Linkage of the current ALS-resistance status with field history information of multiple fields infested with blackgrass (*Alopecurus myosuroides* Huds.) in southern Germany

*Zusammenhang des ALS-Resistenzstatus bei Ackerfuchsschwanz (*Alopecurus myosuroides Huds.*) mit den durchgeführten Bewirtschaftungsmaßnahmen auf mehreren Felder in Süddeutschland*

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Abstract

The repetitive use of herbicides as major tool to control troublesome weeds in agriculture caused an increase in resistant weeds lately, especially when Integrated Weed Management principles were ignored. In a case study approach we sampled blackgrass (*Alopecurus myosuroides* Huds.) in three distinctive locations for at least 3 years. Based on field infestation level, greenhouse biotests and laboratory analyses we grouped 23 fields as resistant (R), 28 fields as less sensitive (I) and 39 fields as sensitive (S) with regard to their ALS resistance status. Field history information was collected for 90 fields. Variables regarding the frequency of 1) summer crops, 2) winter cereals, 3) ploughing, 4) herbicide use, and 5) early versus late seeding were calculated. Fields with a higher frequency of summer crops, ploughing and later sowing dates in the crop rotation were less frequently grouped into R and I. No relationship was found between the number of modes of action used and the resistance status. Intensity of ALS-inhibitor use and use of grass herbicides played a role to distinguish resistant from sensitive fields. Our results suggest that cultural measures to bring the blackgrass population size to lower levels are more important than the selection by the herbicide.

Keywords: ACCase inhibitors, ALS inhibitors, blackgrass, Germany, Herbicide Resistance, Integrated Weed Management

Zusammenfassung

Der übermäßige Einsatz von Herbiziden als einzige Maßnahme zur Reduzierung von Problemunkräutern hat in den letzten Jahren zu einer Zunahme resistenter Unkrautpopulationen geführt. Im Rahmen einer Fallstudie wurde daher Ackerfuchsschwanzsamen (*Alopecurus myosuroides* Huds.) an 3 Standorten über einen Zeitraum von mindestens 3 Jahren beprobt. Basierend auf einer Befallseinschätzung im Feld, Gewächshaustests und Laboranalysen wurden 23 Felder als resistent (R), 28 Felder als vermindert sensitiv (I) und 39 Felder als sensitive Felder (S) gegenüber ALS-Inhibitoren klassifiziert. In Interviews mit den Landwirten konnten die Bewirtschaftungsmaßnahmen für diese 90 Felder erfragt werden. Aus diesen wurden Variablen die den Anteil an 1) der Sommerungen in der Fruchtfolge, 2) des Wintergetreides in der Fruchtfolge, 3) den Pflugeinsatz, 4) den Herbizideinsatz sowie 5) die Saatzeitpunktwahl repräsentieren, generiert. Mit einer Zunahme an Sommerungen in der Fruchtfolge, einem erhöhtem Pflugeinsatz sowie der häufigeren Wahl später Saattermine war eine geringere Häufigkeit an resistenten Feldern zu beobachten. Dagegen wurde kein Zusammenhang zwischen der Anzahl der eingesetzten Wirkstoffklassen und dem Resistenzstatus gefunden. Signifikante Unterschiede beim Einsatz von ALS-Inhibitoren waren nur zwischen S und R erkennbar. Unsere Ergebnisse zeigen dass ackerbauliche Maßnahmen eine stärkere Wirkung auf die Resistenzentwicklung haben als die Häufigkeit und Art des Herbizid-Einsatzes.

Stichwörter: ACCase-Inhibitoren, Ackerfuchsschwanz, ALS-Inhibitoren, Deutschland, Herbizidresistenz, Integrierte Unkrautbekämpfung

Introduction

Weed control to ensure crop yields is essential and herbicides are the primary option used for that purpose. However, due to the overreliance on single Modes of Actions (MoA) and a decrease of additional Integrated Weed Management (IWM) measures, herbicide resistance increased and threatens crop production not only in Europe (HEAP, 2013; POWLES and YU, 2010). Many herbicides against grass weeds currently used in Europe are targeting either the acetyl-coenzyme A carboxylase (ACCase) or the acetolactate synthase (ALS). Focusing on blackgrass (*Alopecurus* *myosuroides* Huds.), an annual weed species, the use pattern of ACCase inhibitors in the 80s and 90s has led to a significant development of resistance to these graminicides in western Europe (DÉLYE et al., 2010; MOSS et al., 2007). Consequently, there is the potential risk that resistance to the ALS- inhibitor family will increase in acreage as well. However, there is a lack of studies focusing on the evolutionary dynamics of resistance at the field level that aim on identifying and quantifying the driving factors behind (NEVE et al., 2014). Investigations into the spatial and temporal development of resistance showed that many individual resistance events occur simultaneously in different fields and that spread of resistance by seeds or pollen plays only a minor role (MENCHARI et al., 2007; BAUCOM and MAURICIO, 2007; HERRMANN et al., 2014). Instead, each resistant population has its own distinct resistance profile composed of different ratios of target-site mutations (HERRMANN et al., 2014).

The aim of this case study approach is to analyze and quantify the variability in resistance patterns among fields within a small scale on nearby fields. Furthermore underlying management and also possible ecological factors shall be identified (HERRMANN et al., 2014). Fields in southern Germany were therefore sampled in consecutive years. Infestation level was assessed and greenhouse bioassays conducted with the seeds of field survivors. To better determine the exact cause of resistance SNP-Analyses were carried out with plants surviving herbicide treatment in the greenhouse. Our hypothesis was that the variation in the resistance level was mainly attributed to a lack of diversity in management, an overreliance on single modes of action and herbicide applications as suggested elsewhere (LUTMAN et al., 2013; POWLES and YU, 2010).

Materials and Methods

Data collection

Seeds of *A. myosuroides* were sampled form fields with different infestation levels in 3 to 5 consecutive years and greenhouse whole plant bioassays and laboratory test were carried out with them. The detailed procedure was reported elsewhere (HERRMANN et al., 2014; HESS et al., 2012). Intensive Interviews with the farmers were carried out to obtain information of the field management for the past 10 years. A complete information set was obtained for 90 fields that are used herein.

Data preparation

To analyze the data in a quantitative form, several indices were calculated (Tab. 1). Based on the observations in HERRMANN et al. (2014) a classification of the resistance status was derived and fields thereafter grouped into resistant (R), less sensitive (I) and sensitive (S) fields. Preliminary analysis showed that fields that were grouped in R had changes in their management (crop rotation, tillage, herbicide use) to counteract the resistance while this was not observed in the other two groups (data not shown). This led to the conclusion that field management data prior to resistance occurrence need to be analyzed for that group. We therefore focused our analysis of the field management on the six years 2005-2010 for the R samples, and 2007-2013 for the I and S samples. Data from 2012 was excluded since it was not considered representative due to a strong winter with crop failure and resulting summer crop seeding.

Statistical analysis

Statistical analysis was carried out with R 3.1.2 (R Core Team, 2015). Frequency data was analyzed using Fishers Exact G-Test with a significance level of $\alpha=0.05$. Cluster analysis was carried out using the function “hclust” with euclidean distance and the “ward.D” method. The classification tree was created with the package “partly” using the “ctree” function (HOTHORN et al., 2006).

Tab. 1 Abbreviations and description of variables created to analyze field management data.

Tab. 1 *Abkürzungen und Beschreibungen der analysierten Bewirtschaftungsvariablen.*

Variable	Explanation
WCereals	The portion of winter cereals in the crop rotation
SCrops	The portion of summer crops in the crop rotation
NCrops	Number of different crops used (winter wheat, triticale and spelt were counted as one) divided by the number of years observed (6 years)
Ploughing	The portion of ploughing in the crop rotation
SeedingDate	The portion of delayed seeding events in the crop rotation. Early, average and late seeding dates for the various crops were determined together with a local crop advisor
UniqueMoA	The number of different Modes of Action used against blackgrass divided by the number of years observed (6 years)
ALOMYHerb	The number of herbicide applications against blackgrass divided by the number of years observed (6 years)
ALOMYGrpB	The number of ALS-Inhibitor applications (HRAC Group B) divided by the number of years observed (6 years)

Results

Variable distribution

Non-Chemical Measures

The portion of WCereals, SCrops, NCrops, Ploughing and SeedingDate were regarded as non-chemical measures (Tab. 2). Winter cereal cropping (WCereals) ranged from 33% to 83% in the crop rotation with a significantly higher portion in fields being classified as R or I compared to the sensitive fields. No difference was observed between resistant and intermediate fields. The portion of summer crops (SCrops) was significantly different between all three groups with the highest portion (64%) occurring in the sensitive fields. No summer crop was grown in most of the resistant fields (87%). The number of crops (NCrops) was significantly different between sensitive and resistant/intermediate fields. The number of different crops grown ranged from two to five. The three fields with only 2 crops had an oilseed rape -winter wheat -winter triticale crop rotation for the resistant field and a maize - winter wheat crop rotation for the two sensitive fields. Note again that winter wheat and triticale were counted as one crop as both were winter sown and treated with an ALS-inhibitor. Crop rotations comprising of five crops had winter wheat, winter barley, spring barley, oilseed rape, sugar beet and oats in the rotation (S). The resistant field with 5 crops was cropped with oilseed rape, winter wheat, oats, winter barley and spring barley. Ploughing ranged from 0/6 (no ploughing) to 6/6 (ploughing every year). A significant difference was only found for the comparison of S/R while the other two comparisons were not significantly different from each other. Seeding dates were found to be significantly different between S/R and I/R but not between S/I. This shows that earlier seeding is especially done in the resistant fields.

Tab. 2 Distribution of frequency of non-chemical management data with total sums of observations (Sum) and the corresponding ALS resistance status (AS). "Pair" indicates the corresponding variable Groupings of AS for Fishers Exact G-Test together with the corresponding p-values.

Tab. 2 *Verteilung der Häufigkeiten der nicht-chemischen Maßnahmen und die Summe der Beobachtungen (Sum) für die verschiedenen ALS-Resistenzausprägungen (AS). „Pair" gibt die verschiedenen Vergleiche der ALS-Resistenzsituation mit dem dazugehörigen p-Wert (p-Value) an.*

Variable	AS	0/6	1/6	2/6	3/6	4/6	5/6	6/6	>6/6	Sum	Pair	p-Value
	S			5	9	21	4			39	S/I	0.0302
WCereals	I			1	2	15	10			28	S/R	0.0001
	R				1	8	14			23	I/R	0.2843
	Total			6	12	44	28			90		
	S	13	16	9	1					39	S/I	0.0457
SCrops	I	16	11	1						28	S/R	0.0002
	R	20	3							23	I/R	0.0421
	Total	49	30	10	1					90		
	S			2	13	21	3			39	S/I	0.0483
NCrops	I				18	8	2			28	S/R	0.0000
	R			1	20	1	1			23	I/R	0.0435
	Total			3	51	30	6			90		
	S	4	4	7	6	12	5	1		39	S/I	0.2489
Ploughing	I	4	5	10	4	3	1	1		28	S/R	0.0146
	R	9	3	7	1	1	1	1		23	I/R	0.5039
	Total	17	12	24	11	16	7	3		90		
	S	16	14	2	4	3				39	S/I	0.4313
SeedingDate	I	12	14	1		1				28	S/R	0.0154
	R	19	3	1						23	I/R	0.0067
	Total	47	31	4	4	4				90		

Chemical measures

Frequency of herbicide applications against grasses (ALOMYHerb) and ALS inhibitors applications against grasses (ALOMYGrpB) together with the number of Modes of Action (UniqueMoA) were regarded as chemical measures against blackgrass. Each field received herbicide treatments as no field was grouped into 0/6 for ALOMYHerb. Significant differences between resistant and sensitive fields were observed for ALOMYHerb and ALOMYGrpB but not for UniqueMoA (Tab. 3). The comparison between sensitive and intermediate fields was only barely significant for ALOMYHerb ($p=0.04$) while it was not significant for UniqueMoA and ALOMYGrpB at $p=0.05$. Significant differences between intermediate and resistant fields where not observed for the three variables (Tab. 3).

Variable correlation

As management factors are typically not independent from each other the correlation between the variables was assessed (Tab. 4). As it can be seen in Table 4 a higher diversity of Crops (NCrops) is negatively correlated with ALS-inhibitor intensity ($r=-0.34$, $N=90$, $p=0.012$). Furthermore the portion of winter cereals and ploughing were negatively correlated ($r=-0.37$, $N=90$, $p=0.0003$). There was also a positive correlation found between the portion of winter cereals and the usage of

ALS inhibitors (p=0.33, N=90, p=0.0017). The overall herbicide intensity seemed to decrease with an increase in summer crops (r=-0.34, N=90, p=0.009). Furthermore we found the number of MoA used to decrease with an increase in summer crops (r=-0.29, N=90, p=0.006). There was also a positive correlation between delayed seeding and increase in summer crops (r=0.49, N=90, p<0.001). With an increase in herbicide intensity there was generally also an increase in ALS-inhibitors observed.

Tab. 3 Distribution of chemical management data with total sums of observations and the corresponding ALS Resistance Status (AS). "Pair" indicates the corresponding variable Groupings for Fishers Exact G-Test are displayed together with the corresponding p-values.

Tab. 3 *Verteilung der Häufigkeiten der chemischen Maßnahmen und die Summe der Beobachtungen (Sum) für die verschiedenen ALS-Resistenzausprägungen (AS). „Pair" gibt die verschiedenen Vergleiche der ALS-Resistenzsituation mit dem dazugehörigen p-Wert (p-Value) an.*

Variable	AS	0/6	<2/6	<4/6	<6/6	<8/6	<10/6	<12/6	>12/6	Sum	Pair	p-value
ALOMYHerb	S			4	9	21	4	1		39	S/I	0.0407
	I				7	10	10	1		28	S/R	0.0266
	R				6	7	5	5		23	I/R	0.2341
	Total			4	22	38	19	7		90		
UniqueMoa	S		4	26	9					39	S/I	0.0726
	I			18	7	3				28	S/R	0.5957
	R		4	13	6					23	I/R	0.0594
	Total		8	57	22	3				90		
ALOMYGrpB	S		23	14	2					39	S/I	0.0935
	I		9	16	3					28	S/R	0.0025
	R		4	13	4	2				23	I/R	0.2768
	Total		36	43	9	2				90		

Tab. 4 Correlation Matrix of the different management factors. Values indicate pearsons correlation coefficient. Significance levels are indicated with p<0.001(***), p<0.01(**) and p<0.05(*).

Tab. 4 *Korrelationsmatrix der verschiedenen Management-Faktoren. Die Werte stellen Pearsons-Korrelations-koeffizienten dar. Signifikanzen sind mit p<0.001(***), p<0.01(**) und p<0.05(*) angegeben.*

	WCereals	SCrops	NCrops	Ploughing	ALOMYHerb	ALOMYGrpB	UniqueMoA
WCereals							
SCrops	-0.46***						
NCrops	-0.61***	0.40***					
Ploughing	-0.37***	0.10	0.23*				
ALOMYHerb	-0.01	-0.34***	0.01	-0.07			
ALOMYGrpB	0.33**	-0.12	-0.34**	-0.23*	0.37***		
UniqueMoA	0.06	-0.29**	0.07	0.04	0.53***	0.01	
SeedingDate	-0.15	0.49***	-0.16	0.12	-0.35***	-0.05	-0.31**

Clustering of groups and feature identification

To analyze the hypothesis that equal management leads to an equal resistance status all fields were grouped based on the eight management variables assessed using cluster analysis. Three distinctive groups (clusters) that show similar values for the variables assessed were extracted from the dataset (Tab. 5). All clusters were of almost equal size. Cluster 1 contains 73% sensitive fields and 10% resistant fields, while cluster 3 only contains 10% sensitive fields but 90% of fields were resistance was detectable (I+R). Cluster 2 contains 48% sensitive fields (Tab. 5) indicating that at least some resistance was detectable in 52% of the fields in this cluster.

Recursive partitioning was done to separate these three clusters and identify the underlying management factors that characterize every group (Fig. 1). It was found that cluster 3 contains fields that are mostly characterized by a high herbicide use with 50% or more of winter cereals in the crop rotation while only 10% of these samples had less than 50% winter cereals in the rotation (Node 8 and 9, Fig. 1). Among the fields in cluster 1 63% showed lower intensity of herbicide use compared to group 3 with higher intensities of ploughing compared to the majority of fields of group 2 (Node 5 and 6, Fig. 1). Group 2 differed from group 3 in a lower herbicide intensity. 59% showed also lower portions of ploughing. However, there was a smaller portion in this group (28%) that was similar to group 1 but showed lower levels of herbicide intensity (Node 5, Fig. 1). Group 2 seems therefore to be a group that is not clearly separable from group 1 which contains mostly sensitive fields and indicates that this type of management does not fully explain the resistance status as we see it at the moment. Management by itself is therefore only one of the aspects resulting in the distribution of fields with different ALS resistance statuses within this group. Other factors not considered within this analysis (quality of the management factors, ecological field factors) might therefore also be of importance.

Tab. 5 Cluster Solution of field management variables and frequency of every ALS Resistance Status (AS) found within each group.

Tab. 5 *Cluster-Gruppierungen der Variablen zur Bewirtschaftung und Häufigkeit des Auftretens nach Status der ALS Resistenz (AS).*

AS	Group 1	Group 2	Group 3	Sum
S	22	14	3	39
I	5	9	14	28
R	3	6	14	23
Total	30	29	31	

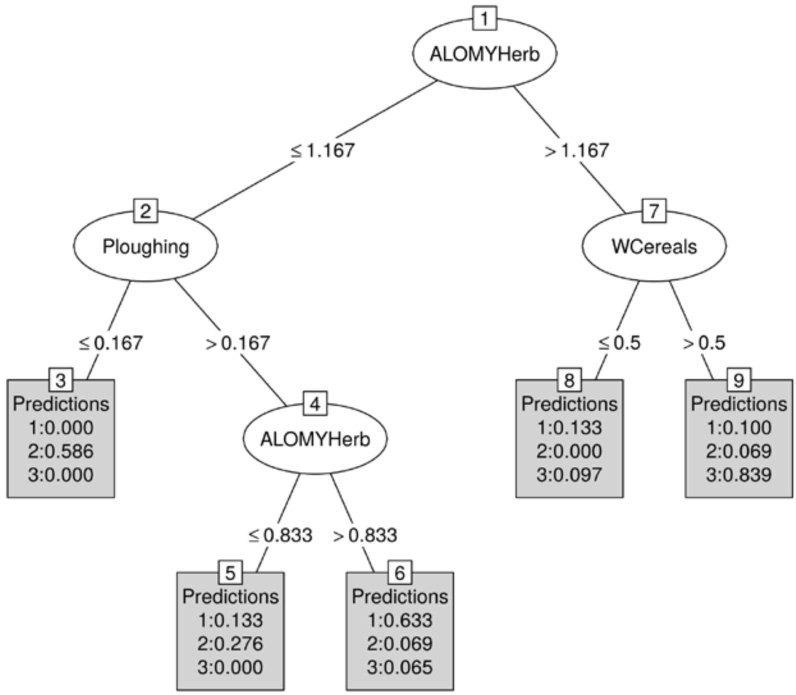

Fig. 1 Separation of the three clusters (groups) identified in Table 5 by management factors. Relative distribution of the three clusters (groups) is indicated by the numbers 1 to 3 in the terminal nodes (grey box).

Abb. 1 *Verteilung der drei Gruppen aus Tabelle 5 nach den Hauptmanagementfaktoren. Die Anteilige Verteilung der Gruppen ist durch die Nummern 1 bis 3 dargestellt (graue Box).*

Discussion

Non-chemical measures in combination with herbicides (Integrated Weed Management) is less frequently used in agriculture in our days because of the uncertainty of its success, its higher time consumption and its often higher costs (LUTMAN et al., 2013). We report here for the first time an analysis of a random field sampling survey that assessed the outcome of different weed management strategies used by farmers under practical conditions over a 6year time frame. As reported by BECKIE (2009) the risk of resistance was highest among rotations comprising four or more cereal crops in a 6 year rotation. Our findings show a similar trend but differences become clearer if the portion of cereals was exceeding four in six years (Tab. 2). Based on fields showing low product efficacy, DÉLYE et al. (2010) found no significant correlation between resistance and the frequency of winter cereals indicating the importance of a true random sampling for assessment of management factors. A lack of diversity in the crop rotation as indicated by a lower number of crops grown and a lower portion of summer crops was more often found in fields being classified as resistant. Similar results were obtained for *Avena fatua*, from a survey by BECKIE et al. (2008). LUTMAN et al. (2013) ranked summer crops as having the strongest effect in reducing the blackgrass population. In our study maize (*Zea mays*) was a very prominent example of a summer crop that due to its biology is harvested later. The correlation between summer crops and late seeding date can be partially explained by the late seeding dates of the subsequent wheat crop following maize (Tab. 4). In addition maize fields were mostly ploughed in autumn which consequently results in several non-chemical measures that are associated with that particular crop. Ploughing by itself, due to its ability to burry (resistant) seeds in deeper soil layers where they cannot germinate, is also reported to reduce the weed seed bank (LUTMAN et al., 2013; BECKIE, 2009).

Our study suggests that diversity in crop rotations is more important to slow down the evolution of resistance than diversity of herbicide use (Tab. 2, 3). This is confirmed by the data of cluster 3 (Fig. 1, Tab. 5) which is characterized by intensive herbicide use together with mostly high portions of winter cereals. Reducing the selection pressure by introducing more non-chemical measures results therefore in a longer availability of this potent tool. JASIENIUK et al. (2008) found that the level of resistance is quite variable and attributed this to the diversity of management systems resulting in differences in herbicide pressure. It is clear from this study that there are management systems that include more than just one tool to fight blackgrass with the success of less resistance cases resulting in these groups (Fig. 1, Tab. 5). However, our study also shows that management practices cannot explain all observations and that there are certain management strategies that work on one field but not the other. The interviews with farmers revealed that most of their troublesome fields were the ones they converted from grassland to arable land the latest due to a historic risk of water logging. It is known that blackgrass prefers high moisture and high clay contents and that on these fields higher population densities are observed. This might mean that in certain locations, higher risks of weed resistance evolution to herbicides are intrinsically present and in these cases IWM measures should be applied with particular care. The analysis of soil maps to further elucidate the contribution of these environmental factors to herbicide resistance evolution is currently in progress. Due to economical constrains, ideal crop rotation might not always be possible. In that case, rotation of mode of action between autumn and spring application is recommended. However, diversifying the toolbox and the use of all measures of integrated weed management is the key to delay resistance in blackgrass and preserve the existing herbicides.

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