Spatial and temporal development of ACCase and ALS resistant Black-grass (*Alopecurus myosuroides* Huds.) populations in neighboring fields in Germany

Räumliche und zeitliche Ausbreitung ACCase und ALS resistenter Ackerfuchsschwanzpopulationen in benachbarten Feldern Deutschlands

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Abstract

The repetitive use of herbicides of a given mode of action as primary tools to control weeds in simplified cropping systems has led to the development of resistant populations.

This study reports preliminary results of the infestation level and the herbicide resistance structure of black-grass (*Alopecurus myosuroides* Huds.) in circa 40 neighboring fields in each of three locations in Southern Germany during 2010-2012. In each location one field with confirmed field resistance served as a starting point to survey the surrounding fields. Field infestation was assessed at the end of the season where seeds were harvested. Based on greenhouse biotests nearly all tested fields showed resistance to ACCase, but the pattern of ACCase mutations and metabolic resistance to fenoxaprop-ethyl showed differences according to space and time. High resistance to ALS was found in several fields in two locations where ALS-resistance was previously confirmed in a single field only leading to severe crop failure. Interestingly, either Pro197 or Trp574 seemed to dominate in these two locations, while almost no ALS target-site mutations were found in the third location. Target-site mutations appear to be the major mechanism for these early cases of ALS-resistance. A few fields also showed non-target site resistant plants to mesosulfuron-methyl. This unique data set provides new insights into the structure and development of 1) an established resistance (ACCase-inhibitors) and 2) a developing resistance (ALS-inhibitors). Multivariate analysis including greenhouse biotests data and target-site analyses corresponding to mutations conferring resistance showed that even spatially closely related fields develop different herbicide resistance patterns. This suggests that weed control measures have a major impact within each field and that resistance can evolve independently. In the current situation black-grass infestations can still be controlled in most of the fields. Integrated Weed Management tools can contribute to keep the selection pressure low and slow down resistance development.

Keywords: ACCase inhibitors, ALS inhibitors, blackgrass, enhanced metabolic resistance, Germany, non-target-site resistance, target-site resistance

Zusammenfassung

Der übermäßige Einsatz von Herbiziden mit gleichen Wirkmechanismen zur primären Unkrautkontrolle in intensiven Fruchtfolgen hat zur Entstehung resistenter Unkrautpopulationen geführt.

Diese Arbeit stellt erste Ergebnisse eines dreijährigen Versuches zum Ackerfuchsschwanz-Befall (*Alopecurus myosuroides* Huds.) und seiner Resistenzstruktur in 3 Regionen Süddeutschlands mit jeweils ca. 40 Feldern vor. Als Ausgangspunkt diente dabei ein Feld mit bekannter Resistenz. Zusätzlich wurden benachbarte Felder beprobt. Die Beprobung und Bestimmung der Befallsintensität wurde jeweils zum Erntezeitpunkt der Kultur vorgenommen. In Gewächshausversuchen konnte in fast allen beprobten Feldern eine ACCase Resistenz in Ackerfuchsschwanz nachgewiesen werden. Das Verhältnis zwischen verschiedenen ACCase-Target-Site Mutationen und metabolischer Resistenz variierte jedoch sowohl in den unterschiedlichen Jahren, sowie zwischen benachbarten Feldern sehr stark. Eine ausgeprägte und agronomisch relevante ALS Resistenz basierend auf den Target-Site Mutationen Pro197 oder Trp574 wurde in einigen Feldern von zwei der drei beprobten Regionen nachgewiesen. In der dritten Region dagegen gab es kaum Pflanzen mit einer ALS Target-Site Resistenz. Target-Site Resistenz scheint daher in diesen frühen Stadien die Hauptursache für ALS-Resistenz zu sein, während einige Proben auch Non Target-Site Resistenz zeigten. Diese Daten geben neue Einblicke in die Struktur und Entwicklung einer 1) etablierten Resistenz (ACCase-Inhibitoren) und 2) einer sich entwickelnden Resistenz (ALS-Inhibitoren). Multivariate Datenanalyse von Gewächshaus- und Target-site-

Analysen der resistenzbedingenden Mutationen zeigen, dass nahe beieinander liegende Felder unterschiedliche Herbizidresistenzstrukturen ausbilden. Dies legt nahe, dass unterschiedliche Unkrautbekämpfungsstrategien, basierend auf unterschiedlichen Fruchtfolgen unterschiedliche Resistenzstrukturen fördern. Zurzeit ist eine erfolgreiche Ackerfuchsschwanz Bekämpfung mit ALS-Inhibitoren in den meisten Fällen noch möglich, benachbarte Felder zeigen aber das alle Möglichkeiten des integrierten Pflanzenschutzes ausgenutzt werden müssen um den Selektionsdruck niedrig zu halten und die weitere Resistenzentwicklung zu verlangsamen.

Stichwörter: ACCase-Inhibitoren, Acker-Fuchsschwanz, ALS-Inhibitoren, Deutschland, metabolische Resistenz, Nicht-Zielortresistenz, Zielortresistenz

Introduction

Weed control is essential to maintain high crop yields and herbicides are primary tools used to control weeds. However the repetitive use of similar modes of action has led to the development of resistant populations (POWLES and YU, 2010) and herbicide resistance is an increasing and significant problem worldwide (HEAP, 2013). Chemical classes targeting acetolactate synthase (ALS) and acetyl-coenzyme A carboxylase (ACCAse) are major herbicides used in European cropping systems in which cereals are key crops (EU, 2013). In the main herbicide resistant monocotyledonous weeds present in cereal fields (*Alopecurus myosuroides*, *Apera spica-venti*, *Bromus* spp., *Lolium* spp.,) mutations of the coding sequences of *ALS* and *ACCase* genes have been extensively characterized as well as increased herbicide metabolism (reviewed by POWLES and YU, 2010). Different approaches to address how herbicides resistance is developing in space and time have been reported (MENCHARI *et al.*, 2006; MARSHALL and MOSS, 2008; PETERSEN *et al.*, 2012). However, instead of following on severe resistance cases or large scale monitoring programs we investigate field relationships in a small regional scale in terms of herbicide resistance development (HESS *et al.*, 2012).

Three locations in proximate distance in Southern Germany were investigated on their occurrence of *A. myosuroides* between 2010 and 2012. Field infestation was assessed at the end of the season when seeds were harvested. In addition to observations of the field infestation level, herbicide resistance was characterized by performing greenhouse biotests. Laboratory tests were carried out on greenhouse survivors to analyze whether the resistance type is either target site or enhanced herbicide metabolism. This unique set of data enabled multivariate analysis using more than 10 variables, including greenhouse biotests and target-site mutations corresponding to mutations conferring resistance, to study the pattern of development of herbicide resistance according to space and time.

Material and Methods

In 2010 three locations in Southern Germany (H, M, and Z) where resistances to either ACCase or ALS inhibiting herbicides were first observed were sampled for *A. myosuroides* seeds from a limited number of fields (ca. 40) surrounding these three original fields. The sampling procedure was extended to more fields from different farmers during 2011 and 2012 in all three locations. *A. myosuroides* samples were harvested by walking every second tractor track and monitoring two borders of the field. It was ensured that only one ear per plant was sampled. Rating of infestation was done as described with the following scale: 0= no ears found; 1 = traces of ears from a few solitary, scattered plants in the field or along field borders; 2 = occasional small patches; 3 = large patches; 4 = widespread throughout the field; and 5 = a dense and serious infestation (Hess *et al.,* 2012). Fields having infestation levels of 4 and 5 with each containing >50ears/m² and more than >200ears/m² respectively were considered to cause yield reductions.

Greenhouse biotests were conducted based on the method described by (MENNE and HOGREFE, 2012) with Fenoxaprop-P-ethyl at 166 g ha^{-1} (Ralon Super®) and mesosulfuron-methyl + iodosulfuron-methyl at, 7.5+1.5, 15+3 to 30+6 g ha^{-1} (Atlantis WG®) as representative ACCase and ALS herbicides respectively. PCR and pyrosequencing procedures used for the mutation analysis were described by BEFFA *et al.* (2012) and 40 plants per field sample were analyzed in average.

Plants surviving the greenhouse treatment with either Atlantis WG® or Ralon Super® but not carrying a target-site mutation were classified as non-target site resistant. Most samples showing resistance in the biotests but no target-site mutations had enhanced metabolism of either mesosulfuron-methyl or fenoxaprop-P-ethyl (BEFFA, personal communication).

Statistical analysis was based on percentage of occurrence of ACCase and ALS target site mutations (SNPs), and percentage of plants that were target-site resistant, non-target site resistant, or sensitive to ACCase or ALS herbicides.

ANOVA and cluster analysis was carried out using the statistics software R. Cluster analysis was done using a Manhattan distance matrix and the ward clustering algorithm for the years 2011 and 2012. The clustering results were compared using the adjusted Rand index (HUBERT and ARABIE, 1985) and a Mantel Test (MANTEL, 1967).

Results

Infestation level with ears of *A. myosuroides* Huds.

The infestation level with blackgrass was significantly different among the three regions ($F_{(2,147)}$=3.59,<0.05) and among the three years ($F_{(2,147)}$=4.4,<0.05) analyzed (Tab. 1). Severe control failure with infestation levels of 4 and 5 were only observed in 2.1%, 11.5% and 5.0% of the fields for 2010, 2011, and 2012, respectively. No blackgrass ears or only traces (infestation levels 0 and 1) were found in 76.8%, 58.3% and 59.9% of all fields analyzed in 2010, 2011, and 2012, respectively, and infestation levels 2 and 3 were found in 21.0%, 30.2%, and 35.1% of the fields in 2010, 2011, and 2012, respectively. This suggests that infestation is still at a tolerable level in the majority of the fields. Results from biotests and laboratory analyses showed that frequencies of fields showing the presence of resistance is higher than the frequency of fields with infestation levels of 4 and 5 (Tab. 1 and Tab. 2). This suggests that these methods can detect herbicide resistance before it causes significant agronomic problems in the fields and provide useful information for weed management.

Tab. 1 Proportion of fields of every site-year with corresponding infestation level (from 0 to 5).

Tab. 1 *Prozentuale Verteilung der Befallsgruppierung (0-5) für jede beprobte Region in den drei Versuchsjahren.*

Location	Year	0	1	2	3	4	5
H	2010	0.80	0.20	0.00	0.00	0.00	0.00
	2011	0.07	0.47	0.27	0.20	0.00	0.00
	2012	0.14	0.57	0.14	0.07	0.00	0.07
M	2010						
	2011	0.07	0.67	0.07	0.00	0.20	0.00
	2012	0.00	0.47	0.27	0.20	0.00	0.07
Z	2010	0.33	0.33	0.11	0.15	0.04	0.04
	2011	0.15	0.44	0.15	0.11	0.07	0.07
	2012	0.00	0.67	0.22	0.04	0.07	0.00

Tab. 2 Proportional characterization of fields in greenhouse bioassays with S (susceptible >80% efficacy), I (intermediate, 50-79% efficacy), R (resistant, <49% efficacy).

Tab. 2 *Prozentuale Gruppierung der analysierten Felder in Gewächshausresistenztests mit S (sensitiv >80 % Wirkung), I (intermediär 50-79 % Wirkung), R (resistent, <49 % Wirkung).*

	Atlantis WG			**Ralon Super**		
	S	**I**	**R**	**S**	**I**	**R**
2010	0.74	0.13	0.13	0	0.03	0.97
2011	0.72	0.05	0.23	0	0.06	0.94
2012	0.77	0.09	0.14	0.02	0.04	0.94

<u>Resistance structure and occurrence of resistance conferring mutations</u>

The average portion of plants carrying at least one target site mutation in ACCase ranged from 22.6±8.0% for Region H in 2011 to 48.9±5.2% for Region M in 2011. The portion of plants having a target site mutation in ALS ranged from 0.1±0.1% in region H in 2011 to 37.7±5.9% in Region M in 2011.

Table 3 presents an overview of the distribution of the individual ACCase and ALS target site mutations in the sampled regions with ACCase resistance being well established. The average number of plants per field showing either TSR or NTSR to Ralon Super was 90% in average (77.5%-97.8%) over all regions and years. In contrast, levels of resistance to Atlantis WG were only 24.0% in average (0.1%-52.2%). However, the portion of fields showing target site or non-target site resistance to ALS seemed to increase over the years and resistance was found in a high number of fields at low levels.

Tab. 3 Mean proportion of plants per field (Mean) with corresponding range and portion of fields sampled (Fields) showing either target site or non-target site resistance to ACCase and ALS. Samples were taken from each of three locations in 2010, 2011 and 2012.

Tab. 3 *Durchschnittliche Anzahl Pflanzen (Mean) pro Feld mit dazugehörigem Intervall und prozentualem Anteil der untersuchten Felder für die Zielort- bzw. bzw. Nicht-Zielortresistente Pflanzen gegenüber ACCase und ALS-Inhibitoren in den drei beprobten Regionen gefunden wurden. Untersucht wurden die Jahre 2010, 2011 und 2012.*

		TSR ACCase		**TSR ALS**		**NTSR ACCase**		**NTSR ALS**	
Location	**Year**	**Mean**	**Fields**	**Mean**	**Fields**	**Mean**	**Fields**	**Mean**	**Fields**
Z	2010	33.6 (5-78)	100	11.6 (0-55)	80	53.7 (12-100)	100	18.8 (0-79)	40
H	2011	22.6 (0-96)	90	0.1 (0-2)	5	75.3 (4-100)	100	0 (0-0)	0
M	2011	48.9 (2-78)	100	37.7 (0-74)	94	45.1 (15-80)	100	14.4 (0-94)	33
Z	2011	44.6 (3-92)	100	14.3 (0-87)	58	49.3 (0-97)	97	8.0 (0-42)	59
H	2012	35.5 (0-100)	89	1.4 (0-74)	21	42.0 (0-94)	93	10.0 (0-55)	86
M	2012	47.7 (12-86)	100	23.5 (0-98)	78	42.3 (0-73)	97	14.8 (0-66)	90
Z	2012	48.5 (4-97)	100	4.2 (0-86)	28	44.5 (0-89)	99	9.1 (0-70)	90

Different mutations seem to be prevalent in the different regions. For ALS we observed mutations at position 547 to be most prevailing in Region M while mutations at position 197 were most prevailing in Region Z. No such conclusion could be drawn for region H due to the very low level of ALS-TSR (data not shown). Mutations in ACCase at position 1781 seemed to be prevailing in Region H, while mutations at position 2078 seem to be prevailing in Region Z. No clear picture could be drawn for region M, but this region showed the highest percentage of mutations at position 2096 (data not shown).

All five mutations already reported in the literature to confer resistance to ACCase herbicides in *A. myosuroides* were found in all three regions. The median number of unique ACCase mutations per field was found to be 1, 4, and 3, and 3, 3.5 and 3 for Region H, M, and Z in 2010 and 2012, respectively. Only a small number of fields contained all 5 mutations conferring resistance to ACCase. In 2012, 2%, 10%, and 8% of the fields of Region H, M, and Z showed all 5 ACCase mutations, respectively.

<u>Spatial and Temporal Analysis of three different regions for 2011, 2012</u>

Cluster analysis revealed that neighboring fields for a given region are not always clustered together (not shown). This is illustrated by Figure 1 showing the variability within Region Z for the prevalent ACCase mutations within the fields analyzed for 2011 and 2012. Furthermore, the clustering of the data for 2011 and 2012 has a low consistency as indicated by a low adjusted Rand index (0.18) indicating variability in between years. Relationship between neighboring fields was analyzed using Mantel's test to test for correlation between the spatial distance and the distance matrix of the resistance patterns as used in the cluster analysis. The Mantel test showed a week correlation for 2011 (r = 0.22, p = <0.01) and for 2012 (r = 0.18, p = <0.01).

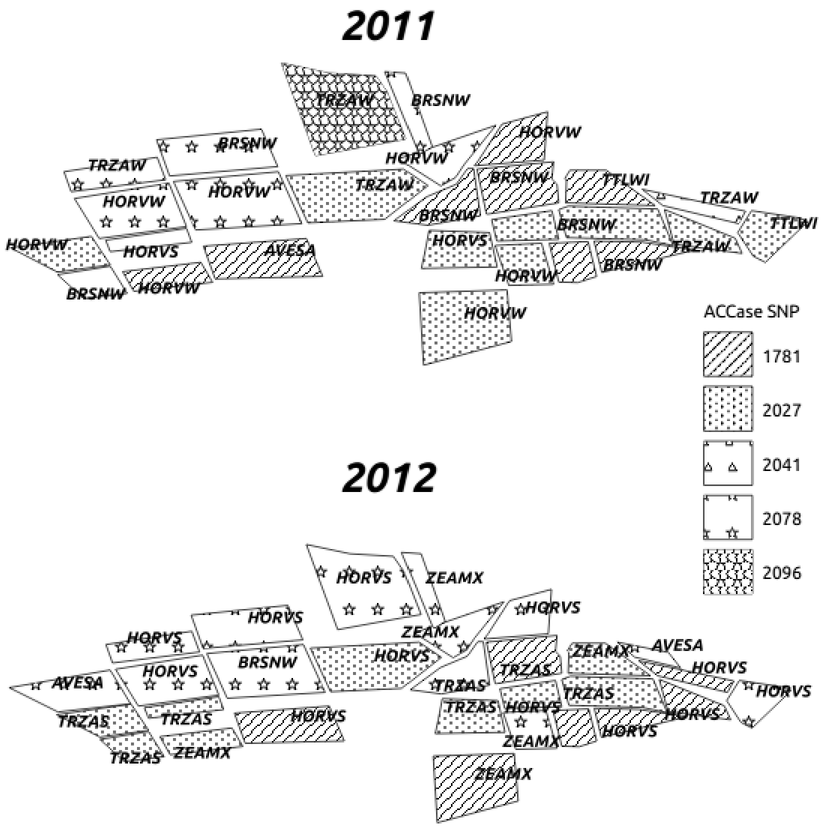

Fig. 1 Prevalent ACCase target-site mutation (SNP) for selected fields of location Z in 2011 (top) and 2012 (bottom). Crop in a given year is abbreviated using EPPO-Code.

Fig. 1 *Dominierende ACCase Zielortmutationen ausgewählter Felder der Region Z aus 2011 (oben) und 2012 (unten). Die dazugehörige Kultur in dem jeweiligen Jahr ist mit dem EPPO-Code abgekürzt.*

Discussion

Infestation level with ears of *A. myosuroides* Huds.

The degree of infestation was recorded at harvest time and therefore represents the number of surviving or unsprayed plants. An assessment of the infestation severity before spraying in fall and spring was not done. Therefore the presented data reflect the result of the selection process done by the farming activity during years. Furthermore the six-rating scale used is not based on individual plant count and classification, and is open to bias, especially at low densities, since individual plants might have been missed. For a more detailed explanation of the rating and collecting method the reader is referred to Hess *et al.* (2012). Table 1 shows that blackgrass in most of the fields is still at low levels and therefore well controlled. The number of fields with heavy infestation is therefore low and represents individual cases. Resistance confirmation tests in the greenhouse provided a deeper insight of the development in the field by being more sensitive. This comparison between field and greenhouse data over several years will contribute to set up earlier management decisions to prevent resistance from building up and take counteractive measures. Due to the strong winter in 2012 the infestation levels of *A. myosuroides* were decreased due to crop failure over the winter and replanting in spring. This presented a rather unique situation with a more intense blackgrass control than in average years. However, this did not seem to have a big impact on overall blackgrass infestation levels.

Resistance structure and occurrence of resistance conferring mutations

In all sampled regions the problem of ACCase resistance was well established with an average of 90% of the plants showing target site and/or non-target-site resistance (Tab. 3).

A majority of the farmers is aware of that problem. ALS resistance is still at a low level with only a small number of fields where low blackgrass control was observed. However, Table 3 shows that either TSR or NTSR to ALS inhibiting herbicides is present at a low level in the majority of the fields.

This means that farmers need to take counteractive measures now in order to prevent resistance development to ALS inhibitors. In the two regions M and Z single mutations at either position 574 and 197 seem to dominate with a very low number of fields where both are present together. We will need to intensify our research, especially in analyzing the field history, to better understand the basis of this.

Spatial and temporal analysis of three different regions for 2011, 2012

Cluster analysis of the seven analyzed target site mutations together with overall portion of target site and non-target site ACCase and ALS plants revealed that:

1) No strong relation between the distance in between fields and the resistance structure can be observed, meaning that neighboring fields in most cases show a different pattern in their resistance structure which is not necessarily attributed to higher or lower sensitivity to ACCase and ALS herbicides. This is exemplified for ACCase conferring target site mutations in Figure1. The hypothesis is therefore that resistance in fields evolves differently due to different selection pressure, occurring due to different farming practices, e. g., crops sown. This agrees with earlier findings by Menchari *et al.* (2006) and Cavan *et al.* (1998). Investigation will be undertaken to examine the genetic relationship in between *A. myosuroides* populations to gain insights into the underlying processes.

2) Overall patterns seem to change with different selection pressure, attributed to the different crops sown in consecutive years and the related weed management. Related to ACCase where mutations are not specific to a given location different mutations dominate in a given year. Interviews with the farmers will hopefully contribute to understand the selection process and enable predictions on resistance patterns. Related to ALS, so far Z and M locations were characterized by the prevalence of one given mutation.

These results show the importance of long term observations to be able to draw accurate conclusions on the resistance status within a given region. Resistance patterns seem to be quite volatile over years. The study is still ongoing but these preliminary results suggest already new insights in the spatial and temporal development of ACCase and ALS resistance. Increasing ALS resistance will lead to a great reduction of available herbicides for use in *A. myosuroides* control. We therefore suggest that all integrated weed management tools are used in combination, in particular crop rotation, rotation of modes of action, soil tillage, as well as use of pre- and post-emergence herbicides.

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