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Impact of weed control strategies on resistance evolution in *Alopecurus myosuroides* – a long-term field trial

Einfluss von Herbizidstrategien auf die Resistenzentwicklung bei Alopecurus myosuroides *– ein Dauerversuch*

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Abstract

The impact of various herbicide strategies on populations of *Alopecurus myosuroides* is investigated in a long-term field trial situated in Wendhausen (Germany) since 2009. In the initial years of the field experiment, resistant populations were selected by means of repeated application of the same herbicide active ingredients. For the selection of different resistance profiles, herbicides with actives from different HRAC groups were used. The herbicide actives flupyrsulfuron, isoproturon und fenoxaprop-P were applied for two years on large plots.

In a succeeding field trial starting in 2011, it was investigated if the now existing resistant field populations could be controlled by various herbicide strategies. Eight different strategies consisting of various herbicide combinations were tested. Resistance evolution was investigated by means of plant counts and molecular genetic analysis.

Keywords: Herbicide resistance, long-term field trial, resistance strategy

Zusammenfassung

In einem Dauerfeldversuch wird am Standort Wendhausen (Nähe Braunschweig) seit dem Jahr 2009 der Einfluss unterschiedlicher Herbizidstrategien auf *Alopecurus myosuroides*-Populationen mit unterschiedlichen Resistenzprofilen untersucht. In den ersten Jahren des Versuches wurden auf dem Standort durch jährliche Applikation derselben herbiziden Wirkstoffe entsprechende resistente Populationen selektiert. Dabei wurden zur Selektion unterschiedlicher Resistenzprofile Herbizide aus unterschiedlichen HRAC-Wirkstoffgruppen verwendet. So wurden die herbiziden Wirkstoffe Flupyrsulfuron, Isoproturon und Fenoxaprop-P in gleichbleibenden Großparzellen über einen Zeitraum von zwei Jahren eingesetzt.

Bei dem in 2011 begonnen Versuch sollte im Anschluss untersucht werden, inwieweit die bestehenden resistenten Populationen mit unterschiedlichen Herbizidstrategien bekämpft werden können. Dabei wurden acht verschiedene Strategien mit unterschiedlichen Herbizidkombinationen getestet. Zudem wurde die Entwicklung der Resistenz anhand von Feldbonituren und molekulargenetischen Analysen untersucht.

Stichwörter: Dauerfeldversuch, Herbizidresistenz, Anti-Resistenzstrategie

Introduction

Herbicide resistance in *Alopecurus myosuroides* is a well-known phenomenon in German arable cropping systems. Due to the low number of selective herbicide active ingredients available for grass weed control in cereals, ACCase and ALS inhibitors are frequently used to control grass weed species such as *A. myosuroides* (Moss et al., 2007). Regarding *A. myosuroides*, winter cereals and ACCase and ALS inhibitors are therefore the crop species and herbicide chemical groups most widely affected by resistance evolution (Petersen, 2014). Anti-resistance strategies including the application of soil-active pre-emergence active ingredients such as flufenacet are now widely adopted by farmers but are often only able to retard but not to completely prevent resistance evolution. Once resistance has occurred on a specific field, the main aim of farmers is to reduce the spreading of resistant individuals in the field and to control the resistant populations in order to minimize the negative impact on yield. However, recent studies have shown that resistance evolution on a field cannot be completely reversed by specific control strategies but that increased control efficacy including control of resistant individuals can be achieved by the adopting of appropriate herbicide strategies including active ingredients not yet affected by resistance (RUMMLAND, 2014).

In this experimental field study, we investigate the effect of different herbicide strategies on *A. myosuroides* populations pre-selected with different active ingredients. Using three different active ingredients, we first pre-selected for distinct reduced herbicide sensitivity in a field *A. myosuroides* population. After a two-year selection period, we tested eight different herbicide strategies (HS) in order to monitor further resistance evolution in the pre-selected *A. myosuroides* populations.

Materials and Methods

Experimental design

A long-term field trial was set up in 2009 at an experimental field in Wendhausen close to Braunschweig, Germany. The site was characterized by a high infestation with *A. myosuroides*. First bioassays prior to the start of the experiments have indicated that the present *A. myosuroides* population may exhibit a reduced sensitivity towards ACCase inhibitors (data not shown). The field was sown with winter wheat each year and early sowing (end of September) was conducted. The initial experimental design consisted of three large neighboring experimental plots (12 x 150 m) treated with three different active ingredients (Tab. 1). The initial herbicide treatments (IHT) were applied in autumn post-emergent each year. The aim of the three IHT was to cause a sensitivity shift in the *A. myosuroides* population present on the field and to select for varying herbicide susceptibility between the plots as a result of the three treatments.

Tab. 1 Initial herbicide treatments (IHT) in the experimental years 2009-2011**.**

Tab. 1 *Herbizidbehandlungen (IHT) in den Versuchsjahren 2009-2011.*

Plot	Herbicide trade name	Active ingredient	Herbicide dose rate
I	Arelon Top	Isoproturon (500 g L^{-1})	3.0 Lha^{-1}
II	Ralon Super	Fenoxaprop-P-Ethyl (69 g L^{-1})	1.2 L ha^{-1}
III	Lexus	Flupyrsulfuron-methyl (500 g L^{-1})	20 g ha^{-1}

In 2011, the experimental design characterized by the three IHT plots (I-III) was altered and new treatments consisting of eight different weed control strategies (WCS no. 1-8) were set up transverse to the three initial plots (Fig. 1).

Parts (12 x 50 m) of the former three large IHT plots were maintained in order to further monitor the impact of the three IHT indicated in Table 1 which were further on applied annually post-emergent in autumn. The new eight herbicide treatments (WCS, Tab. 3) were replicated four times (block a-d) with a plot size of 3 x 36 m. Winter wheat was continuously grown on the experimental plots analogous to the previous experimental period in 2009-2011.

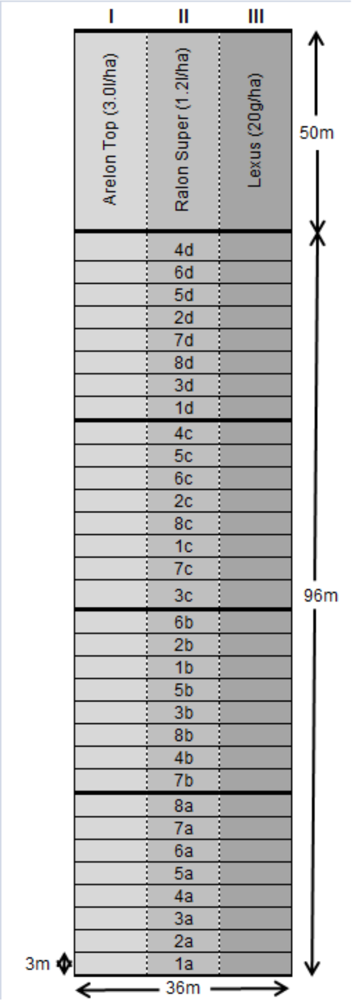

Fig. 1 Experimental set-up in the experimental years 2011-2015.

***Abb. 1** Versuchsdesign in den Versuchsjahren 2011–2015.*

The aim of the altered experimental design was to use the selective effect of the three IHT (Tab. 1) and to test the effect of the eight WCS on the pre-selected populations of *A. myosuroides*. Nine different herbicides were applied as part of the herbicide strategies (Tab. 2).

The eight tested WCS (Tab. 3) included a control treatment with no herbicide application (WCS 1), two strategies using only active ingredients from HRAC group A or B post-emergent (WCS 3 and 4, respectively), one strategy using both HRAC group A or B actives post-emergent (WCS 3) and several different strategies consisting of varying pre-emergence and a post-emergence applications (WCS 2 and 6-8). Herbicides were applied using an experimental field sprayer (Schachtner) with a width of 3 m calibrated to deliver a volume of 300 L/ha

Tab. 2 Herbicides with concentrations of active ingredients and HRAC groups as used as part of the eight weed control strategies (WCS) in the experimental years 2011-2015.

Tab. 2 *Verwendete Herbizide mit Konzentrationsangaben der Wirkstoffe und der Wirkstoffgruppen in den acht unterschiedlichen Unkrautbekämpfungsstrategien (WCS) in den Versuchsjahren 2011-2015.*

Herbicide trade name	Active ingredient	HRAC group
Atlantis WG	Mesosulfuron-methyl (30 g kg^{-1})	B
	Iodosulfuron-methyl-natrium (6 g kg^{-1})	B
	Mefenpyr (90 g kg^{-1})	
Axial 50	Pinoxaden (50 g L^{-1})	A
Bacara Forte	Flufenacet (120 g L^{-1})	K3
	Flurtamone (120 g L^{-1})	F1
	Diflufenican (120 g L^{-1})	F1
Boxer	Prosulfocarb (800 g L^{-1})	N
Cadou SC	Flufenacet (500 g L^{-1})	K3
Herold SC	Flufenacet (400 g L^{-1})	K3
	Diflufenican (200 g L^{-1})	F1
Ralon Super	Fenoxaprop-P-ethyl (69 g L^{-1})	A
Lexus	Flupyrsulfuron-methyl (500 g L^{-1})	B
Traxos	Pinoxaden (25 g L^{-1})	A
	Clodinafop-propargyl (25 g L^{-1})	A

Tab. 3 Weed control strategies (WCS) in the experimental years 2009-2015.

Tab. 3 *Unkrautbekämpfungsstrategien (WCS) in den Versuchsjahren 2009-2015.*

WCS	Autumn		Spring
	Pre-emergent	Post-emergent	Post-emergent
1	-	-	-
2	Cadou SC (0.3 L ha^{-1}) + Bacara Forte (0.75 L ha^{-1})	Traxos (1.2 L ha^{-1})	-
3	-	Lexus (20 g ha^{-1}) + FHS	Atlantis WG (500 g ha^{-1}) + FHS (1.0 L ha^{-1})
4	-	Ralon Super (1.2 L ha^{-1})	Axial 50 (1.2 L ha^{-1})
5	-	Lexus (20 g ha^{-1}) + FHS	Axial 50 (1.2 L ha^{-1})
6	Boxer (2.5 L ha^{-1}) + Herold SC (0.6 L ha^{-1})	Axial 50 (0.9 L ha^{-1})	Axial 50 (1.2 L ha^{-1})
7	Fenikan (2.5 L ha^{-1})	-	Traxos (1.2 L ha^{-1})
8	Cadou SC (0.3 L ha^{-1}) + Bacara Forte (0.75 L ha^{-1})	Traxos (1.2 L ha^{-1})	Traxos (1.2 L ha^{-1})

Bioassays

In order to monitor any change in sensitivity in *A. myosuroides* as a result of the three IHT treatments (IHT I-III: Isoproturon, fenoxaprop-P-ethyl and flupyrsulfuron-methyl, Tab. 1), bioassays with seed samples from the three plots were conducted in different years. In 2010, 2011 and 2015, seed samples from all three plots were taken whereas in 2013 only samples from plots treated with Arelon Top (IHT plot I) and Ralon Super (IHT plot II) were analyzed. *A. myosuroides* seed samples were taken in July when the seeds were fully ripe. Seeds were germinated in petri dishes and transplanted at BBCH 10 into pots containing standardized soil with five plants per pot and four replicates per treatment. At BBCH 12, plants were treated with different herbicides and efficacy was assessed 21 days after treatment. For the seed samples taken in 2010, 2011 and 2015, the herbicide actives Arelon Top, Ralon Super, Lexus and Focus Ultra were tested whereas for the samples taken in 2013, only Arelon Top and Ralon Super were used. Herbicides were applied at the registered dose rates (Tab. 4).

Tab. 4 Herbicides used in the bioassays with content of respective active ingredients and applied dose rates.

Tab. 4 *In den Biotesten verwendete Herbizide mit Konzentrationsangaben der Wirkstoffe und der verwendeten Aufwandmengen.*

Herbicide	Active ingredient	Herbicide dose rate
Arelon Top	Isoproturon (500 g L^{-1})	3.0 l ha^{-1}
Ralon Super	Fenoxaprop-P-ethyl (69 g L^{-1})	1.2 l ha^{-1}
Lexus	Flupyrsulfuron-methyl (500 g kg^{-1})	20 g ha^{-1}
Focus Ultra	Cycloxydim (100 g L^{-1})	2.5 l ha^{-1}

A. myosuroides assessment

Starting in 2011, the occurrence of *A. myosuroides* was assessed in the plots of the eight herbicide strategies only. Plant number of *A. myosuroides* in all plots was assessed once in autumn after the post-emergent herbicide treatment and twice in spring before and after the spring herbicide treatment. Shortly before harvest, the number of *A. myosuroides* heads was additionally counted. Plant and head numbers were counted in quadrats of 0.1 m^{-2} and three quadrats were assessed in each plot. For this analysis, only the number of *A. myosuroides* heads will be analysed.

Statistical analysis

Linear mixed-effects models were fitted in R (R DEVELOPMENT CORE TEAM, 2007) which, according to the split-plot design, incorporated the following error structure (number of levels indicated in parentheses): Block (4) / WCS (8) / IHT (3). As the study was characterised by a balanced and orthogonal design, maximum likelihood was used within the linear mixed effects models. Block was included as a random block factor to account for environmental heterogeneity on the study site. The response variables tested was *A. myosuroides* head number (m^{-2}) at the assessment date before harvest. The appropriateness of the model was checked by plotting standardised residuals against fitted values. Statistically significant effects derived from the model with best fit were further investigated using ANOVA, following the error structure of the linear mixed-effects models and Tukey HSD post hoc tests ($P < 0.05$) on data averaged over the four experimental blocks. Results (significances) of the Tukey HSD post hoc tests are not shown in Table 6-8 due to the high number of factor levels.

Molecular genetic analysis of target-site resistance

In 2014, leave samples for target-site mutation analysis were taken from IHT plots II (Ralon Super) and III (Lexus). Since no survivors were found after application of Arelon Top in the bioassay, no molecular analysis was performed for this treatment. In the following year, bioassay using seed samples from IHT plots II and III were conducted as described above. Survivors from these bioassays that survived 200% herbicide dose were analyzed for potential target-site alterations. For DNA extraction, 0.5 cm of green leaf material was ruptured in a Retch Mill at 30 Hz for 1 min in DNA extraction buffer (100 mM Tris-HCL (pH 9.5), 1 MKCl, 10 mM EDTA). Subsequently, cellular debris was removed by centrifugation and the DNA in the supernatant was precipitated using 100% ethanol. For the leaf samples taken from IHT plot II in the field, target-site mutation analysis for the codon coding for Ile1781 of the ACCase protein was carried out as described by DÉLYE et al. (2002). Since the codon for Ile1781 was not shown to be altered in the leaf samples taken in 2014, this potential mutation site was not analyzed in 2015. Potential mutations in the codons coding for Trp2027, Ile2041, Asp2078 and Gly2096 were determined performing pyrosequencing (Tab. 9). The dCAPS procedure to determine mutations in the codons coding for Pro197 and Trp574 in the ALS protein was performed according to DÉLYE et al. (2008).

Results

Bioassays

The sensitivity analysis of seed samples taken from the three IHT plots showed varying level of resistance toward the tested herbicides (Fig. 2). The efficacy of Arelon Top (isoproturon) was high (> 98%) on all samples tested (Fig. 2 a)-c)). In contrast, the efficacy of Lexus (flupyrsulfuron) was lower (< 90%) especially on samples taken from IHT plot III continuously treated with Lexus (flupyrsulfuron; Fig. 2c)). In addition, the efficacy of Lexus decreased over time especially for samples from IHT plot III where only 6% control was observed for samples taken in 2015. Regarding Ralon Super (fenoxaprop-P), the efficacy was low (0 - 41%) on all samples tested (Fig. 2 a)-c)). No impact of the sampling year was evident but virtually no control by Ralon Super was observed for the seed sample taken from the IHT plot II (Ralon Super) in 2015 (Fig. 2b)) whereas the control was higher for samples from IHT plots I and III (Fig. 2 a) and 2 c)).

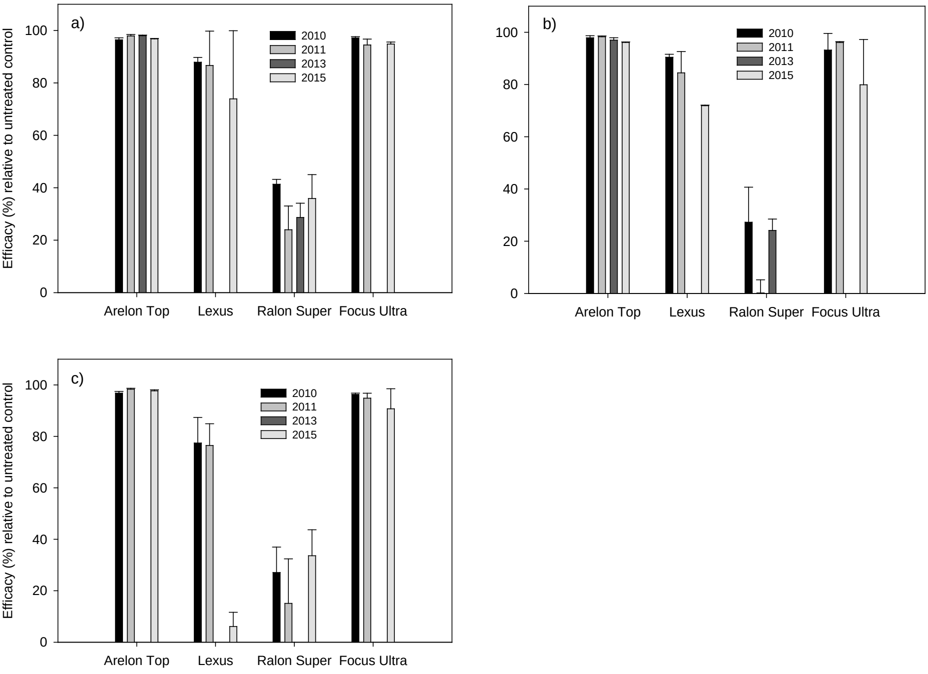

Fig. 2 Results of the bioassay for seed samples derived from IHT plot I (Arelon Top, a), II (Ralon Super, b) and III (Lexus, c).

Abb. 2 *Ergebnisse der Biotests mit Samen, die aus den IHT-Parzellen I (Arelon Top, a), II (Ralon Super, b) und III (Lexus, c) stammen.*

A. myosuroides head number

The number of *A. myosuroides* heads was significantly influenced by WCS in all three experimental years (Tab. 5). The impact of the three initial herbicide treatments (IHT) was only significant in 2014 and 2015. A significant interaction between the two factors was observed in the experimental years 2013 and 2014.

Tab. 5 Effects of WCS and IHT on *A. myosuroides* head number in winter wheat (linear mixed-effects models).

Tab. 5 *Einfluss von WCS und IHT auf die Ährenzahl von A. myosuroides in Winterweizen (lineare gemischte Modelle).*

		2013		**2014**		**2015**	
	d.f.	**MS**	**F**	**MS**	**F**	**MS**	**F**
WCS	7	225529	6.90***	634181	29.28***	405264	10.89***
IHT	2	1970	0.59	21770	6.32**	110545	11.00***
WCS x IHT	14	11498	3.42***	9480	2.75**	16037	1.60
Residuals	28	161515		3446		10054	

WCS, Weed control strategy (no 1-8; 2011-2015); IHT: Initial herbicide treatment (plot I-II, 2009-2011). Significance levels are *P < 0.05; **P < 0.01; ***P < 0.001; NS, not significant.

In 2013, the impact of IHT was not significant (Tab. 6). Therefore, no significant differences in *A. myosuroides* head number were observed between the three IHT levels. High numbers of *A. myosuroides* heads were observed under WCS 4 (Ralon Super in autumn followed by Axial 50 in spring; 435-489 heads/m^2). Number of *A. myosuroides* heads was lowest in response to WCS 3 (Lexus in autumn followed by Atlantis WG in spring, 7-12 heads/m^2) across all IHT level.

Tab. 6 Mean number (mean) and corresponding standard error (SE) of *A. myosuroides* head number in 2013.

Tab. 6 *Durchschnittliche Anzahl (mean) und Standardfehler (SE) der A. myosuroides-Ährenzahl in 2013.*

	Arelon Top (IHT I)		**Ralon Super (IHT II)**		**Lexus (IHT III)**	
WCS	**mean**	**SE**	**mean**	**SE**	**mean**	**SE**
1	322	55.22	473	48.56	270	51.14
2	393	61.95	289	16.35	338	32.04
3	7	2.36	12	7.43	10	8.86
4	477	115.85	435	85.14	489	145.03
5	230	19.34	245	26.44	327	46.51
6	373	52.26	334	39.75	357	72.77
7	379	39.07	383	45.73	453	42.52
8	378	40.52	309	43.83	359	54.15

WCS, Weed control strategy (no 1-8; 2011-2015); IHT: Initial herbicide treatment (plot I-II, 2009-2011).

From 2013 to 2014, a strong increase in *A. myosuroides* head number was observed in the control treatment (WCS 1; Tab. 6 and 7). In 2014 and 2015, the impact of the IHT on *A. myosuroides* head number was significant (Tab. 7 and 8) and differences in the effects of the eight WCS were observed between the three IHT levels. Very high numbers of *A. myosuroides* heads were again observed in the control treatment (WCS 1) with no significant differences between the three IHT in 2014 (751-795 heads/m^2) and in 2015 (743-821 heads/m^2). In 2014, low numbers of *A. myosuroides* heads were counted under WCS 2 (Cadou SC + Bacara Forte in autumn followed by Traxos in spring) with no significant differences between the three IHT in 2014 (23-37 heads/m^2). In contrast, *A. myosuroides* head number was higher under 2 in 2015 (293-556 heads/m^2) with considerable higher numbers under IHT II and IHT III compared to IHT I (but no statistical difference found; Tab. 8).

Tab. 7 Mean number (mean) and corresponding standard error (SE) of *A. myosuroides* head number in 2014.

***Tab. 7** Durchschnittliche Anzahl (mean) und Standardfehler (SE) der A. myosuroides-Ährenzahl in 2014.*

	Arelon Top (IHT I)		**Ralon Super (IHT II)**		**Lexus (IHT III)**	
WCS	**Mean**	**SE**	**Mean**	**SE**	**Mean**	**SE**
1	765	33.39	751	53.47	795	50.99
2	23	10.83	30	6.56	37	10.44
3	155	69.75	77	16.04	75	17.17
4	440	56.27	512	65.23	439	57.59
5	210	52.05	217	25.86	220	22.21
6	359	87.04	376	59.00	328	62.74
7	400	33.57	450	35.35	284	48.73
8	303	56.86	459	59.10	274	41.59

WCS, Weed control strategy (no 1-8; 2011-2015); IHT: Initial herbicide treatment (plot I-II, 2009-2011).

Differences between the IHT were observed in 2014 for WCS 3 (Lexus in autumn followed by Atlantis WG in spring) with *A. myosuroides* head number being about two times higher under the Arelon Top IHT I (155 heads/m^2) compared to the two other IHT (77 and 75 heads/m^2). However, this difference was not statistically significant due to the high variation in *A. myosuroides* head number under IHT I (SE = 69.75). In 2015, this difference was less pronounced and not statistically significant.

Tab. 8 Mean number (mean) and corresponding standard error (SE) of *A. myosuroides* head number in 2015.

***Tab. 8** Durchschnittliche Anzahl (mean) und Standardfehler (SE) der A. myosuroides-Ährenzahl in 2015.*

	Arelon Top (IHT I)		**Ralon Super (IHT II)**		**Lexus (IHT III)**	
WCS	**Mean**	**SE**	**Mean**	**WCS**	**Mean**	**SE**
1	775	18.48	821	34.26	743	19.51
2	293	38.57	531	78.66	556	58.26
3	476	43.24	434	51.94	412	69.46
4	724	99.86	860	139.58	777	122.13
5	267	77.25	378	61.65	338	51.62
6	318	77.57	391	112.33	403	36.12
7	632	95.39	793	54.00	622	106.14
8	325	59.96	539	79.65	497	56.37

WCS, Weed control strategy (no 1-8; 2011-2015); IHT: Initial herbicide treatment (plot I-II, 2009-2011).

Analysis of target-site resistance

Molecular genetic analysis did not reveal a significant contribution of target-site mutations to ACCase inhibitor resistance. Among the survivors of the bioassay, only two plants were found to be heterozygous coding a Asp2078Gly substitution (Tab. 9). In contrast, ALS inhibitor resistance could be attributed to alterations in the codons coding for Pro197 and Trp574, respectively. All analyzed plants were carrying mutations in one or both codons known to confer herbicide resistance.

Tab. 9 *Target*-site mutations determined in the years 2014 and 2015, given in % of the number of analyzed plants.

Tab. 9 *In den Jahren 2014 und 2015 bestimmte Wirkortmutationen, angegeben in % der Anzahl der analysierten Pflanzen.*

		ACCase					ALS	
Year	**IHT**	**Ile1781**	**Trp2027**	**Ile2041**	**Asp2078**	**Gly2096**	**Pro 197**	**Trp574**
2014	II	0	not analyzed					
	III						100	77.7
2015	II	not analyzed	0	0	10,5	0		
	III						18.75	87.5

Discussion

The results show that none of the eight different weed control strategies (WCS) was able to reduce the density of *A. myosuroides* over the three tested experimental years. In contrast, the efficacy ranking for the eight WCS varies between the years. In 2013, head number was lowest in response to WCS 3 (Lexus in autumn followed by Atlantis WG in spring). In 2014, WCS 2 (Cadou SC + Bacara Forte in autumn followed by Traxos in spring) achieved the highest control efficacy whereas in 2015, the highest control efficacy was observed for WCS 5 consisting of an application of Lexus in autumn followed by Axial in spring. These results are in contrast to other studies which have shown that sequence application of pre-emergence application with active ingredients from less resistance-prone HRAC groups such as K1, K3 and F1 followed by an application of post-emergence actives may provide the highest control efficacy (MOSS et al., 2007; GEHRING et al., 2012; GEHRING and THYSSEN, 2014).

The lowest control efficacy was observed for WCS 4 (Ralon Super in autumn followed by Axial 50 in spring) in all three experimental years. This was most likely due to the high level of resistance to ACCase inhibitors that was present already before the start of the field experiment. Corresponding to that, a low efficacy of Ralon Super was also observed in all conducted bioassays. Analysis of possible resistance mechanism showed, that resistance to ACCase inhibitors was not caused by any of the four tested mutations on the ACCase gene. Therefore, non-target-site resistance mechanisms are a possible cause for the low efficacy of ACCase inhibitors. This conclusion is supported by the high efficacy of Focus Ultra (cycloxydim) in the conducted bioassays. As cycloxydim is not or less metabolised by plants with enhanced metabolism, it can be used as an indicator of non-target-site resistance.

Results of the study show that control of *A. myosuroides* by means of herbicides only is not sufficient on field sites with present herbicide resistance. Therefore, non-chemical measures such as diversified crop rotation, delayed drilling and inversion tillage have to be used in order to reduce the overall infestation of *A. myosuroides* (LUTMAN et al., 2013).

The conducted study presents a long-term field study that was conducted under continuous winter wheat. In situation with resistance level such as those observed in the study, other crop species such as oilseed rape can be grown in which other active ingredients such as propyzamide with a high efficacy against *A. myosuroides* can be applied. In addition, non-selective actives (glyphosate) might be used to reduce high densities of *A. myosuroides*.

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