University of Hohenheim
Institute of Phytomedicine (360)
Department of Weed Science
Prof. Dr. Roland Gerhards

Integrated management, analysis of mechanisms and early detection of resistant populations of *Alopecurus myosuroides* HUDS. and *Apera spica-venti* L. Beauv.

Dissertation
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by
Yasmin Isabelle Kaiser
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Examination Committee

Vice Dean: Prof. Dr. Markus Rodehutscord

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Supervisor and Reviewer: Prof. Dr. Roland Gerhards

Co-Reviewer: Prof. Dr. Wilhelm Claupein

Additional Examiner: PD. Dr. Regina Belz

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List of Abbreviations

a.i.	Active ingredient
ACCase	Acetyl CoA carboxylase
ACE	*A. myosuroides* control efficacy
a.i.	Active ingredient
ACCase	Acetyl CoA carboxylase
ALOMY	*Alopecurus myosuroides*
ALS	Acetolactate synthase
ANOVA	Analysis of variances
AOPP	Aryloxyphenoxypropionate
APESV	*Apera spica-venti*
AUC	Area under curve
CCD	Charge-coupled device
CI	Confidence interval
cm	Centimetre
CR	Crop rotation
DAT	Days after treatment
DFG	Deutsche Forschungsgemeinschaft
DNA	Deoxyribonucleic acid
ED50	Effective dosage causing 50% reduction of the plant response
ED90	Effective dosage causing 90% reduction of the plant response
EW	Emulsion, oil in water
F0	Dark fluorescence yield
Fm	Maximal fluorescence yield
FPE	Fenoxaprop-P-ethyl
Fv/Fm	Maximum quantum efficiency of PSII
g	Gram
GIS	Geographic information system
GPS	Global positioning system
h	Hour
ha	Hectare

HAT	Hours after treatment
HPLC	High Performance Liquid Chromatography
HRAC	Herbicide Resistance Action Committee
HS	Herbicide strategy
IWM	Integrated Weed Management
Kg	Kilogram
kPa	Kilo pascal
L	Litre
LC/MS-MS	Liquid chromatography tandem mass spectrometry
LED	Light emitting diodes
M	Maize
M+I	mesosulfuron+iodosulfuron
m	meter
m^{-2}	Square meter
mm	Millimetre
mM	Millimolar
MOA	Mode of action
N	Nitrogen
nm	Nanometer
NS	non-significant
NTSR	Non-target-site resistance
OD	Oil dispersion
OEPP/EPPO	European and Mediterranean Plant Protection Organization
OR	Odds ratio
PCR	Polymerase chain reaction
PPFD	Photosynthetic photon flux density
PSI	Photosystem I
PSII	Photosystem II
PUFAs	Polyunsaturated fatty acids
RF	Resistance factor
ROC	Receiver Operating Characteristic
ROS	Reactive oxygen species
s	Second

SB	Spring barley
SNP	Single nucleotide polymorphism
t	Ton
TSR	Target-site resistance
WG	Water-Dispersible Granules
WW	Winter wheat
µl	Microliter

Chapter I

General Introduction

1 General Introduction

Due to the rising human population, agricultural production must steadily be increased to meet the growing demand on food. By the introduction of high-yielding cultivars, irrigation systems, synthetic fertilizers and pesticides, agronomic systems have been improved resulting in an increase of world food production. Nevertheless, crop yield is still threatened by pests and diseases (Oerke & Dehne 2004). On a global scale, the major biotic cause of yield loss in crops, are due to weeds. Without weed control, the competition for light, space, water and nutrients can result in a 34 % loss of crop yield on average (Oerke 2006). Weed competition can be eliminated chemically, mechanically or by agronomic practices such as crop rotation and soil tillage (Cardina *et al.* 1998, Oerke 2006). A significant contribution to the high productivity of global agriculture can be attributed to herbicides (Powles & Yu 2010). With a control efficacy of up to 99 % on target weeds, herbicides are by far the most effective weed control measure ever developed (Foster *et al.* 1993). Herbicides, also known as reliable and cost-effective tools for weed control, have largely replaced mechanical, human and animal weed control strategies since their introduction (Powles & Yu 2010, Powles & Shaner 2001). The reliance on chemical weed control and the associated selection pressure exerted by herbicides has resulted in the selection of herbicide-resistant weed populations (Powles & Yu 2010). The first herbicide-resistant weed species was found in 1957, less than twenty years after the introduction of synthetic herbicides (Hilton 1957, Oerke 2006). Since then, the number of resistant weed populations is continuously rising (Délye *et al.* 2013). To date 245 weed species (142 dicotyledonous, 103 monocotyledonous) have evolved resistance globally (Heap 2015). *Alopecurus myosuroides* HUDS. (blackgrass) and *Apera spica-venti* L. Beauv. (silky windgrass) are the most important weed species in Europe. In cereal crops, at high infestation rates *A. myosuroides* and *A. spica-venti* may cause yield losses of about 45 % and 25%, respectively (Moss 1987, Gehring *et al.* 2012). There are different mechanisms in the plants which can be responsible for herbicide resistance. An alteration or overexpression of the target site of the herbicide can reduce or prevent herbicide binding, a mechanism called target-site resistance (TSR). Non-target-site resistance (NTSR) comprises mechanisms such as enhanced metabolism of the herbicide or decreased translocation and absorption of the herbicide. Furthermore, a sequestration of the herbicide into the vacuole or the cell wall of a plant can result in resistance, when the concentration reaching the target enzyme is reduced (Heap 2014). In contrast to TSR mechanisms, NTSR mechanisms are far less investigated. The increasing number of putative herbicide-resistant weed populations requires new resistance tests for an accelerated detection as well as alternative non-chemical weed control strategies. Integrated Weed Management (IWM) is an approach with the target to limit weed infestations. This approach is less dependent on chemical measures, however it does not limit productivity and economic performance. Recommendations of IWM include a diverse crop rotation, the use of competitive cultivars, mechanical weed control measures and adjusted soil tillage, among others (Chikowo *et al.* 2009).

1.1 Objectives

The overall aim of this research is first to develop a new methodology for an accelerated detection of herbicide resistance. Second, to investigate the degradation and metabolism of herbicides in *A. myosuroides*, in order to gain knowledge about resistance mechanisms. Third, to test the influence of different agronomic factors such as crop rotation, soil tillage and herbicide strategies on population density and resistance development with regard to the weed species *A. myosuroides* and *A. spica-venti*.

1.2 Structure of the dissertation

The work is presented as a cumulative thesis and consists of four scientific papers. Three papers are published in peer-reviewed journals. The fourth paper is submitted and currently under review.

The first paper titled 'Chlorophyll fluorescence imaging: a new method for rapid detection of herbicide resistance in *Alopecurus myosuroides*' is published in the Weed Research Journal and introduces a new screening method for an accelerated detection of herbicide resistance in *A. myosuroides*. Results are available in a shorter time and using less space compared with standard resistance tests.

The second paper titled 'Degradation and metabolism of fenoxaprop and mesosulfuron + iodosulfuron in multiple resistant blackgrass (*Alopecurus myosuroides*)' is published in the Gesunde Pflanzen Journal and assesses resistance mechanisms. Specifically, the paper focuses on differences in the degradation and metabolism of herbicides between sensitive and resistant *A. myosuroides* populations.

The third paper titled 'Development of a Geo-Referenced Database for Weed Mapping and Analysis of Agronomic Factors Affecting Herbicide Resistance in *Apera spica-venti* L. Beauv. (Silky Windgrass)' is published in the Agronomy Journal and examines the influence of agronomic and biological factors on the probability of resistance occurrence in *A. spica-venti*. Furthermore, a geo-referenced database was developed for mapping the spread of herbicide-resistant *A. spica-venti* populations across Europe.

The fourth paper titled 'Crop rotation and herbicide strategies affecting herbicide resistance and population development of *Alopecurus myosuroides*' is submitted to the Weed Research Journal. The paper investigates how population densities of *A. myosuroides* and herbicide resistance are influenced by crop rotation and different herbicide strategies.

In the thesis, the papers are presented with unified formatting and citation style.

Beyond the publications presented in this thesis, two contributions to conference proceedings of international scientific symposiums were accepted:

- KAISER YI & GERHARDS R (2014) Degradation and metabolism of fenoxaprop-P-ethyl in sensitive and resistant populations of *Alopecurus myosuroides.* Julius-Kühn-Archiv, Braunschweig, Proceedings 26th German Conference on Weed Biology and Weed Control **443**, 52-59.
- KAISER YI & GERHARDS R (2015) Growth and impact of herbicide sensitive and multiple resistant *Alopecurus myosuroides* in winter wheat. Proceedings of the 17th European Weed Research Society Symposium. 'Weed management in changing environments', Montpellier, France.

Chapter II

Publications

2 Publications

2.1 Chlorophyll fluorescence imaging: a new method for rapid detection of herbicide resistance in *Alopecurus myosuroides*.

Yasmin I. Kaiser [1], Alexander Menegat [1] & Roland Gerhards [1]

[1] Department of Weed Science, Institute of Phytomedicine, University of Hohenheim, 70599 Stuttgart, Germany

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The original publication is available at:

http://onlinelibrary.wiley.com/doi/10.1111/wre.12043/abstract

Abstract

Due to the steadily increasing number of presumed herbicide resistant weed populations, the demand for rapid in-season tests is rising. In this study we present a new quantitative herbicide resistance test system based on chlorophyll fluorescence imaging analysis of photosynthesis related parameters. Herbicide resistant and susceptible populations of *Alopecurus myosuroides* (blackgrass) were cultivated in multiwell tissue culture plates containing nutrient agar and different dosages of fenoxaprop-P-ethyl and mesosulfuron+iodosulfuron. The maximum quantum efficiency of the PSI was measured 3 hours after transplanting (HAT) and then for seven days every 24 hours. Data of maximum quantum efficiency of the PSII were compared with conventional whole-plant pot tests and molecular tests for target-site mutations. It was possible to fit dose-response curves and calculate corresponding resistance factors for ED90 for all populations tested using the chlorophyll fluorescence imaging. It was possible to distinguish between resistant and susceptible populations. The results of the chlorophyll fluorescence imaging corresponded well to the conventional whole-plant pot tests in the greenhouse. However, populations with verified target-site mutations did not differ from other herbicide resistant populations in the maximum quantum efficiency values of the PSII. We conclude that the chlorophyll fluorescence imaging provides reliable data on herbicide resistance for both modes of action tested in shorter time and using less space compared with conventional whole-plant pot tests in the greenhouse.

Keywords: chlorophyll fluorescence imaging, herbicide resistance quick test, ALS, ACCase, fenoxaprop-P-ethyl, mesosulfuron+iodosulfuron

2.2 Degradation and metabolism of fenoxaprop and mesosulfuron + iodosulfuron in multiple resistant blackgrass (*Alopecurus myosuroides*)

Yasmin I. Kaiser [1] and Roland Gerhards [1]

[1] Department of Weed Science, Institute of Phytomedicine, University of Hohenheim, 70599 Stuttgart, Germany

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http://link.springer.com/article/10.1007/s10343-015-0343-3

Abstract

Standard greenhouse experiments were conducted to investigate herbicide resistance in selected populations of blackgrass (*Alopecurus myosuroides* HUDS.). Three populations with either target-site resistance (TSR) or non-target-site resistance (NTSR) demonstrated decreased sensitivity to the active ingredients fenoxaprop and mesosulfuron + iodosulfuron. Degradation and metabolism of these herbicides were examined using a liquid chromatography followed by tandem mass spectrometry. Fenoxaprop degraded in resistant and sensitive populations within 144 hours after treatment without significant differences among populations. Fenoxaprop-P, the acid metabolite of fenoxaprop, was detected in all populations. The dynamics of fenoxaprop-P differed significantly with an increased degradation of the substance in NTSR populations. The metabolite 6-chlorobenzoxazol-2(3H)-one could be found in all populations 2 hours after treatment and degraded almost completely within 144 hours. The degradation of mesosulfuron + iodosulfuron lasted over 21 days. It was significantly faster in the NTSR than in the sensitive and the TSR populations. The metabolite metsulfuron was found 7 days after treatment in resistant and sensitive populations, without significant differences in the dynamics. The results clearly demonstrate that herbicide metabolism plays an important role in the evolution of herbicide resistant blackgrass populations.

Keywords: Herbicide resistance, non-target-site resistance, LC/MS-MS, ACCase, ALS, weed

2.3 Development of a Geo-Referenced Database for Weed Mapping and Analysis of Agronomic Factors Affecting Herbicide Resistance in Apera spica-venti L. Beauv. (Silky Windgrass)

Dario Massa [1], Yasmin I. Kaiser [1], Dionisio Andújar-Sánchez [1], Rocío Carmona-Alférez [2], Jörg Mehrtens [3] and Roland Gerhards [1]

[1] Department of Weed Science, Institute of Phytomedicine, University of Hohenheim, 70599 Stuttgart, Germany

[2] Agency for Health Technology Assessment, Institute of Health Carlos III, 28029 Madrid, Spain

[3] Proplanta GmbH & Co. KG, 70599 Stuttgart, Germany

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Abstract

In this study, we evaluate the role of agronomic factors in the selection for herbicide resistance in *Apera spica-venti* L. Beauv. (silky windgrass). During three years, populations were collected in more than 250 conventional fields across Europe and tested for resistance in greenhouse biotests. After recording the field history of locations, a geo-referenced database has been developed to capture the dispersal of herbicide-resistant *A. spica-venti* populations in Europe. A Logistic Regression Model was used to assess whether and to what extent biological and agricultural factors (crop rotation, soil tillage, sowing date, soil texture and weed density) affect the probability of resistance selection apart from the selection pressure due to the application of herbicides. Our results revealed that rotation management and soil tillage are the factors that have the highest influence on the model. Additionally, first order interactions between these two variables were highly significant. Under conventional tillage, a percentage of winter crops in the rotation exceeding 75% resulted in a 1280-times higher risk of resistance selection compared to rotations with less than 50% of winter crops. Under conservation tillage, the adoption of >75% of winter crops increased the risk of resistance 13-times compared to rotations with less than 50% of winter crops. Finally, early sowing and high weed density significantly enhanced the risk of resistance compared to the reference categories (later sowing and low weed density, respectively). Soil texture had no significant impact. The developed model can find application in management programs aimed at preventing the evolution and distribution of herbicide resistance in weed populations.

Keywords: farm management; geographic information system; logistic regression model (LRM)

Nomenclature: *Apera spica-venti* L. Beauv.; Silky windgrass; APESV

2.4 Crop rotation and herbicide strategies affecting herbicide resistance and population development of *Alopecurus myosuroides*

Yasmin I. Kaiser [1] and Roland Gerhards [1]

[1] Department of Weed Science, Institute of Phytomedicine, University of Hohenheim, 70599 Stuttgart, Germany

Submitted to: Weed Research

2.4.1 Abstract

Alopecurus myosuroides (blackgrass) is one of the most abundant weeds in Europe and a competitive grass in winter wheat. Densities increased due to changes in agricultural practice, especially a higher proportion of winter cereals in the crop rotation, earlier sowing dates of winter wheat and a change to non-inversion tillage systems. To investigate the impact of crop rotation and different herbicide strategies on *A. myosuroides*, on-farm studies have been carried out at two locations in Southern Germany. Crop rotations with different proportions of autumn-sown and spring crops were established. Different herbicide strategies were included in each crop to determine the effect of herbicides on resistance development. A rapid increase of *A. myosuroides* densities was observed in continuous winter wheat when no weed control was carried out. Within a period of three years, the population density of *A. myosuroides* changed from 14 ears m^{-2} to 882.5 ears m^{-2}. Winter wheat yield was reduced by 50 % at densities of approximately 1200 ears m^{-2}. Spring crops reduced densities of *A. myosuroides* by 92 % compared to continuous winter-wheat in the plots without herbicide application. A rapid selection of target-site resistant populations against ALS- and ACCase inhibitors was observed over the three years of study. Selection pressure was lower with a consequent change of herbicide mode of action. We conclude that solely the integration of spring crops in the rotation reduced *A. myosuroides* infestations. In combination with rotating herbicide mode of action, *A. myosuroides* can be sufficiently controlled and selection pressure towards resistant *A. myosuroides* is reduced.

Keywords: spring crops, herbicide resistance management, selection pressure, crop sequence, blackgrass

2.4.2 Introduction

Crop rotation has traditionally been considered as an effective strategy to control weeds and pests (Leighty 1938, Froud-Williams 1988, Oerke 2006). With the opportunity to use synthetic fertilizers and pesticides diverse crop rotations have been simplified and narrowed (Froud-Williams *et al.* 1981, Walker & Buchanan 1982, Power & Follett 1987). In Germany and some other western European countries, the cropping pattern has changed from diverse crop rotations to cropping practices with a high percentage of autumn-sown crops. *Alopecurus myosuroides* HUDS. (blackgrass) is a winter annual weed that typically occurs in autumn-sown crops such as winter oilseed rape (*Brassica napus* L.) and winter wheat (Melander 1995). Although it is known that densities of *A. myosuroides* tend to increase with a higher proportion of winter cereals, many farmers prefer the cultivation of more profitable winter cereals instead of spring crops (Zwerger *et al.* 1990, Hurle 1993, Melander 1995). A high proportion of winter wheat in crop rotations in combination with early sowing dates and reduced tillage practices have resulted in the high infestation rates of *A. myosuroides* (Melander 1995). *A. myosuroides* can produce approximately 200 seeds surviving 6-10 years in the soil (Moss 1985). At high infestation rates, *A. myosuroides* may cause yield losses of about 45 % in cereal crops (Moss 1987). Due to continuous applications of herbicides with the same modes of action (MOAs), there is a selection for herbicide resistant weed populations (Powles & Yu 2010). *A. myosuroides* is considered as the most important herbicide-resistant weed in Europe (Moss *et al.* 2007). Several herbicide-resistant *A. myosuroides* populations have been found in the past years, sometimes showing cross or multiple resistance to herbicides with different target sites (Holt *et al.* 1993, De Prado & Franco 2004). Resistance mechanisms such as target-site resistance (TSR) and non-target-site resistance (NTSR) with increased herbicide metabolism, accumulate in a single plant as a result of cross-pollination (Werck-Reichhart *et al.* 2000). Drobny *et al.* (2006) found populations of *A. myosuroides* (Lower Saxony and Schleswig-Holstein) with metabolic herbicide resistance sometimes combined with TSR. On farm studies at two locations in Germany were carried out over three years to investigate the effect of crop rotations in combination with herbicide application on *A. myosuroides* density, crop yield and resistance development in the population. It was hypothesized that (1) densities increase with a high percentage of autumn-sown crops and decrease in crop rotations when spring crops are included. We further hypothesize that (2) population densities rapidly increase, when weed control measures are omitted. Finally, it was hypothesized that (3) herbicide resistance is promoted in crop rotations with a high percentage of autumn-sown crops and continuous use of herbicides with the same MOA.

2.4.3 Materials and Methods

Greenhouse bioassay

At both locations, populations of *A. myosuroides* were investigated in a greenhouse bioassay. Ripe seeds of untreated *A. myosuroides* plants were sampled after the first year. Seeds were sown in vermiculite and cultivated at a temperature regime of 15 °C day and 5°C night with a 12-h photoperiod. At one-leaf stage seedlings were transplanted into 8 x 8 cm Jiffy pots. Plants were sprayed at two-three leaf stage with a precision application chamber using a flat-fan nozzle (8002 EVS, Teejet® Spraying Systems Co., Wheaton, IL, USA). The application chamber was adjusted for a volume of 200 L ha $^{-1}$, at a speed of 800 mm s^{-1}, a spraying pressure of 300 kPa and a distance of the spray nozzle of 500 mm above sprayed surface. Ten herbicides belonging to ACCase inhibitors, ALS inhibitors and a PSII-Inhibitor were used. The active ingredients isoproturon, fenoxaprop, clodinafop, fluazifop, pinoxaden, clethodime, mesosulfuron + iodosulfuron, florasulam + pyroxsulam, flupyrsulfuron and propoxycarbazone were sprayed at the recommended dosages, which were 500 g L^{-1}, 82.8 g L^{-1}, 53.5 g L^{-1}, 125 g L^{-1}, 60 g L^{-1}, 121 g L^{-1}, 12 + 2.4 g kg^{-1}, 15 + 5 g kg^{-1}, 10 g kg^{-1}, 70 g kg^{-1}, respectively. Each treatment was repeated three times. Pots were placed in the greenhouse in a completely randomised design.

Field experiments

Field experiments were established at the experimental station Ihinger Hof, near Renningen (48.74° N, 8.92° E, 478 m altitude) in autumn 2011 and at Wurmberg (48.86° N, 8.83 °E, 450 m altitude) in autumn 2012. Both sites are located in the southwest of Germany. At Ihinger Hof, the average annual temperature was 9.1° C and the annual precipitation 825 mm. At Wurmberg, the average annual temperature was 10.6 °C and precipitation 736 mm. Soil type was clayey loam at both locations. The experiments were designed as a split-plot with four replicates. The main plot factor was crop rotation (CR) and the sub-plot factor was herbicide strategy (HS; Table 1). The size of the sub-plots was 6 x 12 m. The different CR treatments differed in the proportion of winter annual crops. CR 1 was a winter wheat (*Triticum aestivum* L.) monoculture, in CR 2, spring barley (*Hordeum vulgare* L.) was grown in the third year instead of winter wheat, in CR 3 winter wheat was followed by maize (*Zea mays* L.) in the second year and spring barley in the third year. For the sub-plot factor HS, four different treatments were investigated (Table 1). In the first year, ALS inhibitors were applied in HS 2, HS 3 and HS 4 to produce an uniform selection pressure on *A. myosuroides* population. All herbicides were applied at the recommended rate (Table 2).

Table 1 Crop rotations (CR) tested to suppress *A. myosuroides* densities and herbicide strategies (HS) in each crop.

	Year		
Crop rotation (CR)	1	2	3
1	winter wheat	winter wheat	winter wheat
2	winter wheat	winter wheat	spring barley
3	winter wheat	maize	spring barley
Herbicide strategy (HS)			
1	untreated control*		
2	consequent change of herbicide MOA		
3	herbicide application according to recommendation of local plant protection services		
4	continuous use of herbicides with only one mode of action (HRAC A or B depending on crop)		

*only to control broad-leaved weeds

At Ihinger Hof, cv. Schamane (Saatzucht Streng, Uffenheim) was used for winter wheat, cv. Torres (KWS, Saat AG, Einbeck) for maize and cv. Grace (Baywa, München) for spring barley. In total, 170 kg N ha^{-1} of nitrogen fertilizer were applied in winter wheat in the first and second year and 185 kg N ha^{-1} in the third year, in maize and spring barley, 140 kg N ha^{-1} of nitrogen granulated were spread. At Wurmberg, cv. Pamier (Syngenta Cereals GmbH, Hanstedt) was used for winter wheat and Torres (KWS Saat AG, Einbeck) for maize. In total, 199 kg N ha^{-1} were applied in winter wheat and 147 kg N ha^{-1} in maize. At both locations, cereals were sown with a single disc drill (row distance 0.12 cm) and maize with a precision air seeder (row distance 0.75 m). Before the spring crops were sown, lacy phacelia *(Phacelia tanacetifolia)* was cultivated as a cover crop (cv. Julia, Feldsaaten Freudenberger, Magdeburg). After cover cropping in spring, glyphosate (1440 g a.i. ha^{-1}, Clinic®, 360 g a.i. L^{-1}, Nufarm) was sprayed prior to sowing. Throughout the three-year study, only reduced tillage operations not deeper than 0.12 m were carried out.

Herbicides were applied with a self-propelled plot sprayer (Schachtner-Fahrzeug- und Gerätetechnik, Ludwigsburg), which was calibrated for a volume of 200 L ha^{-1} water. Crop yield was recorded in 4 x 6 m sub-plots using a using a plot combine harvester.

Assessments of *A. myosuroides*

The response of *A. myosuroides* was assessed by counting ears of *A. myosuroides* in each crop and year, approximately five weeks after treatment at BBCH 61-77. Five randomly placed counts (0.4 m^{-2}) per plot were made. *A. myosuroides* control efficacy (ACE) was calculated using equation 1:

$$ACE\ (\%) = \left[\frac{(A-B)}{A}\right] * 100 \qquad (1)$$

ACE represents the control efficacy of *A. myosuroides*; A represents the number of *A. myosuroides* ears m^{-2} in the untreated control plots (HS 1), and B represents the number of *A. myosuroides* ears m^{-2} the different HS plots.

Additionally, leaf samples of surviving *A. myosuroides* plants were taken for molecular genetic analysis three to four weeks after herbicide treatment. Eight leaf samples were sampled randomly in HS treatments 2, 3 and 4 of each crop and year to record the TSR pattern in the treatments. For each leaf sample, 7 gene positions were investigated, which are most commonly affected in target-site resistant *A. myosuroides*. The ACCase loci tested were 1781, 2027, 2041, 2078, 2096 and the ALS loci were 197 and 574. In year 2, after the isoproturon treatment in winter wheat, leaf samples were also tested for mutations in the PSII gene (gene loci: 219, 220, 251, 255, 256, 264, 265, 266, 275). However, no mutation was found in the PSII gene. PCR assays and pyrosequencing procedures were conducted by IDENTXX GmbH (Stuttgart, Germany).

Table 2 1 Herbicides applied in different crops and years to control *A. myosuroides*.

		control of *A. myosuroides*					
year	crop	application time	HS	herbicide	active ingredient	HRAC - Code	dose (g a.i. ha^{-1})
1	WW	–	1	–	–		–
		spring	2,3,4	Broadway® + Adj.[1]	pyroxsulam + florasulam*	B	15 + 5*
2	WW	–	1	–	–	–	–
		spring	2	Arelon TOP®[3]	isoproturon	C1	1500
		spring	3	Atlantis® WG + Adj.[2]	mesosulfuron + iodosulfuron	B	9 + 1.8
		spring	4	Broadway® + Adj.[1]	pyroxsulam + florasulam*	B	15 + 5*
	M	–	1	–	–	–	–
		spring	2	Laudis®[3]	tembotrione	F2	88
		spring	3	Kelvin®[4]	nicosulfuron	B	32
		spring	4	Elumis®[5]	mesotrione* + nicosulfuron	F2* + B	93.8* + 37.5
3	WW	–	1	–	–	–	–
		autumn; spring	2	Herold® SC[2]; Traxos®[5]	flufenacet + diflufenican; clodinafop + pinoxaden	K3 + F1; A	240 + 120; 30 + 30
		autumn	3	Herold® SC[2]	flufenacet + diflufenican	K3 + F1	240 + 120
		spring	4	Broadway® + Adj.[1]	pyroxsulam + florasulam*	B	15 + 5*
	SB	–	1	–	–	–	–
		spring	2	Axial 50®[5]	pinoxaden	A	60
		spring	3	Axial 50®[5]	pinoxaden	A	60
		spring	4	Axial 50®[5]	pinoxaden	A	60

* No control efficacy towards *A. myosuroides*, Adj.; adjuvant, [1] DOW Agro Sciences, [2] Bayer CropScience , [3] Cheminova,[4] BASF Plant Protection, [5] Syngenta Agro Gmbh

Statistical analyses

Analyses were performed with the statistical software R® 3.0.2 (R Development Core Team 2011).

In the greenhouse bioassay, visual scores (% herbicide damage) were evaluated 28 days after herbicide treatment compared the untreated control and a sensitive reference population (Herbiseed, Twyford, UK). Degrees of resistance were classified according to the R rating system of Moss *et al.* (1999).

For the analysis of the field experiments, year 1 has been taken out from data set, because of the uniform herbicide treatment as it was described before. Due to the different experimental starting times, data of the locations were analysed separately. A linear mixed effect model was used to evaluate the responses of *A. myosuroides*. Crop yield (t ha^{-1}) was evaluated in each year separately, performing an ANOVA. Common yield loss models (Cousens 1985, Kropff & Spitters 1991) were tested to evaluate the influence of *A. myosuroides* on winter wheat yield, but data fitted best to the linear regression model (Equation 2).

$$y = mx + c, \qquad (2)$$

where y is the winter wheat yield, m is the slope of the regression line, x is the number of *A. myosuroides* ears m^{-2} and c is maximum yield in the absence of *A. myosuroides.*

Prior to every final analysis, requirements were proven. Thereupon, the dataset of *A. myosuroides* from Ihinger Hof was log transformed to homogenize variances and to normalize the distribution. In the results section, back transformed means are shown.

TSR frequency in the different HS treatments was expressed in %. Therefore, the molecular genetic results of all tested gene loci were summarized and frequency of TSR affected plants was calculated.

2.4.4 Results

Herbicide resistance status of investigated *A. myosuroides*

Bioassays investigating the *A. myosuroides* population of Ihinger Hof (Figure 1) revealed that except for fenoxaprop, there was a sufficient control by all herbicides (between 92 and 100 %). A lower efficacy of 77% (R?) was observed for fenoxaprop. The control efficacy of the Wurmberg population ranged from 94 to 100 % after treatment with fluazifop, pinoxaden, clethodime and mesosulfuron + iodosulfuron (Figure 2). Population was also sensitive to isoproturon, clodinafop, florasulam + pyroxsulam, flupyrsulfuron and propoxycarbazone, however efficacy was lower with 85-87 %. Resistance was prooved for fenoxaprop with an efficacy of 70 % (RR) compared to the untreated control.

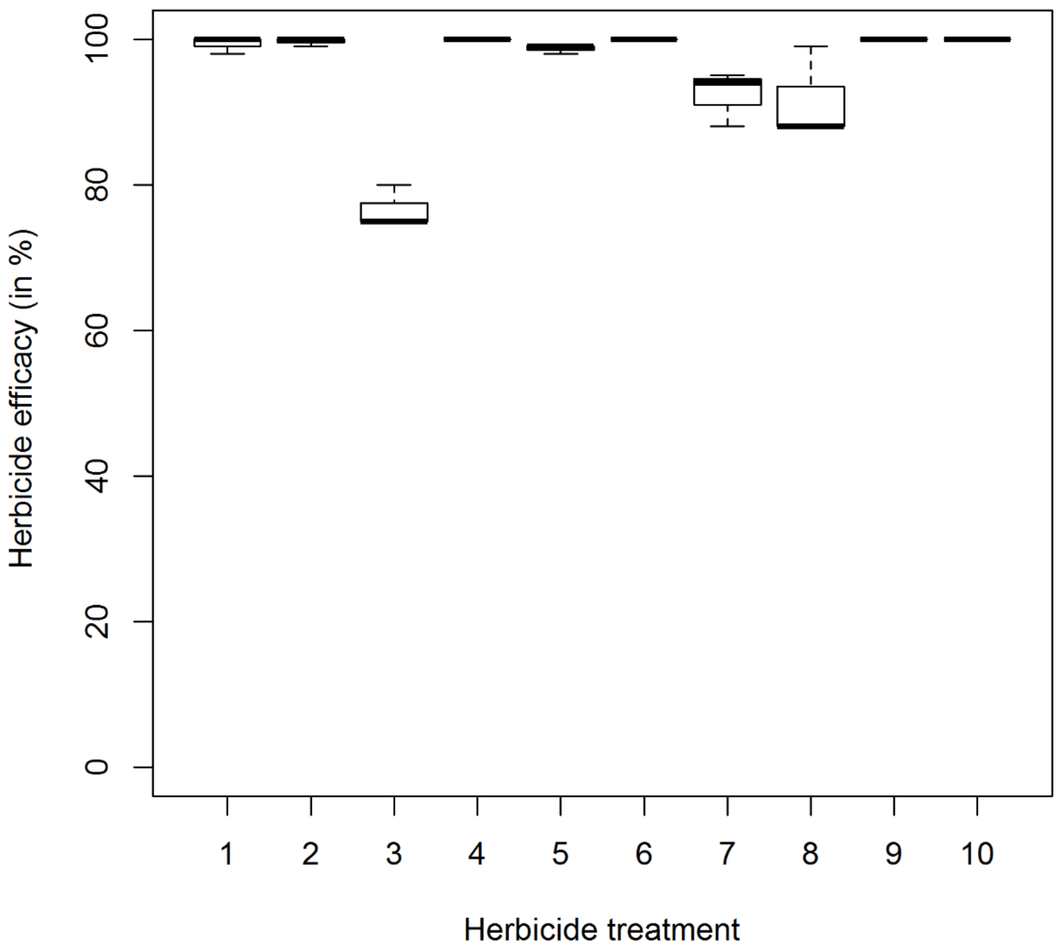

Figure 1 Results of the greenhouse biotest investigating the *A. myosuroides* population of Ihinger Hof at beginning of the field studies. Herbicide treatments: 1, mesosulfuron + iodosulfuron; 2, isoproturon; 3, fenoxaprop; 4, fluazifop; 5, pinoxaden; 6, clethodime; 7, flupyrsulfuron; 8, clodinafop; 9, florasulam + pyroxsulam; 10, propoxycarbazone.

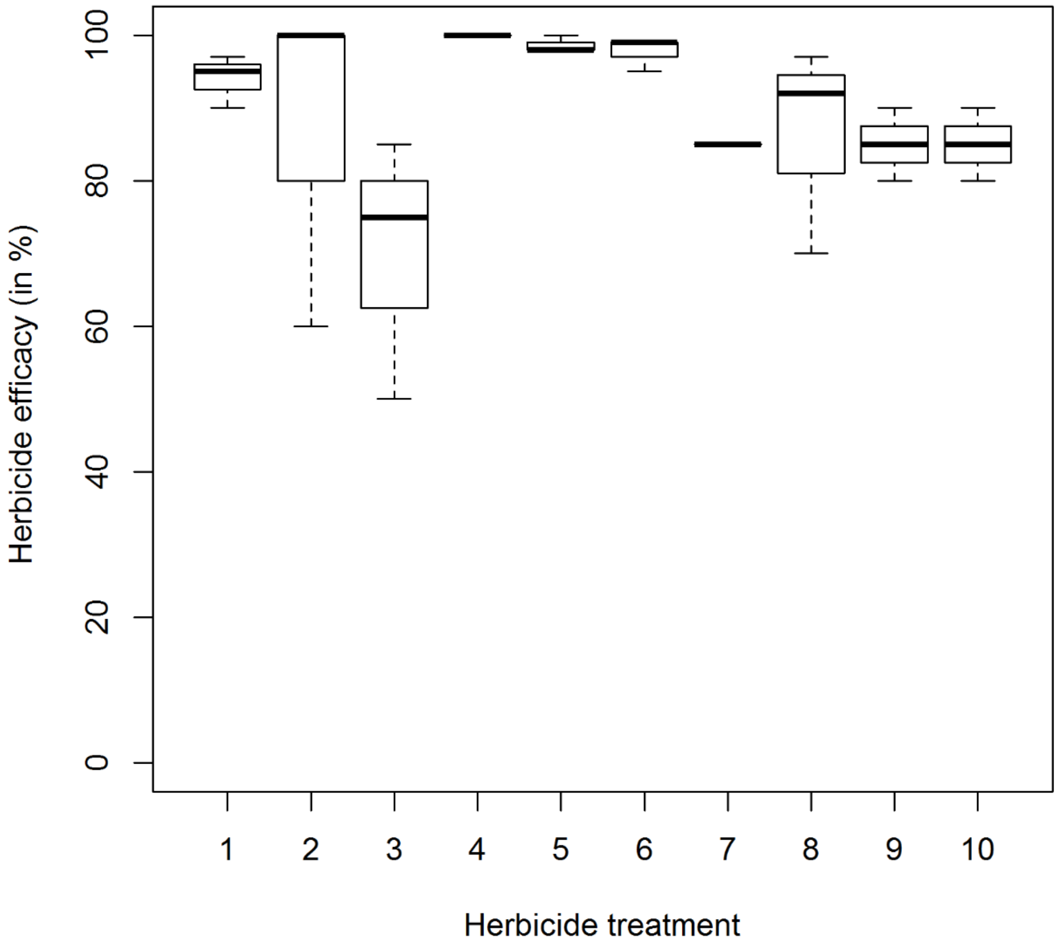

Figure 2 Results of the greenhouse biotest investigating the *A. myosuroides* population of Wurmberg at beginning of the field studies. Herbicide treatments: 1, mesosulfuron + iodosulfuron; 2, isoproturon; 3, fenoxaprop; 4, fluazifop; 5, pinoxaden; 6, clethodime; 7, flupyrsulfuron; 8, clodinafop; 9, florasulam + pyroxsulam; 10, propoxycarbazone**.**

Influence of crop rotation and herbicide strategy on *A. myosuroides* density

Density of *A. myosuroides* at the beginning of this study was lower at Ihinger Hof than at Wurmberg. At Ihinger Hof, 14 ears m^{-2} were counted in winter wheat in the first year when averaged over all untreated plots (HS 1). The influence of CR and HS on *A. myosuroides* and the direction of effects are shown in Table 3. In CR 1, with winter wheat monoculture, density of *A. myosuroides* was significantly increased compared to CR 2 and CR 3 with one and two spring crops after winter wheat. The highest number of *A. myosuroides* ears m^{-2} was counted in HS 1, in the absence of herbicide, and the lowest number in HS 4, the treatment with only one MOA. The effects of HS 2 performing a consequent change of herbicide MOA and HS 3, using herbicide applications according to recommendation of local plant protection services, were statistically equal and ranged between HS 1 and HS 4. The factor year was significant, with more *A. myosuroides* ears m^{-2} in the third, than in the second year. The interaction of CR, HS and year also had a significant influence (Table 3). Mean values of *A. myosuroides* ears m^{-2} and letter display in the different treatments and years can be found in Table 4.

Table 3 Effects of crop rotation (CR), herbicide strategy (HS), year and interactions on density of *A. myosuroides* ears at Ihinger Hof and Wurmberg (linear mixed-effect models).

Location	Parameter	Significance level	Direction of effect
Ihinger Hof	CR	***	CR 1 > CR 2 > CR 3
	HS	***	HS 1 > HS 2 = HS 3 > HS 4
	Year	**	Year 3 > Year 2
	CR x HS	**	
	CR x HS x Year	**	(see table)
	Parameter	Significance level	Direction of effect
Wurmberg	CR	**	CR 1 = CR 2 > CR 3
	HS	***	HS 1 > HS 2 > HS 3 = HS 4
	CR x HS	NS	

CR, crop rotation; HS, herbicide strategy; CR x HS, interaction effect of crop rotation and herbicide strategy; CR x HS x Year, interaction effect of crop rotation; Significance levels are *P < 0.05; **P < 0.01; ***P < 0.001; NS, not significant. Direction of effect based on Tukey's HSD method at the P = 0.05 significance level

Table 4 Response of *A. myosuroides*, expressed in average ears m^{-2} and target-site resistance (TSR) frequency in the treatments and years at Ihinger Hof.

CR x HS		Year 2		Year 3	
		Ears m^{-2} in winter wheat	TSR frequency	Ears m^{-2} in winter wheat	TSR frequency
1	1	222.44 ab	–	882.5 a	–
	2	19.51 de	50%	32.50 cde	25%
	3	10.38 efg	0%	78.75 bcd	25%
	4	12.035 efg	75%	5.63 fgh	38%
		Ears m^{-2} in winter wheat	TSR frequency	Ears m^{-2} in spring barley	TSR frequency
2	1	165.59 abc	–	64.5 bcd	–
	2	24.90 def	50%	0.25 gh	13%
	3	22.83 bcd	0%	0.75 gh	25%
	4	16.19 def	75%	0.88 gh	38%
		Ears m^{-2} in maize	TSR frequency	Ears m^{-2} in spring barley	TSR frequency
3	1	75.40 bcd	–	62.13 bcd	–
	2	17.6 de	50%	0 gh	13%
	3	0 h	NT	0 gh	25%
	4	0 h	NT	0.25 gh	38%

CR, crop rotation; HS, herbicide strategy; CR x HS x year, interaction effect of crop rotation, herbicide strategy and year; HS 1, untreated control; HS 2, consequent change of herbicide MOA; HS 3, herbicide application according to recommendation of local plant protection services; HS 4, continuous use of herbicides with only one MOA (HRAC A or B depending on crop); Means followed by the same letter are not significantly different according to Tukey's HSD method at the P = 0.05 significance level; Interaction effect of CR x HS x year was significant (<0.05); Tested loci for TSR frequency: ACCase 1781, 2027, 2041, 2078, 2096. ALS 197, 574.

Table 5 Response of *A. myosuroides*, expressed in average ears m^{-2} and target-site resistance (TSR) frequency in the treatments in year 2 at Wurmberg.

CR	HS	Year 2	
		Ears m^{-2} in winter wheat	TSR frequency
1	1	1341.5	–
	2	374.0	25%
	3	136.25	25%
	4	146.25	38%
		Ears m^{-2} in winter wheat	TSR frequency
2	1	1602.5	–
	2	707.5	25%
	3	186.5	25%
	4	252.5	38%
		Ears m^{-2} in maize	TSR frequency
3	1	941.86	–
	2	231.25	0%
	3	0.75	NT
	4	0	NT

CR, crop rotation; HS, herbicide strategy; HS 1, untreated control; HS 2, consequent change of herbicide MOA; HS 3, herbicide application according to recommendation of local plant protection services; HS 4, continuous use of herbicides with only one MOA (HRAC A or B depending on crop); Main effects CR and HS significant (<0.05), see Table 3; Tested loci for TSR frequency: ACCase 1781, 2027, 2041, 2078, 2096. ALS 197, 574.

In the rotations including spring barley (CR 2) as well as maize and spring barley (CR 3), the number of *A. myosuroides* ears m^{-2} was reduced by 92 % without herbicide input (HS 1).

At Wurmberg, a mean number of 188.4 ears m^{-2} was observed in the untreated winter wheat plots in the first year (HS 1). There were significantly more *A. myosuroides* ears m^{-2} in winter wheat (CR 1 + CR 2) than in maize (Table 5, Figure 4). All HS differed significantly from untreated HS 1, when regarding *A. myosuroides* ears m^{-2}. In HS 2 in winter wheat, the infestation of *A. myosuroides* was reduced with an ACE of about 65 - 70 % (Table 5). HS 3 and HS 4 had an ACE of 80 to 90 % in winter wheat. In maize (CR 3), control efficacy of HS 2 was about 75 %. In HS 3 and HS 4, ACE was almost 100 %. In the untreated control (HS 1), the density of *A. myosuroides* has increased from 188.4 ears m^{-2} to 1472 ears m^{-2} (averaged) in winter wheat and to 941.86 ears m^{-2} in maize.

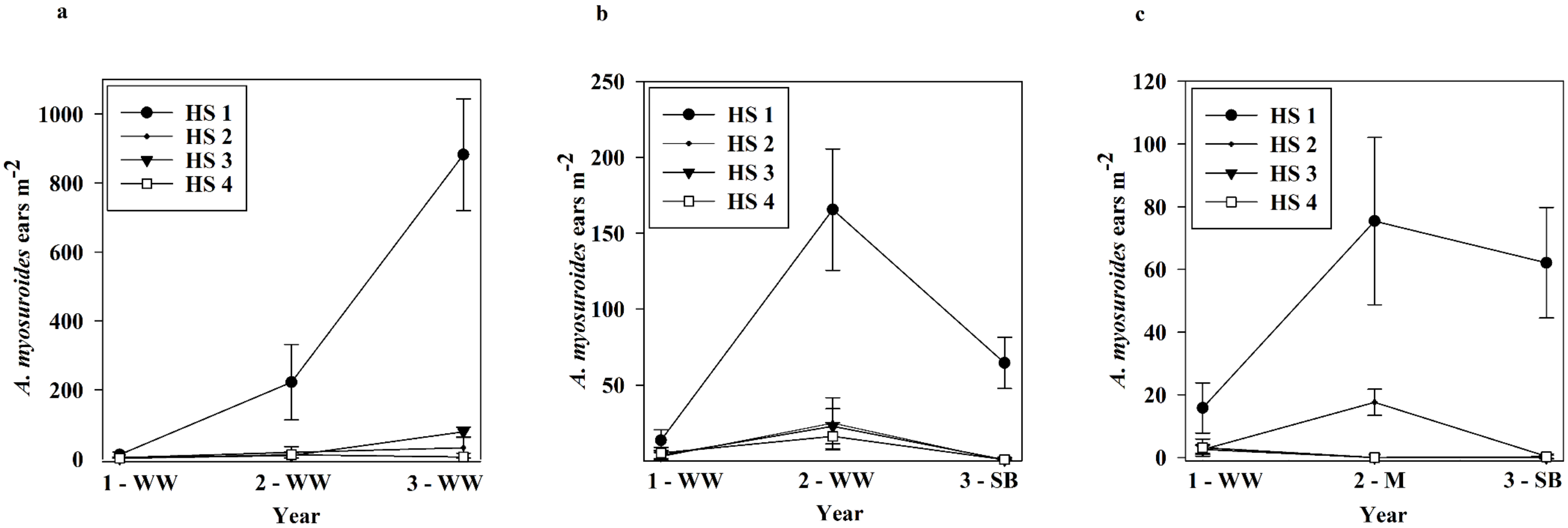

Figure 3 Average density of *A. myosuroides* ears m^{-2} in different crop rotations (CR) and herbicide strategies (HS) at Ihinger Hof. Significant effects were shown for the main effects CR and HS and their interaction (see Table 3).

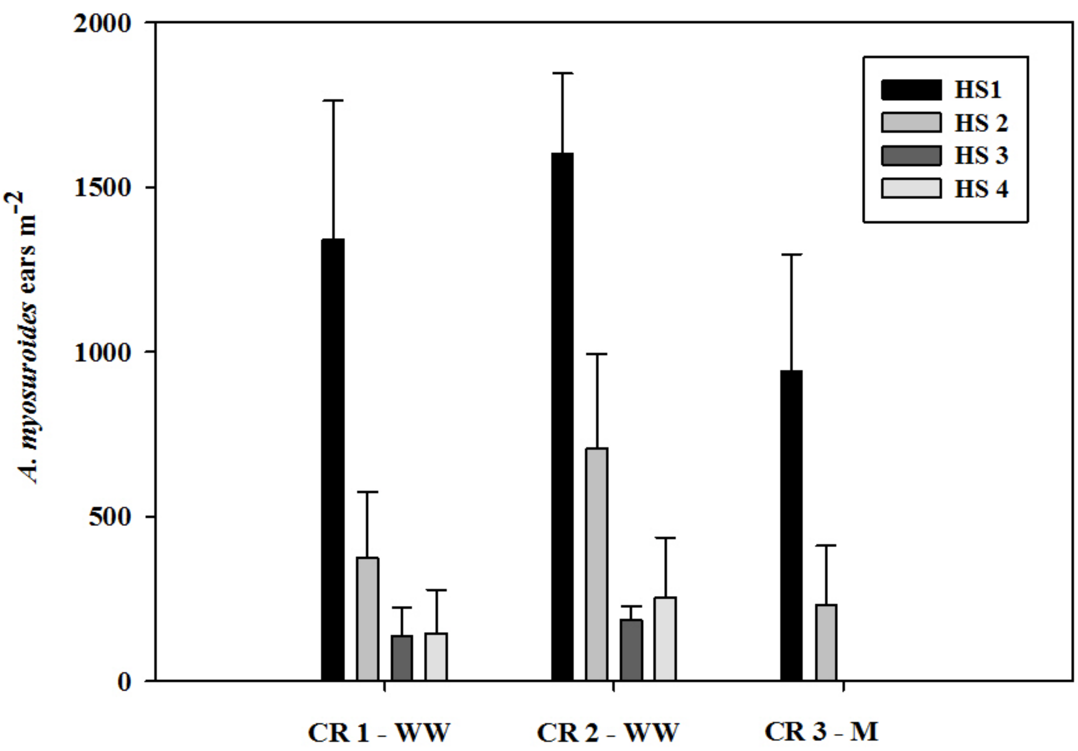

Figure 4 Average density of *A. myosuroides* ears m^{-2} in different crop rotations (CR) and herbicide strategies (HS) at Wurmberg in the second year of study. Significant effects were shown for the main effects CR and HS. Direction of effects was CR1 = CR2 > CR 3; HS 1 > HS 2 > HS 3=HS 4.

Influence of crop rotation and herbicide strategy on TSR frequency

In winter wheat, TSR frequency was highest in HS 4, using only one herbicide MOA in the second year at both locations (Table 4 + 5). TSR frequency was reduced in HS 2 and HS 3. In maize, no plants could be sampled in HS 3 and HS 4 due to the high ACE. In the third year (Ihinger Hof), HS 4 supplied the highest TSR frequency in winter wheat and in summer barley. A lower frequency was observed in HS 2 and HS 3.

Influence of crop rotation and herbicide strategy on crop yield

At Ihinger Hof, winter wheat yield was almost the same in all HS treatments ranging from 7.3 to 8.9 t ha^{-1} in year 2 (Table 6). In maize, yield was significantly reduced in HS 1(1.5 t ha^{-1}) compared to the HS treatments 2, 3 and 4 (7.0 - 7.7 t ha^{-1}). In year 3, a significant lower winter wheat yield of 5.6 t ha^{-1} was recorded in HS 1 compared to the yields of the HS treatments (between 8.8 and 9.3 t ha^{-1}). Yield of spring barley was statistically equal in all HS treatments and ranged from 6.0 to 7.5 t ha^{-1}. At location Wurmberg, winter wheat yield was significantly reduced in HS 1 (2.1 and 3.8 t ha^{-1}). A higher yield was observed in HS 2 (5.3 - 7.4 t ha^{-1}) and in HS 3 and 4 (7.5 - 8.9 t ha^{-1}).In maize, yield was significantly lower in HS 1 (8.4 t ha^{-1}) than in the HS treatments 2,3 and 4 (19.7 - 20.2 t ha^{-1}).

Table 6 Crop yield in dependency of crop rotation, herbicide strategy and year at Ihinger Hof and Wurmberg.

Ihinger Hof Crop yield (t ha^{-1})				
Year	HS	CR 1	CR 2	CR 3
		WW	WW	M
2	1	8.62 (0.96) a	8.88 (0.69) a	1.51 (0.18) b
	2	8.39 (1.13) ab	8.24 (0.98) a	6.91 (0.78) a
	3	7.61 (1.97) ab	7.99 (0.88) a	7.65 (0.87) a
	4	7.30 (1.67) b	8.24 (1.14) a	6.96 (1.08) a
		WW	SB	SB
3	1	5.6 (2.15) b	6.66 (1.75) a	6.52 (1.34) a
	2	8.95 (1.32) a	6.77 (1.81) a	7.44 (0.82) a
	3	8.77 (2.29) a	6.94 (1.83) a	7.47 (1.43) a
	4	9.33 (2.55) a	6.96 (2.36) a	5.99 (1.19) a

Wurmberg Crop yield (t ha^{-1})				
Year	HS	CR 1	CR 2	CR 3
		WW	WW	M
2	1	2.07 (0.58) c	3.77 (1.4) b	8.37 (1.94) b
	2	5.31 (0.73) b	7.39 (1.52) a	19.72 (0.92) a
	3	7.69 (1.31) a	8.21 (1.12) a	20.18 (1.07) a
	4	7.51 (2.02) a	8.86 (0.82) a	19.99 (0.59) a

CR, crop rotation; HS, herbicide strategy; WW, winter wheat; M, maize; SB, spring barley; HS 1, untreated control; HS 2, consequent change of herbicide MOA; HS 3, herbicide application according to recommendation of local plant protection services; HS 4, continuous use of herbicides with only one MOA (HRAC A or B depending on crop); Means within columns followed by the same letter are not significantly different according to Tukey's HSD method at the P = 0.05 significance level.

Figure 5 represents the linear relationship of winter wheat yield and *A. myosuroides* for both locations. The regression analysis showed that the slope is significant (m= - 0.003 ± 0.003). A maximum winter wheat yield of 7.787 t ha^{-1} in the absence of *A. myosuroides* was calculated. The resulting equation is therefore y = 7.787 - 0.003 x.

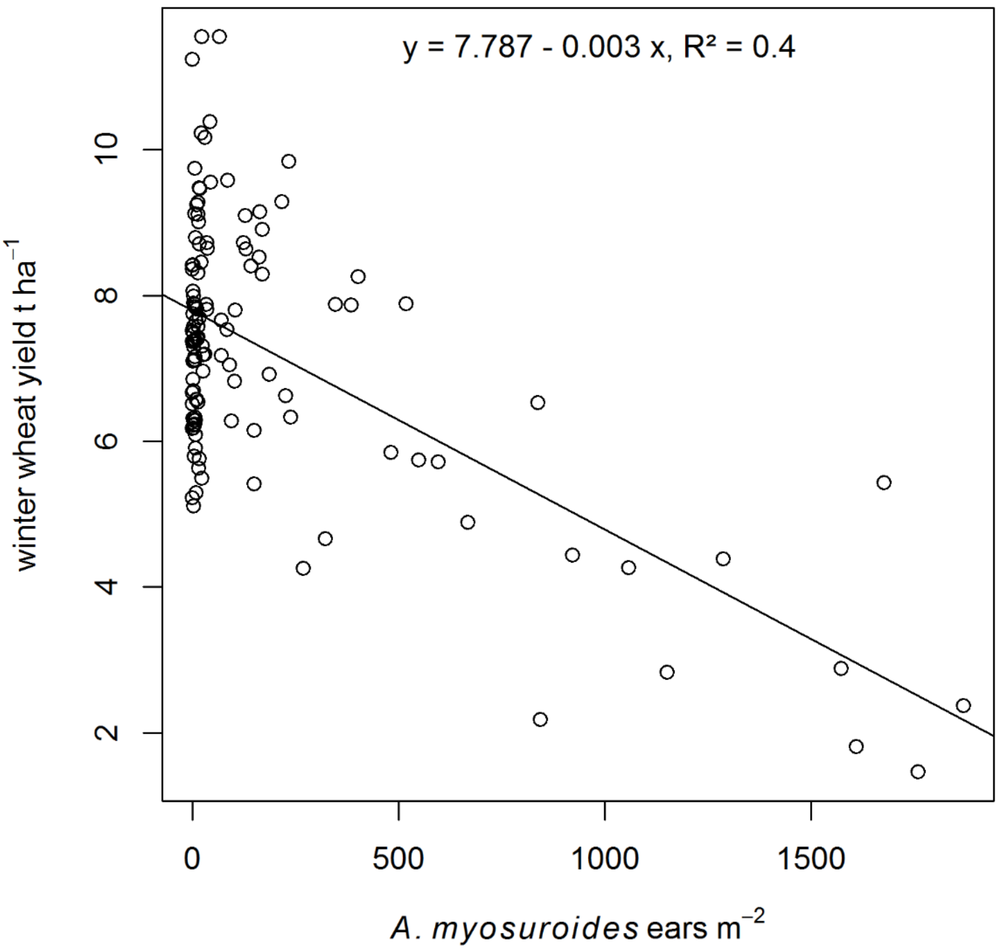

Figure 5 The relationship between *A. myosuroides* (ears m^{-2}) and winter wheat yield (t ha^{-1}).

2.4.5 Discussion

Location Ihinger Hof has not been considered as a problematic site at the beginning of the experiment. Densities were beyond the economic weed threshold and no reduction in weed control efficacy was observed in the previous years. At Wurmberg, the *A. myosuroides* densities exceeded economic threshold since the beginning of the experiment. The results have shown, that a high percentage of winter wheat strongly promoted the abundance of *A. myosuroides* and enabled the establishment of high densities, when no control measures have been carried out and in case when herbicide measures have not been successful. Therefore, hypothesis one and two are confirmed. The introduction of maize and barley strongly reduced *A. myosuroides* densities. Studies of Lutman *et al.* (2013) also showed the ability of spring crops to suppress *A.*

myosuroides. In their studies spring wheat reduced *A. myosuroides* densities by 88 % compared to winter wheat. When spring crops are included in the rotation, cover crops can be grown between winter wheat and spring crop. In our study phacelia was grown after winter wheat and cover crop residues were left on the field until sowing of spring barley and maize. We assume that suppression of *A. myosuroides* was a combined effect of crop rotation and cover cropping. Brust *et al.* (2014) also found that cover crops, besides their positive influence on the agro ecosystem, significantly suppressed weeds. The consequent change of herbicide MOA, showed the lowest ACE in all CR treatments in year 2. This can be explained by the fact, that isoproturon, which was applied in HS 2 in winter wheat, had a poor efficacy especially at Wurmberg. We assume that environmental conditions, such as low soil water content after herbicide application was responsible for low weed control efficacy of isoproturon (Blair 1985). Another reason for the low control efficacy in HS 2 was due to tembotrione used in maize at both locations. In comparison to the active ingredients of HS 3 and HS 4 (see Table 2) tembotrione is known to have only moderate control efficacy against *A. myosuroides.* Nevertheless, it was used for this experiment to allow a consequent change of herbicide MOA. The control efficacy however was mostly higher in HS 3 and HS 4. In these treatments more ALS inhibitors were used. ALS-inhibitors have high efficacy against *A. myosuroides* if the population has not yet developed herbicide resistance. Nevertheless, this herbicide group is considered as a high resistance-risk MOA. At the beginning of the study, ALS inhibitors were still very effective against *A. myosuroides* of both locations (see results of bioassay). However, there was a strong selection pressure in these treatments, resulting in highest TSR frequency in HS 4 with the use of only one MOA (Table 4 and 5). Therefore hypothesis 3 was confirmed. A lower TSR frequency could be shown in the treatments where herbicide MOAs were changed in every year. This observation clearly demonstrates, that a consequent change of herbicide MOA is essential for the prevention of herbicide resistance. With the analysis of TSR frequency, we could exclusively demonstrate the influence of herbicide strategies on TSR development. The influence on NTSR development could not been tested, due to a lack in testing procedure. However, researchers monitored that mutations in the ALS-gene do rarely occur alone. In the majority of cases they appear in combination with NTSR mechanisms, such as enhanced metabolism rate (Sievernich *et al.* 2013). Therefore, it should be assumed that NTSR also was promoted in *A. myosuroides,* although it was not tested in this study.

The investigation of crop yield has shown, that especially in year 2 in maize *A. myosuroides* caused yield losses. As described by Oerke (2006) maize is a low competitive species in the juvenile stage. The presence of *A. myosuroides* in high densities affected maize development and yield parameters at both locations. In a normal field situation, additionally mechanical weed control measures, such as hoeing, could have been performed to eliminate the weeds and the competition to maize. At Wurmberg winter wheat yield was already strongly influenced by *A. myosuroides* in the second year, at Ihinger Hof this effect was observed in the third year. The regression analysis revealed that approximately 1200 *A. myosuroides* ears m^{-2} reduced winter wheat yield by 50%. These findings are in line with Moss (1987), showing that at high infestation rates *A. myosuroides* may cause substantial yield losses of about 45 %. The yield of summer barley was not significantly influenced by *A. myosuroides.*

The results of the present field studies are in line with the observations of Liebman & Dick (1993), who found that a diverse crop rotation can prevent the propagation of particular weed species. Already in 1982, Walker & Buchanan advised farmers not to rely only on herbicides for weed control. Integrated Weed Management (IWM) combines preventive methods, such as diverse crop rotations, the use of competitive cultivars and soil tillage with chemical and non-chemical weed control strategies to control weed populations (Chikowo *et al.* 2009). This approach is less dependent on chemical measures, still guarantees high productivity and prevents the selection of herbicide resistant weed populations.

2.4.6. Conclusions

Our on-farm study revealed, that densities of *A. myosuroides* tremendously increased if weed control measures were ineffective or if they were omitted. Within a period of three years we noticed a rapid build-up of *A. myosuroides* densities affecting crop yield, even for locations which have not been considered as a problematic site. Herbicide resistance was promoted by using only one MOA. Selection pressure could be reduced by realizing a consequent change of herbicide MOA. Spring crops had the ability to reduce *A. myosuroides* densities even without herbicide input. In combination with mechanical weed control measures and a strategic selection of herbicides, crop rotations including spring crops can be seen as an effective agronomic factor to prevent high densities of *A. myosuroides* and associating yield loss.

Acknowledgements

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Chapter III

General Discussion

3 General Discussion

In this study, investigations on herbicide resistance in the weed species *Alopecurus myosuroides* HUDS. and *Apera spica-venti* L. Beauv. have been carried out. Therefore experiments in the laboratory, in the greenhouse and in the field were conducted. The results are presented in four papers which are part of this thesis. In this chapter, the main outcomes of these experiments are summarized and discussed and future prospects are given.

Detection of herbicide resistance and investigation of resistance mechanisms

The classical approach to screen for herbicide resistance is to gather seeds from surviving plants in suspected fields, grow these in pots in the greenhouse, and apply the herbicides of interest. In these classical greenhouse assays, herbicides are usually sprayed at a single dose, mainly the recommended field dose. After herbicide treatment, the response of putative resistant samples is compared with that of a selected sensitive reference standard and respective untreated control plants (Burgos *et al.* 2013). Degrees of resistance can be classified according to the R rating system of Moss et al. (1999). The evaluation of a resistance level among populations can be realized by applying several doses and creating dose response curves, relative to a sensitive reference standard. Comparison between populations is most commonly done by determination of ED50 and ED90 values, the dose at which the plant response is reduced by 50 % and 90 %, respectively. Additionally, the calculation of resistance factors (ratio Resistant/Sensitive) is a useful tool to quantify the magnitude of resistance (Burgos *et al.* 2013). Greenhouse assays proved a powerful and practical tool for the investigation of sensitivity of weed populations to different herbicides (Streibig 1988). Disadvantages are the need of a lot of space in the greenhouse and the duration of up to two months until results are available. Due to the steadily increasing number of putative herbicide resistant weed populations, the demand for rapid in-season tests is rising. In the first paper of this dissertation, titled 'Chlorophyll fluorescence imaging: a new method for rapid detection of herbicide resistance in *Alopecurus myosuroides*' a new resistance quick test was developed. In the experiments of the paper sensitive and resistant populations of *A. myosuroides* were transplanted into multiwell tissue culture plates containing nutrient agar and different dosages of fenoxaprop-P-ethyl and mesosulfuron + iodosulfuron. The parameter used for the evaluation of herbicide resistance was the maximum quantum efficiency of the PSII, measured by a sensor. It was possible to fit dose-response curves and calculate corresponding resistance factors for all populations tested using the chlorophyll fluorescence imaging. The results corresponded well to the standard whole-plant pot tests in the greenhouse. The new test needed less time and space than for the standard test in the greenhouse. Results were available 96 hours after transplanting at the last (depending on active ingredient). Including time for plant cultivation, the whole testing procedure can be realized within 10 to 14 days. As well as in greenhouse assays, the chlorophyll fluorescence imaging was able to detect the resistance of target-site resistant (TSR) and non-target-site resistant (NTSR) populations. We consider this study as an initial investigation to identify herbicide resistant weed populations. The results showed that the test proved suitable for the investigated ACCase inhibitor fenoxaprop-P-ethyl and the ALS inhibitor mesosulfuron + iodosulfuron. Beside the experiments presented in the paper, the suitability of the chlorophyll fluorescence imaging was assessed for further weed species (i.a. *A. spica-venti*, *Matricaria* spp., *Papaver rhoeas*, *Stellaria media*), active ingredients and modes of action (HRAC A, B, C, G, O). The chlorophyll fluorescence imaging might also be used as an in-season field test. For this application, weed seedlings could be transplanted into multiwell plates treated with different herbicides and the response could be measured after 48-96 hours in relation to an untreated control. Furthermore, based on the presented test, a system to transfer chlorophyll fluorescence measurements into the field is currently in the development. Therefore, a field sensor, named ‘Weed PAM’ and the associated software has already been developed. With the aim to calibrate the system and to realize a resistance classification at the end of the project, numerous data are recorded at present.

For the investigation of herbicide resistance at the DNA level, SNP (single nucleotide polymorphism)-assays and DNA sequencing procedures have been standardized. Based on the polymerase chain reaction (PCR) these tests investigate the base sequence of the target genes and provide results within a day. Approaches at the DNA level are limited to the mechanism of target-site resistance (Burgos *et al.* 2013). For the investigation of non-target site resistance several methods have been described as well. To investigate uptake, translocation and metabolism of active ingredients in plants the use of radioactive-labelled (^{14}C) herbicides is an appropriate method (McIntyre 1962, Manley *et al.* 1999, Wakelin *et al.* 2004, Lycan & Hart 2006, Pester *et al.* 2009). These procedures provide accurate results, however dedicated isotope laboratories are needed. Another option to investigate herbicide metabolism is via High Performance Liquid Chromatography (HPLC) and combined Liquid Chromatography/Mass Spectrometry (LC/MS). The determination of the amount of active ingredient and their metabolites over time enables to detect differences in herbicide metabolism between sensitive and resistant plants. In the second paper, titled 'Degradation and metabolism of fenoxaprop and mesosulfuron + iodosulfuron in multiple resistant blackgrass (*Alopecurus myosuroides*)' measurements were carried out by using Liquid Chromatography/tandem Mass Spectrometry (LC/MS-MS). This methodology allows the detection of substances even at very low amounts as it is necessary for the investigation of ALS-inhibitors (notably sulfonylureas) due to its low dose rates. The results showed that degradation and metabolism of herbicides differed in sensitive and resistant populations of *A. myosuroides*. Differences in the metabolism of the investigated ACCase herbicide fenoxaprop-P-ethyl were shown with regard to the acid metabolite, namely fenoxaprop-P. The dynamics differed between sensitive, TSR and NTSR *A. myosuroides*. With regard to the investigated ALS inhibitor mesosulfuron + iodosulfuron differences were shown in the degradation. In the resistant, in particular the non-target-site resistant populations, mesosulfuron + iodosulfuron degraded significantly faster than in sensitive and target-site resistant *A. myosuroides.*

The LC/MS-MS measuring technique proved suitable for the studies, because even low contents could be detected. However, for the preparation of laboratory samples, there was a high demand on plant biomass (two grams fresh weight per sample). That means that it was not feasible to investigate single plants. As it is described by Délye (2013) weed populations always consist of different individuals, some being sensitive and some being resistant. Although whole populations and no single plants were investigated in the experiments, differences between populations became clearly evident. It can, however, be assumed that differences between sensitive, NTSR and TSR *A. myosuroides* are even more clear, when analysing single plants or clones. In this respect, further studies could be conducted investigating genetically identical individuals (clones), for example by vegetative propagation. In order to gain even more knowledge on NTSR, further studies investigating more populations, different weed species as well as further metabolites would be helpful.

Factors affecting population density and herbicide resistance

The effect of repetitive application of herbicides with the same mode of action in the evolution of resistant weed populations has been widely documented (Holt 1992, Maxwell & Mortimer 1994, Powles & Yu 2010). Agricultural management measures and biological factors are also known to play a crucial role in the risk for resistance selection (Holt *et al.* 1993, Murphy & Lemerle 2006). In the third paper, titled 'Development of a Geo-Referenced Database for Weed Mapping and Analysis of Agronomic Factors Affecting Herbicide Resistance in *Apera spica-venti* L. Beauv. (Silky Windgrass)' a risk assessment model and a database for mapping the distribution of herbicide-resistant *A. spica-venti* populations in Europe were developed. The geo-referenced database represents a useful tool to evaluate relationships between field history and resistance spread over time and space. An application in other fields beyond weed science, such as phytopathology, is conceivable due to the flexibility deriving from the multiple features of the database. The statistical analysis was performed on a sample of 263 *A. spica-venti* populations origination from Europe. The analysis of influence factors revealed a statistically significant effect for crop rotation, soil tillage, sowing date, weed density and the interaction between crop rotation and soil tillage. Soil texture had no significant influence. Crop rotation, soil tillage and their interactions were the most important factors. A high percentage of winter crops in the crop rotation (>75%), together with conservation tillage, early sowing dates and high population density increased the occurrence of herbicide resistance in *A. spica-venti*. The results correspond with most existing literature. In continuous cropping systems, species that have phenological and physiological similarities to the crop are promoted (e.g. grass weeds in cereals) (Bárberi 2003, Cardina *et al*. 1998). Therefore, *A. spica-venti* with its winter growth habit and preference for autumn germination is favored in autumn-sown crops, mainly winter cereals and winter oilseed rape (Hurle 1993, Melander *et al.* 2008). Early sowing dates strengthen the effect by providing more time for establishment until the beginning of dormancy period. With regard on crop rotation and soil tillage the results correspond to the observations of Melander *et al*. (2008) where *A. spica-venti* particularly was promoted in crop rotations exclusively consisting of autumn-sown crops and with non-inversion tillage. Severe *A. spica-venti* infestations are mainly documented for light textured soils such as moist sand and light loam (Rola 1990, Northam & Callihan 1992) . The impact of soil texture on the probability of resistance selection could not be confirmed in the studies. Most infested fields were characterized by a middle soil texture whereas light soils and heavy soils were under-represented. The huge influence of high weed densities in the risk of resistance selection could be affirmed in the studies. In accordance to many references (e.g. Powles & Yu 2010), high weed densities increase the risk of herbicide resistance development. For the prevention of the evolution and spread of herbicide resistance, the developed model can find application in resistance management programs.

As another important weed in terms of crop yield reducing ability and herbicide resistance, *A. myosuroides* can be mentioned. Similar to *A. spica-venti*, densities of *A. myosuroides* have increased in Europe in the past three decades probably due to a higher proportion of winter cereals in the crop rotation, earlier sowing dates of winter wheat (Melander 1995) and a change to reduced tillage systems (Melander *et al.* 2008). These cropping practices have caused a greater reliance on herbicides, which can support the selection of herbicide resistant *A. myosuroides* populations. For the fourth paper, titled 'Crop rotation and herbicide strategies affecting herbicide resistance and population development of *Alopecurus myosuroides*', field experiments have been carried out over three years. For the study, two locations have been selected with regard to *A. myosuroides* infestation. Location Ihinger Hof has not been considered as a problematic site at the beginning of the experiment. Densities were beyond the economic weed threshold and no reduction in weed control efficacy was observed in the previous years. At Wurmberg, the *A. myosuroides* densities exceeded the economic threshold since the beginning of the experiment. The results showed that densities of *A. myosuroides* were significantly influenced by the factors crop rotation and herbicide strategy and the interaction of the factors. The results have shown, that continuous winter wheat strongly promoted the abundance of *A. myosuroides* and enabled the establishment of high densities, when no control measures have been carried. In the absence of herbicides, the spring crops maize and barley reduced *A. myosuroides* densities by 92 %. Lutman *et al.* (2013) also ascertained that spring crops were able to suppress *A. myosuroides*. In their studies spring wheat achieved an 88% reduction in *A. myosuroides* densities compared to winter wheat. By evaluating the frequency of TSR plants, it was shown that within the experimental time herbicide resistance was demonstrably promoted when there was a continuous use of herbicides with only one mode of action (HRAC A or B depending on crop). The selection pressure was reduced by using herbicides with different modes of action, resulting in a lower frequency of TSR plants. These findings confirm that a consequent use of herbicides with the same modes of action results in the selection of resistant weed populations, as it is widely documented in literature (Holt 1992, Maxwell & Mortimer 1994, Powles & Yu 2010). The notorious crop yield reducing ability of *A. myosuroides* was confirmed in the experiments. In particular the crop yield of winter wheat and maize was reduced at high *A. myosuroides* densities. Approximately 1200 *A. myosuroides* ears m^{-2} reduced winter wheat yield by 50%. These findings are in line with Moss (1987), showing that at high infestation rates *A. myosuroides* may cause substantial yield losses of about 45 %. As a prospect it can be said, that the field experiments are continued in the two following years. After 5 years of experimental time in total, clear and meaningful results can be expected that evaluate the impact of crop rotation and herbicide strategies on *A. myosuroides.*

Overall, the results of the third and the fourth paper demonstrate the huge influence of agronomic factors on the weed species *A. spica-venti* and *A. myosuroides*, in particular population densities and development of herbicide resistance. Before herbicides became available on the marketplace, crop rotation has traditionally been considered as an essential strategy for the control of weeds (Leighty 1938, Froud-Williams 1988, Oerke 2006). The possibility to suppress weeds by non-chemical means such as soil tillage among others, have been extensively investigated (e.g. Holt *et al*. 1993). Nevertheless, the control of weeds by chemical means is still dominant. With regard on herbicide resistance, alternative non-chemical weed control strategies, should come as a matter of course again. For farmers it will become essential to consider the cost of herbicide resistance in future (i.e. yield loss of crops).

Summary

Zusammenfassung

4 Summary

The control of pests is one of the major challenges in agricultural production worldwide. Especially weeds cause severe yield losses by competing with crops for light, space, water and nutrients. Due to the relatively low costs for acquisition and application of herbicides and a high control efficacy, chemical measures are predominantly applied to control weeds. In Europe, *Alopecurus myosuroides* HUDS. (blackgrass) and *Apera spica-venti* L. Beauv. (silky windgrass) are major weeds especially in winter wheat. The occurrence at high population densities in combination with a consequent use of herbicides with the same modes of action has resulted in the selection of resistant populations. Populations with target-site resistance (TSR) as well as non-target-site resistance (NTSR) could be confirmed for *A. myosuroides* and *A. spica-venti.* In contrast to the mechanisms of TSR, NTSR mechanisms are less investigated. Due to the steadily increasing number of putative herbicide resistant weed populations, the demand for rapid resistance tests is rising. The papers of the dissertation focus on the integrated management, the investigation of resistance mechanisms and the detection of herbicide resistant weed populations. The following research objectives have been examined within the four work packages (papers):

- To develop a new methodology for a rapid detection of herbicide resistance and to confirm that results are comparable with classical greenhouse approaches
- To investigate metabolism of herbicides in sensitive and resistant populations of *A. myosuroides* to gain comprehensive knowledge on resistance mechanisms
- To evaluate the influence of agronomic factors on the probability of resistance occurrence and to develop a geo-referenced database for mapping the spread of herbicide-resistant *A. spica-venti* populations across Europe
- To assess the influence of crop rotation and herbicide strategies on population development and herbicide resistance of *A. myosuroides* and crop yield

The four papers come to the following results regarding the main research objectives:

<u>1st paper:</u> A laboratory test was developed to accelerate the detection of herbicide resistance. Therefore, *A. myosuroides* was cultivated in wellplates containing nutrient agar and herbicides. The evaluation of herbicide resistance was conducted by a sensor, measuring chlorophyll fluorescence. The results of the developed test corresponded well

to the standard whole-plant pot tests in the greenhouse. In both tests sensitive and resistant populations were identified, however results of the Chlorophyll Fluorescence Imaging were available earlier.

2nd paper: Metabolism of herbicides was investigated in populations of *A. myosuroides* by using liquid chromatography - tandem mass spectrometry (LC-MS/MS) to gain comprehensive knowledge on mechanisms of herbicide resistance. NTSR populations differed from sensitive and TSR *A. myosuroides* in form of an enhanced degradation of the active ingredient or metabolite, depending on the investigated herbicide. For the investigated herbicides (inhibition of ACCase and ALS) it was shown that herbicide metabolism plays an important role regarding herbicide resistance in *A. myosuroides.*

3rd paper: To evaluate the influence of agronomic factors on the probability of resistance occurrence in *A. spica-venti*, numerous populations were screened in the greenhouse. The corresponding field history obtained from questionnaires and the results of greenhouse assays were used to develop a GIS-database in which herbicide-resistant *A. spica-venti* populations were mapped. The statistical analysis revealed that a high percentage of winter crops in the crop rotation, together with conservation tillage, early sowing dates and high population density increased the occurrence of herbicide resistance in *A. spica-venti.*

4th paper: To assess the impact of crop rotation and herbicide strategies on *A. myosuroides*, field studies at two locations in Southern Germany have been carried out. Results show that densities of *A. myosuroides* increased in continuous winter wheat. The introduction of spring crops significantly reduced densities, even without using herbicides. Furthermore it has been shown that the risk of herbicide resistance was reduced when performing a consequent change of herbicide mode of action. The use of herbicides with only one mode of action increased the number of herbicide resistant plants. Crop yield was notably influenced by *A. myosuroides* in winter wheat.

The overall results of this dissertation showed the great impact of agricultural measures on herbicide resistance in *A. myosuroides* and *A. spica-venti* and demonstrated opportunities for prevention and management. The developed resistance quick test provides an accelerated detection of herbicide resistance and therefore the chance to initiate resistance management strategies much earlier.

5 Zusammenfassung

Die Bekämpfung von Schaderregern ist weltweit eine der größten Herausforderungen in der landwirtschaftlichen Produktion. Unkräuter verursachen die höchsten Ertragsverluste von allen Schaderregerklassen, indem sie mit Kulturpflanzen um Licht, Raum, Wasser und Nährstoffe konkurrieren. Chemische Unkrautregulierungsmaßnahmen dominieren aufgrund der relativ geringen Kosten für die Anschaffung und Ausbringung, sowie der hohen Wirkungsgrade der Herbizide. In Europa sind vor allem die Ungräser *Alopecurus myosuroides* HUDS. (Ackerfuchsschwanz) und *Apera spica-venti* L. Beauv. (Gemeiner Windhalm) von Bedeutung. Die Kombination eines kontinuierlichen Einsatzes von Herbiziden aus gleichen Wirkstoffgruppen mit hohen Populationsdichten hat zur Selektion von herbizidresistenten Populationen geführt. Von *A. myosuroides* und *A. spica-venti* wurden sowohl Populationen mit Zielortresistenz (TSR) als auch mit Nicht-Zielortresistenz (NTSR) nachgewiesen. Im Gegensatz zum Mechanismus TSR ist die NTSR weniger erforscht. Durch die stetig zunehmende Zahl vermeintlich resistenter Unkrautpopulationen wächst der Bedarf nach Schnelltests zum Nachweis der Herbizidresistenz. In den Veröffentlichungen dieser Dissertation liegt der Schwerpunkt auf der integrierten Bekämpfung, der Untersuchung von Resistenzmechanismen und der Detektion von resistenten Unkrautpopulationen. Die vier Arbeitspakete hatten folgende Zielsetzungen:

– Die Entwicklung eines Schnelltests zum Nachweis der Herbizidresistenz sowie die Überprüfung der Eignung durch den Vergleich mit klassischen Verfahren
– Die Untersuchung des Metabolismus von Herbiziden in sensitiven und resistenten Populationen von *A. myosuroides*, um Erkenntnisse hinsichtlich unterschiedlicher Resistenzmechanismen zu erlangen
– Die Bewertung des Einflusses produktionstechnischer Faktoren auf die Herbizidresistenz und die Entwicklung einer geo-referenzierten Datenbank zur Dokumentation der Verbreitung von *A. spica-venti* Populationen in Europa
– Die Untersuchung des Einflusses von Fruchtfolge und Herbizidstrategie auf die Populationsdichte und die Entwicklung von Herbizidresistenz auf *A. myosuroides* und den Ertrag der Kulturpflanzen

Folgende Ergebnisse wurden hinsichtlich der Hauptzielsetzungen ermittelt:

Veröffentlichung 1: Für einen beschleunigten Nachweis der Herbizidresistenz wurde ein Schnelltest im Labor entwickelt. Dafür wurden Populationen von *A. myosuroides* in Wellplates mit Nährstoffagar und Herbiziden kultiviert. Die Bewertung der Herbizidresistenz erfolgte mittels Sensor, welcher die Chlorophyllfluoreszenz misst. Die Ergebnisse des entwickelten Tests zeigten eine gute Übereinstimmung mit klassischen Gewächshausstudien. Sensitive und resistente Populationen konnten mit beiden Methoden identifiziert werden, im Chlorophyll Fluorescence Imaging waren die Ergebnisse jedoch schneller verfügbar.

Veröffentlichung 2: Um neue Erkenntnisse hinsichtlich der Resistenzmechanismen in *A. myosuroides* zu gewinnen, wurde der Metabolismus von Herbiziden mittels Flüssigkeits-Chromatographie-Massenspektometrie/Massenspektometrie (LC-MS/MS) untersucht. Populationen mit einer NTSR unterschieden sich von sensitiven und TSR Populationen, je nach Herbizid, durch einen beschleunigten Abbau des Wirkstoffes bzw. Metaboliten. Für die untersuchten Herbizide (ACCase- und ALS- Inhibitoren), hat sich gezeigt, dass der Mechanismus eines erhöhten Metabolismus für die Herbizidresistenz in *A. myosuroides* eine wichtige Rolle spielt.

Veröffentlichung 3: Um den Einfluss produktionstechnischer Faktoren auf das Auftreten von Herbizidresistenz in *A. spica-venti* zu bewerten, wurden zahlreiche Populationen im Gewächshaus untersucht. Die zugehörige Historie der Herkunftsflächen dieser Populationen, wurde für die Entwicklung einer GIS-basierten Datenbank verwendet, welche es möglich macht, resistente Populationen von *A. spica-venti* zu kartieren. Die statistische Auswertung ergab, dass das Auftreten der Herbizidresistenz bei einem hohen Anteil von Winterungen in der Fruchtfolge, in Kombination mit reduzierter Bodenbearbeitung, Frühsaatterminen und hohen Populationsdichten signifikant erhöht war.

Veröffentlichung 4: Um den Einfluss von Fruchtfolge und Herbizidstrategie auf die Populationsdichte und die Entwicklung von Herbizidresistenz in *A. myosuroides* zu untersuchen, wurden Feldversuche an zwei Standorten in Süddeutschland angelegt. Nach wiederholtem Anbau von Winterweizen hat die Dichte von *A. myosuroides* stark zugenommen. Die Eingliederung von Sommerungen in die Fruchtfolge konnte die Dichte signifikant reduzieren. Mit einem konsequenten Wirkstoffwechsel konnte das Risiko von Herbizidresistenz vermindert werden. Der kontinuierliche Einsatz derselben Wirkstoffe führte nachweislich zur Selektion resistenter Pflanzen. Der Kulturpflanzenertrag wurde durch die Anwesenheit von *A. myosuroides* besonders in Winterweizen negativ beeinflusst.

Die Ergebnisse dieser Dissertation demonstrieren den großen Einfluss produktionstechnischer Maßnahmen auf die Herbizidresistenz in *A. myosuroides* und *A. spica-venti* und zeigen Möglichkeiten für die Vorbeugung sowie das Management auf. Der entwickelte Resistenz-Schnelltest ermöglicht einen beschleunigten Nachweis der Herbizidresistenz und damit die Gelegenheit Resistenz-Managementstrategien früher einleiten zu können.

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Curriculum vitae

Personal Data

Name	Yasmin Isabelle Kaiser
Date and Place of Birth	February 7th 1986, Stuttgart, Germany

University Education

April 2012 – to date	Doctorate candidate of the department of Weed Science,
	Institute of Phytomedicine, University of Hohenheim
April 2010 – April 2012	Studies in Agricultural Science, University of Hohenheim
	Master of Science (M.Sc.)

School Education

1996 – 2006	Hermann – Hesse Gymnasium Calw
	Abitur
1992 – 1996	Primary school, Hirsau

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Yasmin Kaiser

Stuttgart-Hohenheim, 27. April 2015

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‘Integrated management, analysis of mechanisms and early detection of resistant populations of *Alopecurus myosuroides* HUDS. and *Apera spica-venti* L. Beauv.’

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Yasmin Kaiser

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