistence even when effective herbicides were applied in every year. However, no additional weed patches occurred at locations with low infestation levels. This indicates that patches of *A. myosuroides* persist with few individuals escaping weed control and producing new seeds. Otherwise the population would have been eradicated after a ten years of effective weed control since seeds survive a maximum period of eight years in the soil (Gerhards and Christensen 2003). Herbicide resistance reduces efficacy of chemical weed control and thus increases weed population density in patches. Dunker et al. (2002) used a population dynamic model to predict how *A. myosuroides* will spread within arable fields when individual plants will not be controlled and produce new seeds. Depending on other mortality effects due to crop rotation and soil tillage it take up to 20 years until the total field was heavily infested with *A. myosuroides*. This can be one aspect for modelling the distribution of resistant weeds in the future.

The investigations in 2006 (Figure 5.1) showed first after seeding of sugar beets a decrease in the amount of *A. myosuroides* in May within the patches, but an increase in July. This could be the consequence of the weather conditions (data not shown), which results in a poor weed control due to dry soil conditions. Nevertheless the investigated biotype shows resistance to clethodim, which was applied at low doses to the field. Also this fact could have been led to the increasing amount of *A. myosuroides* plants in July, due to not sufficient grass weed control. *A. myosuroides* patches have a relative spatial stability over the two investigated years. The persistence of patches has been confirmed by investigations of Wilson and Brain (1991) and Krohmann et al. (2006).

Weed density, also within the patches depends on the crop and can be influenced by the crop rotation and the herbicides which are applied within the rotation. In sugar beet *A. myosuroides* has only a slight effect on the yield. But it is possible that the application of low doses of clethodim enhance the development of resistant plants, or the emerged *A. myosuroides* plants were not successfully controlled, respectively. *A. myosuroides* plants show in dose-response relationships a resistance factor of about eight, the control with the normal application rate is therefore not possible. So the application of clethodim can lead

to a selection of a higher amount, or of non-reduction of herbicides resistant *A. myosuroides* plants in the field, respectively.

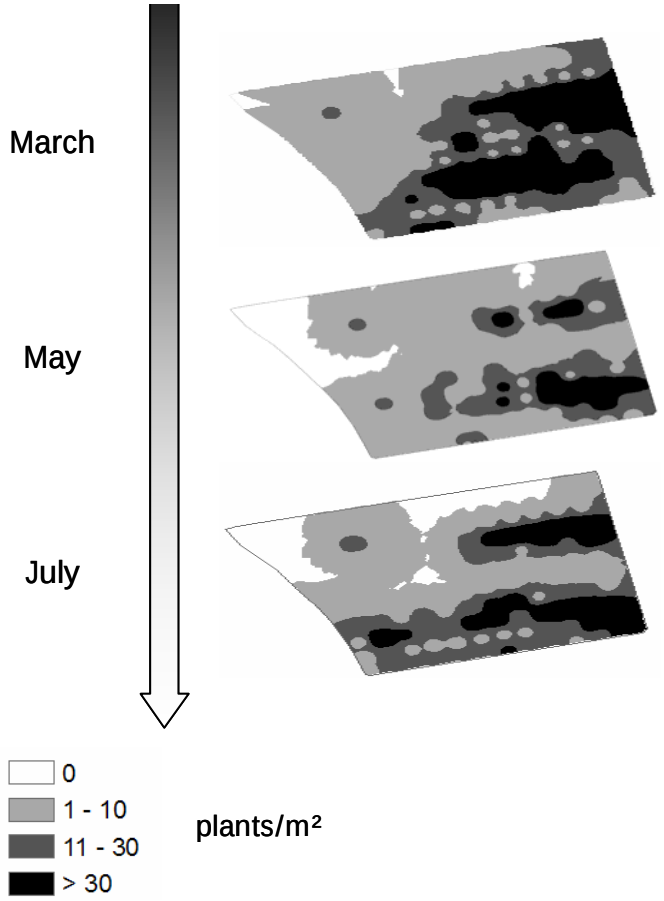

Figure 5.1: Spatial distribution of *A. myosuroides* in sugar beets in 2006 (plants/m²).

In 2007 not only the plant density was assessed, but also the genetic background of sampled *A. myosuroides* plants was analysed and pictured (Figure 5.2). In comparison to 2006 *A. myosuroides* amount is increasing first, because the seedlings emerge at the same time as winter wheat and control took place at the begin of April first. But with the application of sulfonylurea containing herbicides, the amount of *A. myosuroides* is decreasing. Control of this biotype is therefore possible with herbicides containing other active ingredients than ACCase inhibiting herbicides. In dose-response relationships the biotype showed no resistance against glyphosate, which is used in favour with non-tillage and - much more important for management strategies - no resistance against flupyrsulfuron and isoproturon (Balgheim et al. 2006). So these herbicides can be used for

herbicide resistant weed management strategies, where biotypes evolved resistance against ACCase inhibiting herbicides.

In addition Figure 5.2 shows the spatial distribution of the investigated plants in 2007 split up in the distribution of the total plant amount and the amount of the homo- and heterozygous resistant and sensitive plants. A reduction of the total amount of collected *A. myosuroides* is visible. But the amount of homozygous resistant plants increases within the patches, whereas the total amount decreases.

The distribution of the resistant plants depends on the appearance of the *A. myosuroides* patches. If there is a high amount of weeds there will be a high proportion of resistant plants. Successful application of herbicides is depending on several factors and, among others, on the weed density (within the patches). The phytotoxicity of herbicides decreased as plant density increased, because less herbicide is taken up by plant (Weidenhamer et al. 1989). Neve and Powles (2005b) describe the rapid evolution of herbicides resistance under the application of reduced herbicide rates, which is similar to application on a high amount of plant, because the uptake will be similar. This can be one further explanation for the occurrence of the resistant plants within the patches.

So this study gives information about the spatial distribution of herbicides resistant plants and about possibilities for further resistant management ideas. The distribution of resistance alleles in weed populations has been studied for larger geographical areas until now just for *A. myosuroides* (Menchari et al. 2006). To date, there has been no documentation of the spatial scale of *A. myosuroides* on single field level at which herbicide resistance alleles evolve.

- Acknowledgements - We would like to thank Mr. Rothacker for his cooperation and furthermore Ernst Tränkle and Cathrin Reichert for their assistance in sampling and guidance.

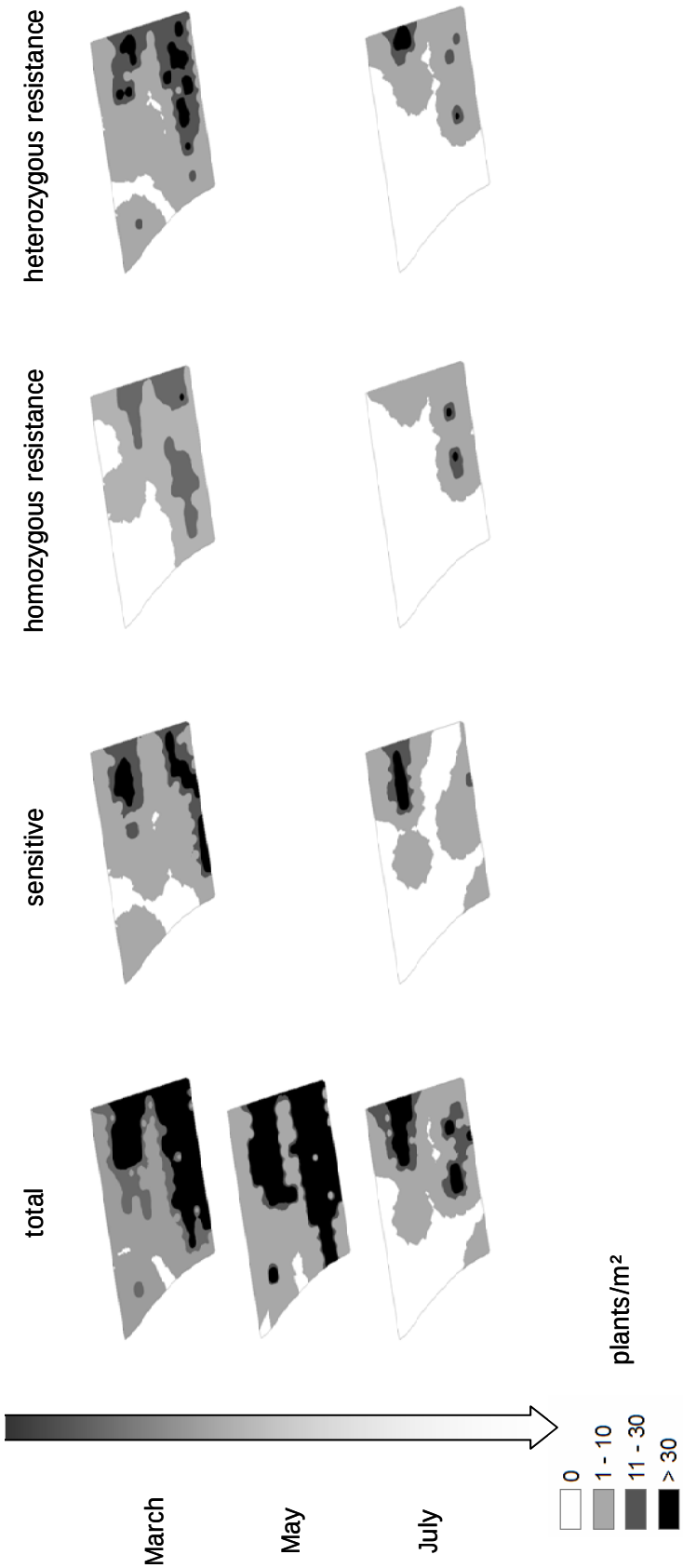

Figure 5.2: Spatial distribution of *A. myosuroides* in winter wheat in 2007, split in the total weed density, amount of sensitive, and homozygous and heterozygous resistant plants (plants/m²).

CHAPTER VI

General Discussion

Natalie Balgheim

6 General Discussion

Herbicide resistance is the challenge for today's agriculture. For economic reasons the use of herbicides is indispensable in the developed world. As there is a heavy reliance on them it is necessary to preserve them, especially because there are no new modes of action in prospect. Therefore profound knowledge about the reasons and backgrounds of herbicide resistance is necessary. Thus, the main objectives of these experiments were to examine different herbicide resistant grass weed species, to quantify their resistance, to analyse the underlying resistance mechanisms and to develop tools for detecting them. Thereby a better understanding of the spatial and temporal distribution of herbicide resistance in fields will be generated.

In this chapter the main results of this work are discussed in comparison to the status quo of herbicide resistance to generate a far ranging knowledge, necessary for developing and implementing appropriate management strategies.

6.1 Herbicide resistance, their evolution and mechanisms

The dynamics of herbicide resistance evolution are governed by the biology of weedy plant species, by the genetic determination of the resistance trait and by herbicide characteristics and use patterns (Neve and Powles 2005a). Thus, this evolution is associated with herbicide intensive agricultural production systems, such as monoculture or short rotation cropping systems (e.g. wheat - rotational crop or fallow wheat) and the frequent use of herbicides of the same mode of action (Beckie and Gill 2006; Ross and Lembi 2009). The number of herbicide applications required to select herbicide resistant weed biotypes depends on herbicide chemical properties (e.g. target-site or soil persistence), the weed species, and the specific agronomic practices mentioned before (Thill and Lemerle 2001).

The selection pressure exposed on target weed species by a herbicide mode of action is the most important factor affecting the rate of evolution resistance (Beckie and Gill 2006). It increases by the long residual activity of many herbicides, i.e. first sulfonylurea and imidazolinone herbicides had an exceedingly high persistence, often into the following season, causing damage to the susceptible rotational crop, which also contributes to a rapid development of resistance (Saari et al. 1994; Gressel 2002). Previous research has also indicated a dose effect in herbicide resistance development, where high dose application

tends to promote target-site resistance development, and low dose application tends to promote non-target site resistance (Yuan et al. 2006; Gressel 2002).

Anyhow Beckie and Gill (2006) summarised that single mutations can confer resistance to single-site-of-action herbicides, multiple mutations within a plant are often needed to confer resistance to herbicides with more than one site of action. Fitting mutations are more probable for non-competitive inhibitors of target site enzymes, such as ACCase and ALS inhibitors, where the herbicide binding site is different from active site. These herbicides are rather presumed to have a high potential to endow resistance evolution, whereas herbicides like glyphosate and glufosinat has a lover potential to cause herbicide resistance (Heap 2009).

Informal surveys showed up Europe's worst grass weeds related with herbicide resistance: *A. myosuroides* and *A. spica-venti* (Naylor and Lutman 2002). Biotypes of both species being resistant to ACCase and ALS inhibiting herbicides were examined in this work. In both investigated *A. myosuroides* biotypes a resistance against ACCase inhibiting herbicides was proved, whereas the *A. spica-venti* biotype showed resistance against ALS inhibiting herbicides. The evolved resistance is supposed to be due to multiple applications of same or similar modes of action. Over a period of several years *A. myosuroides* biotypes were exposed to the repeated use of ACCase inhibiting herbicide. Both species developed a target-site base resistance. The two *A. myosuroides* biotypes showed a SNP on positions 1781 and 2096, respectively. The $Ile_{1781}Leu$ mutation of the biotype $BR_{(R)}$ confers cross-resistance against APPs and CHDs, whereas the $Ala_{2096}Gly$ substitution of biotype $BL_{(R)}$ confers resistance against APPs only. These resistance patterns are well described in literature and were proved in this study once more.

Also the *A. spica-venti* biotype was selected by long term and multiple use of ALS inhibiting herbicides; the ALS inhibitor resistance of the investigated is due to a $Pro_{197}Trp$ substitution.

Conspicuous is the different evolution of herbicide resistance in *A. myosuroides* and *A. spica-venti*, examined in this work on biotypes with target-site resistance: *A. myosuroides* against ACCase inhibitors and *A. spica-venti* to ALS inhibiting herbicides. Whereas *A. myosuroides* populations usually evolve resistance against ACCase inhibiting herbicides, few biotypes are known to evolve resistance against ALS inhibitors as well (Niemann et al. 2002; Marshall and Moss 2008; Heap 2009). German *A. spica-venti* biotypes are found to be mainly resistant against ALS inhibitors only (Heap 2009).

This contrary evolution is supposed to be due to the different preferences of both species and different herbicide strategies. Whereas *A. myosuroides* was found on heavy soils and narrow crop rotations with high proportions on cereals, *A. spica-venti* prefers light loamy soils and extended crop rotations (Niemann and Zwerger 2006). Both grass weeds are highly adapted to winter cereals. However, reduced tillage practices lead to the evolution of herbicide resistance in *A. myosuroides*. But because *A. spica-venti* shares many characteristics with *A. myosuroides*, it may respond similarly to the adoption of reduced tillage systems (Melander at al. 2008). In 1999 Pallutt proved the dependence of the occurrence of *A. spica-venti* on the proportion of winter cereals in crop rotations and the practiced tillage system. Higher proportion of winter cereals and non tillage systems increases the infestation level of *A. spica-venti*. In addition, yield losses caused by *A. spica-venti* exceeding those of *A. myosuroides* (Melander 1995). But because much more mode of actions are available for controlling *A. spica-venti* than for controlling *A. myosuroides*, resistance development is less rapid in *A. spica-venti*.

Surveys among Canadian farmers of Beckie et al. (2008) proved that the risk of herbicide resistance was greatest in fields with cereal-based rotations and least in fields with forage crops, fallow, or where three or more crop types were grown. The results of this study identify cropping system diversity as the foundation of a proactive weed resistance management (Beckie et al. 2008). Comparing the influences of agricultural production systems on the evolution of herbicide resistant biotypes, it is rather presumed that the investigated *A. myosuroides* biotypes were selected by continuous treatments with ACCase inhibiting herbicides and minimum tillage practice using glyphosate. Biotype $BR_{(R)}$ occurs within narrow crop rotation of two years winter wheat followed by one year sugar beet. In both crops ACCase inhibitors were used over a period of ten years. Since 2004 wheat crop is treated with ALS inhibitors. Similar evolution background show biotype $BL_{(R)}$ which occurs in a wheat monoculture treated for eight seasons with ACCase inhibiting herbicides, followed by applications with ALS inhibitors since 2001. The dependence of the occurrence of herbicide resistance weeds on agricultural production practices are therefore proved once more.

6.2 Screening for herbicide resistance

Diagnosing herbicide resistance in weeds is a very time and cost intensive procedure. For first characterising a herbicide resistant biotype detailed dose response studies are required

(Beckie et al. 2000). Seeds have to be collected from invested fields, germinated and transplanted to set up dose response experiments with different sites of action. So, different resistance patterns exhibit by different resistance mechanisms can be examined. If the resistance mechanism is quiet investigated, different methods can be developed using the basic results to detect resistance less time and cost intensive.

In this work carried out greenhouse dose response studies serve as basis for characterising the biotypes and for proving the resistance patterns described in literature (Délye et al. 2008; Tranel et al. 2009). All three investigated biotypes showed strong resistance, whereas no cross resistance to other sites of action was found. The *A. spica-venti* biotype was found to be resistance against ALS inhibiting herbicides, whereas biotype $BR_{(R)}$ showed resistance against APPs and CHDs and biotype $BL_{(R)}$ to APPs only. The derived resistance ratios of dose response studies showed high values for cycloxydim and fenoxaprop for the target-site resistant *A. myosuroides* biotype $BR_{(R)}$. This proved results obtained by Cocker et al. (1999) who found similar resistance ratios in biotypes with the same substitutions. Thus, the level of resistance due to an $Ile_{1781}Leu$ substitution seems to be comparable between biotypes of different origins. Also results which were obtained for the second investigated biotype $BL_{(R)}$ with the $Ala_{2096}Trp$ substitution are described in literature. Délye et al. (2004) showed resistance ratios for the enzyme activity of *A. myosuroides* biotypes similar to the one found in this work.

Therefore it is rather presumed that derived resistance ratios can be compared. The resistance characteristics are just depending on resistance mechanism and in case of target-site resistance on the substitution and their position on the coding gene.

Such bioassays are simple, but generally do not readily differentiate between different resistance mechanisms. Moreover they are time consuming and labour intensive and often do not allow an informed choice of an appropriate management program within the growing season (Kaundun and Windass 2006). Clear results will be maintained by DNA analyses only which might detect target-site resistance as the endowing resistance mechanism. As target-site resistance occurs as the changes of the herbicide binding site, caused by a single nucleotide polymorphism (SNP) (Cocker et al. 1999; Heap and LeBaron 2001), these might be detected by several methods.

Carried out DNA sequencing reactions detected SNPs resulting in amino acid substitutions conferring ACCase inhibitor resistance in *A. myosuroides* and ALS inhibitor resistance in *A. spica-venti*.

Results obtained during DNA sequencing generate much more information than required for resistance diagnosis; even so clear results might not be obtained in every reaction because of technical difficulties (Corbett and Tardif 2006). But they serve as basis for the further development of different marker systems which fulfil easy and cheap detection of resistance mechanisms.

So, given the prevalence of target-site based resistance, DNA based tests have the potential to provide an accurate and rapid diagnosis of resistance (Corbett and Tardif 2006).

In the last few years several molecular marker technologies found their way into weed science. Already in 1998 Neff et al. rather presumed the dCAPS technology as a useful technique for detecting known mutations in segregating plant populations. Kaundun and Windass (2006) as well as Délye and Boucansaud (2008) proved their benefit for weed science for detecting target-site based resistance in several grass weeds. Based on these studies, dCAPS marker were developed in this work to distinct clearly between target-site resistant biotypes of *A. myosuroides* and *A. spica-venti* by restriction endonuclease digestion of specific PCR products. The advantages of this technology are: the discrimination between heterozygous and homozygous resistant biotypes and rapid detection of the underlying resistance mechanism, within the growing season.

The development of such markers is not easy to handle, because of missing recognition sites and restriction enzymes, but the results assumed a high throughput procedure which preserves clear results about resistance mechanisms and their underlying SNPs.

Moreover Pyrosequencing^{TM}, a rapid real time DNA sequencing method was used in this study (Balgheim et al 2008; Wagner unpublished). There usage for SNP detecting was well proven by the authors. This technique differentiates also between homozygous and heterozygous resistant alleles, but it is not feasible in every lab because of the expensive basic equipment and high operating costs.

Summarized, it can be assumed, that cross-resistance patterns due to a specific mutation are similar between biotypes with different origins, and the response to herbicides can be transferred to other target-site resistant biotypes with the same substitutions.

6.3 Spatial and temporal distribution of herbicide resistant *A. myosuroides*

Results of the field mappings of herbicide resistant *A. myosuroides* proved its heterogeneous distribution which was described by Marshall (1988) before. According to Wilson and Brain (1991) and Krohmann et al. (2006) weed patches have a 10-year long persistence even as effective herbicides were applied in every year. This has been proved once more in the presented study. Over the three investigated years results show the occurrence of resistant *A. myosuroides* in high proportions within patches of high densities. Weed density, also within the patches depends on the crop and can be influenced by the crop rotation and the herbicides which are applied within the rotation. However, the amount of homozygous resistant plants increases within the patches, whereas the total amount decreases. The occurrence of weeds in patches rather presumed the appliance of site specific weed management for controlling resistant grass weeds. Beckie and Gill (2006) already suggested GPS as being a useful tool for monitoring and site specific weed control as management tool for controlling herbicide resistant weeds. This can reduce the selection pressure on the whole field. But otherwise the exerted selection pressure on infested weed patches is similar to that of blanket applications. Letting maintain a source of susceptible plants on unsprayed areas, as it might be the case at site specific herbicide application, to dilute the frequency of resistant plants is not likely to be effective for reducing the amount of resistant plants within the field (Jasieniuk et al. 1996). Indeed, the total amount of allied herbicides can be reduced by site specific weed management, but selection pressure will still exert on herbicide resistant weeds.

6.4 How to manage herbicide resistant weeds

As herbicides are the factor which exert the highest selection pressure on weeds, reliance on a single herbicide mode of action in combination with monoculture has been associated with most cases of resistance. These cultivation methods are still increasing. Therefore, ACCase and ALS inhibitor resistant *A. myosuroides* biotypes and ALS inhibitor resistant *A. spica-venti* biotypes are steadily infesting Europe's arable area. Both herbicidal groups are, because of the restricted use of isoproturon, the only remaining modes of action to control *A. myosuroides* and *A. spica-venti* post emerge. Therefore the remaining active ingredients have to be conserved and their efficacy has to be guaranteed. Low herbicide

use rates, bad weather- and soil conditions, wrong application dates and application technique are several factors that might influence herbicide efficacy. To conserve the remaining active ingredients, full use rates have to be applied, to keep herbicides effective for as long as possible (Balgheim 2006).

Related to the pesticide reduction programs (BMVEL 2005) Schröder et al. (2004) recommend a 25 % reduction of the use rates of ALS inhibiting herbicides for controlling *A. spica-venti*, but under optimised conditions only. On the other hand, Neve and Powles (2005a) proved the rapid evolution of herbicide resistance in grass weed species by the application of low herbicide use rates. They showed furthermore that low application rates forced the evolution of non-target site resistance. Compared with target-site resistance, non-target site resistance might pose a greater threat to agriculture because of the often unexpected multi- herbicide resistance (Yuan et al. 2006). So, non-target site resistance in grass weeds might confer resistance to substituted ureas, ACCase and ALS inhibitors (DePrado and Franco 2004). The existence of cross resistance patterns within resistant biotypes dramatically reduces the number of efficient herbicides and therefore the use of non-chemical cultural practices is required (Chauvel et al. 2001). Thus, if herbicide resistant grass weeds have rather infested a field, successful weed management strategies have to be implemented in the agricultural productions systems: changes in the tillage system to ploughing, the cultivation of spring sown and competitive crops and late sowing dates of winter cereals are the main crop management strategies (Balgheim 2006). Obviously this might be combined with reasonable herbicide management strategies. Thus, active ingredients shall alternate between and within crops.

6.5 Conclusions and future prospects

Years ago several authors postulate clear characterization of the biochemical basis of herbicide resistance in weeds (Cocker et al. 1999). Today the development of strategies for preventing and managing herbicide resistance should be an approach by integrating knowledge from population und evolutionary biology into weed science (Neve 2007).

This thesis provides basic knowledge about target-site resistance in different grass weed species und their distribution within arable fields. The development of marker technologies enables the detection of resistance mechanisms in resistant grass weed populations, which allows the implementation of convenient management strategies within the growing

season. Although it is postulated by Orson (1999) that prevention can cost significantly less than dealing with resistance once fully developed, implementing these resistance management strategies has proven to be the most difficult step. Most growers still consider herbicide resistance avoidance a low priority because it is a very slow shifting process. So they do not change their weed control programs to avoid the development of herbicide resistant grass weeds (Heap and LeBaron 2001).

Since there will be no new modes of action, the saving of active ingredients is the main requirement for the next years. The results of this thesis might contribute to an extended knowledge of herbicide resistance to be aware of the appearing challenges.

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Summary

Zusammenfassung

Natalie Balgheim

Summary

Weeds are one of the most troublesome threats for farmers, causing high yield losses and serving as hosts for pathogens and insect pests. Since the introduction of chemical weed control agricultural production systems have changed. During the last years the number of herbicide resistant grass weeds is steadily increasing especially in cereal monocultures. These monocultures are characterised by the repeated use of herbicides with the same modes of action and minimum-tillage practices. All these factors can one by one or all together lead to the development of herbicide resistant grass weeds. In general herbicide resistance is the result of heritable changes to biochemical processes that enable plant survival when treated with herbicides. Two different mechanisms are commonly known to confer resistance: target-site resistance and non-target-site resistance. First is the result of an altered target enzyme, where a single point mutation is changing the amino acid structure and exclude herbicide from effectively binding to the target enzyme. The second one, non-target-site resistance, can be summarised as the mechanisms which includes all other mechanisms than target-site resistance, for example rapid metabolic degradation or translocation of herbicides.

In Germany, the most trouble causing weeds associated with target-site resistance are the grass weeds *Alopecurus myosuroides* Huds. and *A. spica-venti* L. Beauv.. All investigations carried out during this thesis are dealing with those two weed species. Therefore the main objectives of this thesis are the following:

- To characterise the resistance levels and patterns of both species.
- To identify the underlying resistance mechanisms.
- To develop molecular markers for rapid detection of target-site based resistance.
- To get an idea of the spatial and temporal distribution of herbicide resistant grass weeds in arable fields.

Both investigated species are highly adapted to cereals and developed resistance against ACCase and ALS inhibiting herbicides. So they are an increasing problem for German farmers and in consideration of the fact, that both weeds have developed multiple

resistances, detecting and management strategies for controlling and preventing of these weeds are absolutely necessary.

Carried out dose response relationships proved strong resistance of the *A. myosuroides* biotype $BR_{(R)}$ against cycloxydim (RF = 130) and fenoxaprop (RF = 52), where low resistance was expressed against clethodim. However, biotype $BL_{(R)}$ showed resistance to fenoxaprop (RF = 6.35) and clodinafop (RF = 34) only.

Dose response experiments carried out with the *A. spica-venti* biotype showed resistance to sulfosulfuron (RF = 83.9) and iodo-/mesosulfuron (RF = 10.9). No cross resistances could be detected in both species.

The carried out DNA analysis revealed target-site resistance as the underlying resistance mechanism. $BR_{(R)}$ and $BL_{(R)}$ showed well known substitutions: an amino acid change on position 1781 with in the CT domain result in a change of Leu to Ile which confers resistance to APPs and CHDs in the biotype $BR_{(R)}$. The mutation of Gly to Ala on position 2096 within the CT domain causes resistance to APPs only. Also in the *A. spica-venti* biotype a amino acid change is the responsible resistance mechanism: a change of Pro to Thr at position 197.

These sequencing results serve as basis for the development molecular markers. Designed markers based on dCAPS technology. Such markers were developed to detect SNPs which can cause amino acid changes on the constitutive enzymes. Developed markers can rather differentiate between heterozygous and homozygous resistant alleles. Their technology is based on the fact that restriction endonucleases can cut DNA strands on specific recognition sites. This fact can be used for developing markers which are cutting the DNA in a previously generated PCR fragment on the mutation or wild-type sites, respectively. If there is no recognition site, it can be implemented by specific primers during the PCR. By these markers suspicious samples can be analysed and the results give an advice for management strategies, because target- and non-target-site resistance need different controlling strategies.

Investigations on the spatial and temporal distribution of weed populations where carried out on an arable field, invested with herbicide resistant *A. myosuroides*. Collected and analysed leave samples give information about the spatial dynamics of homozygous, heterozygous and sensitive plants in the field. Results show that the distribution of resistant plants depends on the weed density. Besides the weeds are distributed heterogeneous on the field and occur in patches that are persistent over several years. This example revealed

that herbicide resistance is rather associated with crop cultivation measurements. Changes in herbicidal and cultivation measurements shall be practiced to control and to prevent the occurrence of herbicide resistant grass weeds.

Zusammenfassung

Unkräuter stellen die wirtschaftlich bedeutendste Gruppe der Schadorganismen dar. Sie verursachen hohe Ertragsverluste und dienen zudem als Wirte für Pathogene und Insekten. Seit Einführung der ersten systemischen Herbizide vollzieht sich ein stetiger Wandel in der Agrarproduktion. Heutzutage ist der Anbau von Agrarprodukten ohne den Einsatz von chemischen Pflanzenschutzmitteln nicht vorstellbar. Aus wirtschaftlichen Gründen hat sich der Pflanzenbau stark gewandelt. Besonders Monokulturen, enge Fruchtfolgen, die wiederholte Anwendung von Herbiziden mit dem gleichen Wirkungsmechanismus und reduzierte Bodenbearbeitung kennzeichnen diese Entwicklung und stellen den Hauptgrund für immer höhere Unkrautdichten dar. Zudem wird immer häufiger über Minderwirkungen von Pflanzenschutzmitteln berichtet. In Europa haben sich vor allem Populationen von *Alopecurus myosuroides* und *Apera spica-venti* mit Herbizidresistenzen gegenüber ACCase- und ALS-Inhibitoren selektiert. Da über die beiden Ungrasarten auch in Deutschland zunehmend in Zusammenhang mit Herbizidresistenz berichtet wird, wurden speziell diese Ungräser auf ihre Resistenzentwicklung hin untersucht. Damit ergeben sich für diese Arbeit folgende Aufgabenstellungen:

- Charakterisierung der Resistenz ausgewählter *A. myosuroides* und *A. spica-venti* Biotypen und die Feststellung ihrer Resistenzausprägung gegenüber verschiedenen herbiziden Wirkstoffgruppen
- Bestimmung der zugrunde liegenden Resistenzmechanismen: Wirkortspezifische oder wirkortunspezifische Resistenz
- Entwicklung von molekularen Markern zum schnellen und einfachen Nachweis wirkortspezifischer Resistenz und den verursachenden Allelen
- Anlage und Prüfung eines neuen Versuchsdesigns für Langzeit-Feldversuche zur Untersuchung der zeitlichen und räumlichen Ausbreitung von herbizidresistenten Ungräsern innerhalb einer Praxisfläche.

Die Ergebnisse der Untersuchung der Resistenzausprägung bestätigten signifikante Unterschiede der resistenten Biotypen gegenüber den sensitiven Biotypen. Dosis-

Wirkungsexperimente zeigten eine eindeutige Resistenz des *A. myosuroides* Biotyps $BR_{(R)}$ gegenüber FOP und DIM Herbiziden. Die ermittelten Resistenzfaktoren lagen zwischen RF = 8.8 für Clethodim und RF = 130 für Cycloxydim. Der Biotype $BL_{(R)}$ hingegen weist nur eine Resistenz gegenüber den FOP Herbiziden auf. Die für den *A. spica-venti* ermittelten Resistenzfaktoren lagen bei RF = 83.9 für Sulfosulfuron und bei RF = 10.9 im Fall von Iodo-/Mesosulfuron. Eine Kreuzresistenz gegenüber Herbiziden mit anderen Wirkorten konnte ausgeschlossen werden.

Um die zugrunde liegenden Resistenzmechanismen zu ermitteln, wurden DNA Sequenzierungen durchgeführt. Dabei wurde für alle drei Biotypen eine wirkortspezifische Mutation nachgewiesen. Ein Aminosäureaustausch von Leucin zu Isoleucin an Position 1781 wurde im $BR_{(R)}$ Biotyp nachgewiesen. Dieser verursacht eine Resistenz gegenüber FOPs und DIMs. Die nur gegen die FOPs ausgeprägte Resistenz von $BL_{(R)}$ wird durch eine Veränderung von Glycin zu Alanin an Position 2096 verursacht. Beide Aminosäureveränderungen führen also zu unterschiedlichen Resistenzausprägungen.

Auch der Resistenz des *A. spica-venti* Biotyps liegt eine Wirkortveränderung zu Grunde. Ein Austausch der Aminosäuren von Prolin zu Threonin an Position 197 des für die ALS kodieren Gens ist die Ursache dieser Resistenz. Dies konnte durch einen ALS-Enzym-Assay der sensitiven und resistenten Biotypen belegt werden, bei dem die Enzymaktivität durch den ALS-Wirkstoff im resistenten Biotype deutlich weniger beeinflusst wird.

Auf den Sequenzierergebnissen beruhend wurden molekulare Marker anhand der dCAPS Methode entwickelt. Im Gegensatz zur DNA Sequenzierung, ist diese Methode sehr schnell und kostengünstig, zudem lassen sich auch heterozygote Resistenzen eindeutig nachweisen.

Um die Populationsdynamik von Biotypen mit einer wirkortspezifischen Resistenz zu untersuchen, wurde eine Praxisfläche mit einem natürlichen Vorkommen einer resistenten Ackerfuchsschwanzpopulation beobachtet. Die Ergebnisse zeigten eine Persistenz der Ungrasnester über den untersuchten Zeitraum. Die Unkrautdichte, hing von den angebauten Kulturpflanzen und dem Ungrasmanagement ab. Die Analyse und Darstellung der Genotypen gesammelter Einzelpflanzen zeigte eine positive Korrelation zwischen der Unkrautdichte und dem Anteil an resistenten Ackerfuchsschwanzpflanzen.

Die erzielten Ergebnisse sollen einem besseren Verständnis der Einflußfaktoren auf die Entstehung von Herbizidresistenzen bei Ungräser dienen und somit zukünftige Managementmaßnahmen unterstützen.

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Acknowledgements

Herein I want to express my thanks to the following people:

Entire my supervisor Prof. Dr. Roland Gerhards for extraordinary guiding and making me my own way on herbicide resistance.

Thanks to Prof. Dr. Wilhelm Claupein for co-reviewing this thesis.

Special thanks are attended to Dr. Jean Wagner for extensive discussions.

The ones who helped me work during lab, greenhouse, and field trials: Alexandra Heyn and Helena Mastel who made my lab and greenhouse days and Cathrin Reichert, Ernst Tränkle and Stefan Knapp who assisted me at the field trials.
My colleagues at the Institute of Phytomedicine for their great fellowship and for sometimes becoming friends where nobody was thinking of.

And almost, last but not least, my family and friends who were supporting me during this special time.

Finally I want to thank Nufarm Deutschland GmbH for founding this research.