Discovering the mechanism of enhanced metabolism in flufenacet resistant grass weeds

Untersuchung des Mechanismus zum schnelleren Flufenacet-Abbau in resistenten Ungräsern

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DOI 10.5073/jka.2016.452.005

Abstract

Inhibitors of very long chain fatty acid synthesis (VLCFAs, HRAC group K3) including flufenacet-based products are key herbicides in weed management strategies in particular of Western European cereal growing areas. They offer an alternative mode of action to those of frequently applied post-emergence active ingredients e.g. ACCase and ALS chemistries. In comparison, herbicide resistance to VLCFAs inhibitors develops considerably slower. Yet, resistance to the 'low-risk' herbicide flufenacet was documented in multi-resistant ryegrass (*Lolium* spp.) in the northwestern United States. A 46-fold resistance shift in a population from Washington State was observed in a dose-response bioassay. The resistance levels described in black-grass (*Alopecurus myosuroides* Huds.) field populations are lower and generally within the range of environmentally caused inconsistency in efficacy. An artificial selection of two UK multi-resistant black-grass populations with flufenacet applied annually for eight successive years resulted in resistant progeny surviving the field rate. Besides characterizing the degree of resistance in black-grass and ryegrass populations, we demonstrated that flufenacet resistance in these populations was caused by enhanced metabolism. No cross-resistance between flufenacet and pyroxasulfone, the newest VLCFAs-inhibiting herbicide, occurred in the ryegrass population. A good understanding of the resistance mechanism and early diagnostics can help preserve the efficacy of flufenacet.

Keywords: Enhanced metabolism, flufenacet, grass weeds, pre-emergence herbicides, resistance mechanisms

Zusammenfassung

Inhibitoren der Synthese sehr langkettiger Fettsäuren (VLCFAs, HRAC-GruppeK3) wie z.B. Flufenacetprodukte gehören vor allem in Westeuropa mit zu den entscheidenden Herbiziden der Unkrautbekämpfungsstrategien in Getreide. VLCFAs-Inhibitoren stellen einen alternativen Wirkmechanismus zu den häufig applizierten Wirkstoffen der ACCase- oder ALS-Inhibitoren dar und bieten den Vorteil, dass sich ihnen gegenüber Resistenzen wesentlich langsamer entwickeln. Auch wenn das Resistenzrisiko dieser Herbizide als niedrig eingestuft wird, konnten beispielsweise Resistenzen in Weidelgras-Populationen (*Lolium* spp.) in den nordwestlichen USA festgestellt werden. Bei einer Population aus Washington State konnte in einem Dosis-Wirkungsversuch eine 46-fache Verschiebung des Resistenzniveaus beobachtet werden. Die Resistenzniveaus, die bei Ackerfuchsschwanz (*Alopecurus myosuroides* Huds.) in Feldpopulationen beobachtet werden sind jedoch wesentlich geringer und liegen innerhalb der für Vorauflaufherbizide charakteristischen Wirkungsbreite. Durch künstliche Selektion zweier multi-resistenter *A. myosuroides*-Populationen mit jährlich applizierten Flufenacetdosen konnten in acht aufeinanderfolgenden Jahren resistente Nachkommen selektiert werden, die die empfohlene Feldaufwandmenge überlebten. Wir konnten in dieser Studie das Resistenzlevel der genannten *A. myosuroides*- und *Lolium*-Populationen charakterisieren und den hauptsächlich zugrundeliegenden Resistenzmechanismus als metabolische Resistenz identifizieren. Ein weiteres Experiment zeigte, dass in der getesteten *Lolium*-Population keine Kreuzresistenz mit dem neusten VLCFAs-Hemmer Pyroxasulfon besteht. Ein gutes Verständnis der Resistenzmechanismen und frühe Resistenzdiagnose können dazu beitragen, die Wirksamkeit von Flufenacet aufrechtzuerhalten.

Stichwörter: Erhöhter Metabolismus, Flufenacet, Resistenzmechanismen, Ungräser, Vorauflaufherbizide

Introduction

The evolution of herbicide resistance is an example of fast adaptation in plant populations. Herbicide resistance, particularly to post-emergence herbicides, including the majority of ACCase and ALS chemistries has been described for many weeds in several cropping systems in the world (HEAP, 2015). To better manage this resistance, one tool among several others, is the use of pre-emergence herbicides (MENNE et al., 2012). Among those, flufenacet-based products are widely recommended and used (KLINGENHAGEN, 2012; HULL et al., 2014a). As an oxyacetamide, belonging to the herbicide class active on very long chain fatty acid elongases (VLCFAs), flufenacet adds a different mode of action (VLCFAs, K3) and improves grass weed control in fields where resistance to inhibitors acting on other targets is observed. Although VLCFAs-inhibitors have widely been spared from the development of herbicide resistance since their market introduction over 50 years ago, in a few occasions weed resistance to these herbicides has been described. The weed species *Lolium rigidum* (Gaud.), *Alopecurus myosuroides* (Huds.), *Echinochloa crus-galli* (L.) and *Lolium multiflorum* (Lam.) are reported to be resistant to this group of herbicides (HEAP, 2015). Reduced efficacy of flufenacet has been described in multi-resistant populations of *L. multiflorum* in the North Western USA as well as in multi-resistant populations of *A. myosuroides* in several European locations (RAUCH et al., 2010; HULL and MOSS, 2012). The appearance of multiple herbicide resistance has frequently been explained by detoxification (enhanced plant metabolism) of the active ingredient (CUMMINS et al., 2013, YU and POWLES, 2014). It is expected that various enzymes involved in the condensing steps of VLCFA synthesis are inhibited. Non-target site resistance, e.g. enhanced metabolism, appears to be the most likely mechanism of resistance as mutations of the active sites of several of these enzymes conferring resistance to VCLFAs-inhibitors appears unlikely (BÖGER et al., 2000). Detoxification of VLCFAs inhibitors is already known from the tolerance of some crops e.g. maize (BIESELER et al., 1997). The present study provides further evidence for enhanced metabolism to be one major mechanism of flufenacet resistance in grasses using black-grass and Italian ryegrass as models.

Materials and Methods

Plant material and growing conditions

In this study populations of different origins and resistance levels were used as research material. The term 'population' herein below is generally used for plant seeds collected in one location or within one cross in the breeding process. A flufenacet resistant population of *Lolium multiflorum* was collected from a field in Walla Walla, WA (USA) previously described by RAUCH et al. (2010). Three additional reference populations were used: A sensitive *L. multiflorum* population 'LOLMU', a sensitive *L. rigidum* population 'LOLRI' and the multiple resistant *L. rigidum* population 'VLR69'. The population 'VLR69' was described as resistant to at least nine different chemical classes of herbicides, including VLCFAs-inhibitors (BURNET et al., 1994 a, b). It comprises metabolic resistance at least to photosystem II inhibitors, ALS inhibitors and ACCase inhibitors (PRESTON et al., 1996). In this study 'VLR69' was used as a positive control. An additional set of experiments was carried out with United Kingdom *A. myosuroides* populations collected 1) from Peldon, Essex in 2003 ('Peldon03'), initially resistant to pendimethalin, and 2) Colsterworth, Lincolnshire in 2005 ('Colsterworth05'). Both populations had previously shown evidence of resistance to pendimethalin in petri-dish assays, and were selected with flufenacet (180 g a.i. ha^{-1}) in outdoor containers for eight generations using the methods described by HULL and MOSS in 2012. A sensitive population from Rothamsted, Hertfordshire ('Rothamsted05') was collected in 2005 from a long-term field trial never treated with herbicides and was used as a reference (MOSS et al., 2004). For all pot experiments, seeds were sown in petri-dishes containing 0.7% agar type A and 0.2 M KNO_3 in order to synchronize germination. Petri-dishes were sealed with Parafilm® and stored in the dark at 4 °C for 3 (ryegrass) or 5 (black-grass) days. Afterwards, plants were transferred to the

greenhouse to induce germination at a 16-h photoperiod with a light intensity of > 55 000 Lux at 22 °C and at an 8-h dark period at 16 °C.

Greenhouse bioassays

For dose-response bioassays seedlings were transplanted at BBCH growth stage 05 to 07 into 2 cm diameter pots containing sandy loam with 2.2% organic matter sandy loam and covered with a thin layer of coarse sand. One experiment (Tab. 1) with *Lolium* was conducted with 40 plants per population and treatment with flufenacet (Tiara® 500 SC). Besides that, the other dose-response experiments were performed using 6-8 plants per populations and dose rate, testing pyroxasulfone (Sakura® 20 WP) on *Lolium* and flufenacet (Tiara® 500 SC) on *A. myosuroides*. The plants were sprayed at different dose rates at a volume of 300 L ha^{-1} on the same day and subsequently watered. Foliage fresh weight and number of surviving plants were assessed 21 days after treatment. The dose-response data were analysed with a three-parameter model according to RITZ and STREIBIG (2005), using the statistical software R (version 3.0.1, R Core Team, 2013).

Metabolism analysis by HPLC

Incubation was done using glass vials filled with 4 mL of coarse sand and 1.2 mL incubation solution. The incubation solution had a concentration of 0.02 M KNO_3 and 1.000.000 dpm mL^{-1} ($7.43_{*}10^{-3}$ M) deriving from ^{14}C-radiolabelled flufenacet. The plantlets were placed upright onto the sand and covered with additional 1.5 mL of coarse sand and kept in a climate chamber for 14 h at 17 °C in the light and 10 h at 11 °C in the dark. After various time points the plantlets were washed in 50% acetone and each eight plants were pooled to eight samples per time point and population. The extraction of the radiolabelled compounds was carried out as described by COLLAVO et al. (2012), except for an additional extraction step with 600 µL 90% acetonitrile. The extract was re-suspended in 80% acetone and injected with a volume of about 90µL into a HPLC-system with a 250 x 4.6 mm Synergi™ 4 µm Hydro-RP 80 Å, LC Column (Phenomenex, Aschaffenburg, Germany). Solvents for the mobile phase were 0.04% HCl (mobile phase A), acetonitrile (mobile phase B) and methanol (mobile phase C) at a flow rate of 0.5 mL min^{-1}. The gradient was performed over 40 min as follows: 0.0 min, 70.0% A, 23.0% B, 7.0% C; 1.0 min, 65.5% A, 27.5% B, 7% C; 16.0 min, 55% A, 45% B; 27.0 min, 5.0% A, 95.0% B; 32.0 min, 5.0% A, 95.0% B; 35.0 min, 70.0% A, 23.0% B, 7.0% C; 40 min, 70.0% A, 23.0% B, 7.0% C. The remaining active ingredient was analysed with a log-logistic sigmoidal three-parameter model according to RITZ and STREIBIG (2005), using the statistical software R (version 3.0.1, R Core Team, 2013).

Results

Response of selected Lolium populations to flufenacet and pyroxasulfone

The flufenacet rate that reduced the biomass by 90% (ED_{90}) exceeded, with 17218 g a.i. ha^{-1}, a 45-fold of the US-field rate in wheat (Tab. 1). The effective dose that reduced biomass by 50% (ED_{50}) was 276 g a.i. ha^{-1}, 46-fold more than the respective dose for the sensitive LOLMU and LOLRI populations. The ED_{50}-value of population Walla Walla differed significantly from the ED_{50}-values of the populations it was compared with. The multi-resistant population VLR69 showed much lower levels of resistance to flufenacet with an ED_{50}-value of 15 g a.i. ha^{-1}, which was significantly different from both the sensitive reference populations as well as the highly resistant Walla Walla population. The amount of pyroxasulfone necessary to reduce the foliage fresh weight of the *Lolium* populations LOLMU, LOLRI and Walla Walla by 50% ranged from 9.83 to 12.72 g a.i. ha^{-1} and ED_{90}-values ranged from 30.63 to 49.51 g a.i. ha^{-1}. The differences between populations were not statistically significant.

Tab. 1 Effectiveness of flufenacet (F) and pyroxasulfone (P) on selected *Lolium* populations characterized by the effective dose rate (in g ha-1) necessary to reduce the biomass by 50% (ED_{50}) and 90% (ED_{90}) ± standard error and the resistance factors of ED50-values. Different letters represent significant differences ($p<0.05$).

Tab. 1 *Wirksamkeit von Flufenacet auf ausgewählte* Lolium-*Populationen, charakterisiert durch die Effektivdosen, die zu 50 % (ED_{50}) und 90 % (ED_{90}) Biomassereduktion führen ± Standardfehler, sowie Resistenzfaktoren (RF) der entsprechenden ED50-Werte. Unterschiedliche Buchstaben kennzeichnen signifikante Unterschiede ($p<0,05$).*

	Flufenacet				Pyroxasulfone			
Population	**ED_{50F}**		**RF_F**	**ED_{90F}**	**ED_{50P}**		**RF_P**	**ED_{90P}**
LOLMU	6 ± 0.8	a	1	24 ± 4.9	11 ± 2.9	a	1.1	48 ± 3.2
LOLRI	7 ± 1.0	a	1	33 ± 7.3	10 ± 2.0	a	1.0	31 ± 5.2
VLR69	15 ± 3.0	b	2	191 ± 60.3	-		-	-
Walla Walla	276 ± 75.2	c	46	17218 ± 7423.8	13 ± 5.2	a	1.3	50 ± 61.7

Flufenacet metabolism in selected Lolium populations

The tested populations needed 0.23 to 19.91 hours in order to degrade 50% of the initial amount of ^{14}C-radio labeled flufenacet (Tab. 2). Population LOLRI degraded 2.22 times faster than the most sensitive population LOLMU and the multiple-resistant population VLR69 degraded the herbicide 6.05 times faster than LOLMU. A T_{50}-value of 86.57, representing the parent compound half-life in the plant tissue, was calculated for population Walla Walla. Each population differed significantly from the other populations tested.

Tab. 2 Flufenacet metabolism in selected *Lolium* populations characterized by time in hours necessary to reduce the initial amount of ^{14}C-radiolabelled flufenacet by 50% (T_{50}) ± standard error, and a metabolism factor (MF) of the respective T_{50}-value in relation to the most sensitive population. Different letters represent significant differences ($p<0.05$).

Tab. 2 *Flufenacet-Metabolismus in ausgewählten* Lolium-*Populationen, charakterisiert durch benötigte Zeit in Stunden um 50% der applizierten Menge ^{14}C-radiomarkierten Flufenacets abzubauen (T_{50}) ± Standardfehler, sowie Metabolismusfaktoren (MF) der entsprechenden T_{50}-Werte in Relation zur sensitivsten Population. Unterschiedliche Buchstaben kennzeichnen signifikante Unterschiede ($p<0,05$).*

Population	**T_{50}**	**Significance**	**MF (T_{50})**
LOLMU	19.9 ± 1.5	d	1
LOLRI	9.0 ± 1.1	c	2
VLR69	3.2 ± 3.3	b	6
Walla Walla	0.2 ± 0.3	a	87

Response of selected *A. myosuroides* populations to flufenacet

In this experiment with 6-8 individuals per populations and treatment dose, the ED_{50}-values of the sensitive reference population Rothamsted05 differed significantly from those of the other populations tested. In order to reduce the biomass of the selected population Colsterworth14 by 50% a 2.4-fold higher dose of flufenacet was necessary in comparison to the original population Colsterworth05. In order to reduce the biomass of the selected population Peldon14 by 50% a 7.1-fold greater dose of flufenacet was necessary in comparison to Peldon03. The ED_{50}-values differed significantly between the Colsterworth50 and Colsterworth14 over years. For a biomass reduction of 90% of the selected populations Peldon14 and Colsterworth14 a multiple of the recommended field rate (776 and 3075 g a.i.ha^{-1}) needed to be sprayed.

Tab. 3 Effectiveness of flufenacet on selected *A. myosuroides* populations characterized as described in Table 1. Resistance factors (RF) were calculated by the division of the ED_{50}-value of the selected population by the ED_{50}-value of the original population. Different letters represent significant differences (p<0.05).

Tab. 3 *Wirksamkeit von Flufenacet auf ausgewählte* A. myosuroides-*Populationen, charakterisiert wie in Tabelle 1 beschrieben. Resistenzfaktoren (RF) wurden durch Teilung der ED_{50}-Werte der selektierten Population durch die ED_{50}-Werte der Ausgangspopulationen errechnet. Unterschiedliche Buchstaben kennzeichnen signifikante Unterschiede (p<0,05).*

Population	ED_{50}	Significance	RF (ED_{50})	ED_{90}
Rothamsted05	17 ± 2.3	a	-	34 ± 10.3
Colsterworth05	83 ± 15.6	bc	1.0	173 ± 62.5
Colsterworth14	201 ± 60.7	bc	2.4	776 ± 548.9
Peldon03	64 ± 19.6	b	1.0	147 ± 69.4
Peldon14	456 ± 201.6	c	7.1	3075 ± 3753.0

Flufenacet metabolism in selected *A. myosuroides* populations

The degradation of flufenacet was characterized in seedlings of five selected *A. myosuroides* populations using HPLC-methods (Tab. 4). These populations needed 0.6 to 28.9 hours in order to metabolize 50% of the initial amount of ^{14}C-radio labeled flufenacet. The selected populations Colsterworth14 and Peldon14 metabolized the applied flufenacet significantly faster (6.2 and 5.6 times, respectively) in comparison to their respective original population.

Tab. 4 Flufenacet metabolism in selected *A. myosuroides* populations, characterized as described in Table 2. Different letters represent significant differences (p<0.05).

Tab. 4 *Flufenacet-Metabolismus in ausgewählten* A. myosuroides-*Populationen, charakterisiert wie in Tabelle 2 beschrieben. Unterschiedliche Buchstaben kennzeichnen signifikante Unterschiede (p<0,05).*

Population	T_{50}	Significance	MF (T_{50})
Rothamsted05	28.9 ± 2.6	d	-
Colsterworth05	8.1 ± 1.2	c	1.0
Colsterworth14	1.3 ± 0.5	ab	6.2
Peldon03	3.4 ± 1.0	b	1.0
Peldon14	0.6 ± 0.4	a	5.6

Discussion

Since their market introduction, inhibitors of VLCFAs have mostly been spared from the development of weed resistance. Yet, field resistance to flufenacet was observed in *Lolium* spp. in the U.S. states Washington, Oregon and Idaho in the North West of the country (RAUCH et al., 2010; HEAP, 2015). A dose-response bioassay conducted in the greenhouse confirmed that a resistant *Lolium* population from Walla Walla is able to survive a multiple of the recommended field rate in winter wheat of about 380 g flufenacet ha^{-1}. The ED_{90} with a value of 17218 g a.i. ha^{-1} exceeded the 45-fold of the field rate. Also to achieve 50% biomass reduction the 46-fold of the rate necessary to control the sensitive reference population LOLMU had to be applied. Even the multiple and metabolically resistant population VLR69 which was never exposed to flufenacet survived significantly higher dose rates than the sensitive reference populations. VLR69 showed also significantly enhanced flufenacet metabolism in comparison to the sensitive reference populations LOLMU and LOLRI and the most resistant population Walla Walla had degraded more than 80% of the initially applied herbicide within 4 hours after application. The metabolic half-time (T_{50}) shows that Walla Walla degraded flufenacet 87 times faster than the sensitive population LOLMU. The close relationships between the speed of flufenacet degradation and the response in

the greenhouse suggest that enhanced metabolism is one main factor in flufenacet resistance. The Walla Walla population is also able to degrade herbicides belonging to different chemical classes faster (RAUCH et al., 2010; DÜCKER, 2014), whereas it could be controlled with low dose-rates of the latest VLCFAs-inhibiting herbicide, pyroxasulfone. This is a further argument supporting non-target-site resistance and the observation of enhanced metabolic resistance as a cause of the survival of flufenacet resistant weeds. This is in agreement with the hypothesis of BÖGER et al. (2000) which excludes target-site alterations as a cause of flufenacet resistance. The good performance of pyroxasulfone, an isoxazoline herbicide, on resistant *Lolium* (WALSH et al., 2011) could be explained by its different chemical structure. The introduction of new chemistries e.g. pyroxasulfone could reduce the selection pressure that lasts on flufenacet in some areas of Europe with resistance problems, where other modes of actions do not cushion the selection pressure anymore or to a lesser extent. Although among pre-emergence herbicides flufenacet shows to have superior efficacy even on multi-resistant field populations of *A. myosuroides*, shift of activity or resistance at the disputed low-level range (HEAP, 2005) could be observed (KLINGENHAGEN, 2012; PETERSEN and OLF, 2014). The resistance levels described in *A. myosuroides* field populations are lower and generally within the range of environmentally caused inconsistency in efficacy. In another approach, HULL and MOSS (2012) could demonstrate in an experiment started in 2003 how multi-resistant *A. myosuroides* populations can develop flufenacet resistance by recurrent selection with repeated annual treatment with the herbicide. In a dose-response experiment it was shown how these initially pendimethalin resistant populations Peldon03 and Colsterwort05 underwent a 2.4-fold (Colsterworth05) and a 7.1-fold (Peldon03) resistance shift until 2014. The differences between the original populations and the populations evolved by recurrent selection become even higher considering the ED_{90} which was below the UK field rate of 240 g a.i. ha^{-1} in case of the original populations, whereas multiples of the field rate had to be applied to control 90% of the selected populations. These results underpin the findings about successive reduction of flufenacet efficacy over about eight years of recurrent selection (HULL and MOSS, 2012), whereas a much slower shift of efficacy can be generally observed in the field (HULL et al. 2014a). The decreased efficacy of flufenacet was correlated with a significantly faster degradation of applied radiolabel led flufenacet in both of the selected populations Peldon14 and Colsterworth14 in comparison to their respective original populations Peldon03 and Colsterworth05. In addition, all four populations degraded the herbicide significantly faster than the sensitive population Rothamsted05. This shows that enhanced flufenacet metabolism could be increased by recurrent selection with repeated herbicide applications as previously described for other herbicides (NEVE and POWLES, 2005; BUSI et al., 2012). In conclusion, it is important to develop the tools to analyse the flufenacet resistance evolution in order to take, when necessary, fast countermeasures to delay its evolution e.g. the application of full dose rates and mixtures (e.g. flufenacet + diflufenican) in combination with well-known measures of cultural control introducing as much diversity as possible in the system. Nevertheless, analyses of *A. myosuroides* field populations confirmed that resistance to pre-emergence herbicides can occur, but resistance tends to be partial and does not appear to increase very rapidly (HULL et al., 2014a and b).

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