Degradation and metabolism of fenoxaprop-P-ethyl in sensitive and resistant populations of *Alopecurus myosuroides*

Abbau und Metabolismus von Fenoxaprop-P-ethyl in sensitiven und resistenten Populationen von Alopecurus myosuroides

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Abstract

Blackgrass (*Alopecurus myosuroides)* is one of the most economically important weeds in Europe. Because of the development and distribution of herbicide resistant populations the control of this weed has become a serious challenge in agriculture. In recent years a reduced efficacy of fenoxaprop-P-ethyl towards *A. myosuroides* has been observed. To investigate the degradation of the active substance fenoxaprop-P-ethyl in *A. myosuroides*, four populations were grown in the greenhouse: One herbicide sensitive population, two populations with a non-target-site resistance (NTSR) and one population with a target-site resistance (TSR). For dose response studies the plants were treated with different dosages of fenoxaprop-P-ethyl and dry weight was determined after three weeks. For the NTSR populations resistance factors of 76 and 2 could be calculated based on ED50 values. The development of the TSR plants was not restricted by the herbicide treatment, not even with the highest dosage.

For the degradation and metabolism studies plants were treated with fenoxaprop-P-ethyl and harvested for laboratory analysis 2, 8, 24, 48 and 96 hours after treatment. The active substance degraded within 96 hours without any significant differences between the populations. Two hours after herbicide treatment a metabolite could be identified and quantified in all populations. The mean contents at eight and 24 hours after treatment differed significantly between the populations. Results have shown that the metabolism of fenoxaprop-P-ethyl to fenoxaprop-P is very similar in the tested populations although they have different resistance mechanisms.

Further studies are intended to show if the populations differ in the formation of other unknown metabolites.

Keywords: ACCase resistance, aryloxyphenoxypropionates (AOPPs), herbicide metabolism, non-target-site resistance (NTSR)

Zusammenfassung

Ackerfuchsschwanz (*Alopecurus myosuroides)* ist in Europa eines der Ungräser mit der größten wirtschaftlichen Bedeutung. Durch die Entwicklung und Ausbreitung resistenter Populationen wurde die Bekämpfung zu einer ernstzunehmenden Herausforderung in der Landwirtschaft.

Seit einigen Jahren wird eine zunehmende Wirkungsschwäche von Fenoxaprop-P-ethyl gegenüber *A. myosuroides* beobachtet.

Um das Abbauverhalten des Wirkstoffes in *A. myosuroides* zu untersuchen, wurden vier Populationen im Gewächshaus angezogen. Neben einem sensitiven Standard wurden für diese Untersuchung zwei Populationen mit einer nicht-wirkortspezifischen Resistenz (NTSR), sowie eine Population mit einer wirkortspezifischen Resistenz (TSR) verwendet. Für Dosis-Wirkungsversuche wurden die Pflanzen mit unterschiedlichen Konzentrationen des Wirkstoffs Fenoxaprop-P-ethyl behandelt und das Trockengewicht nach drei Wochen ermittelt. Basierend auf ED50-Werten konnten Resistenzfaktoren von 76 und 2 für die NTSR Populationen berechnet werden. Die Entwicklung der TSR Pflanzen wurde durch die Herbizid Behandlung nicht nachweisbar beeinflusst.

Für die Abbau- und Metabolismusstudien wurden die Pflanzen mit Fenoxaprop-P-ethyl behandelt und anschließend nach 2, 8, 24, 48 und 96 Stunden geerntet und im Labor aufgearbeitet. Der Wirkstoff wurde nach 96 Stunden fast vollständig abgebaut ohne signifikante Unterschiede zwischen den Populationen. Zwei Stunden nach Herbizid Applikation konnte ein Metabolit des Wirkstoffes in allen Populationen identifiziert und quantifiziert werden. Die Gehalte des Metaboliten in den Populationen unterscheiden sich nach 8 und nach 24 Stunden signifikant voneinander. Die Ergebnisse zeigen, dass der Metabolismus von Fenoxaprop-P-ethyl zu Fenoxaprop-P, unabhängig vom Resistenzmechanismus sehr ähnlich ist.

Weitere Studien sollen zeigen, ob sich die Populationen durch die Bildung von anderen, unbekannten Metaboliten unterscheiden.

Stichwörter: ACCase-Resistenz, Aryloxyphenoxypropionate (AOPPs), Metabolismus, nicht-wirkortspezifischen Resistenz (NTSR)

Introduction

The use of herbicides is still the most extensively used weed control measure in agriculture (MASSA *et al.,* 2013). By the opportunity to remove weeds selectively in a relatively rapid and easy way, herbicides are an important tool in plant protection.

The recurrent application of herbicides with the same modes of action in combination with simple cropping systems and high population densities led to the selection of resistant weed populations (BECKIE, 2006). One of the most economically important herbicide resistant weeds in Europe is the annual, monocotyledonous weed blackgrass (*Alopecurus myosuroides* HUDS*)*. In Germany the first resistant population was recorded in 1983 (HEAP, 2013). Today it is considered as a problem in many countries of North and Central Europe (MOSS *et al.,* 2007). Because of the drastic decline in available herbicides and the fact that no new herbicide mode of action has been introduced into the marketplace for over 20 years, the possibility to control weeds chemically is limited (BECKIE and TARDIF, 2012). Aryloxyphenoxypropionate herbicides (AOPPs) inhibit the first step of fatty acid synthesis by blocking the enzyme acetyl coenzyme-A carboxylase (ACCase) and are high efficacious herbicides for the control of *A. myosuroides* (COCKER, 1999).

The AOPP herbicide fenoxaprop-P-ethyl is available since the 1980s and has been widely used for the control of *A. myosuroides* (XU *et al*., 2013). In 1983 the first fenoxaprop-P-ethyl resistant population of blackgrass was encountered in Germany (HEAP, 2013). Resistance mechanisms are mainly a modification in the target site enzyme or an enhanced detoxification of fenoxaprop-P-ethyl (POWLES and YU, 2010).

In resistant weeds as well as in cereal plants, there are two types of enzymes, glutathione S-transferases (GSTs) and cytochrome P450 monooxygenases, that are most often found to be responsible for the metabolic detoxification of herbicides (COCKER, 1999).

In *Triticum* species it has been shown that GSTs detoxify fenoxaprop-P-ethyl by catalyzing their conjugation with the tripeptide glutathione (OLE, 1996; COCKER, 1999). In soil and wheat the degradation of fenoxaprop-P-ethyl seems to be mainly hydrolysis to the metabolite fenoxaprop-P (CHEN *et al.*, 2011).

Because of the fact that there is still a lack of understanding non-target-site-based resistance mechanisms, aim of the study was to investigate if the metabolism of fenoxaprop-P-ethyl differs in sensitive and resistant populations and in populations with different resistance mechanisms.

Material und Methods

Plant material

For the experiments one herbicide-sensitive (ALOMY-S) and three herbicide resistant populations of *A. myosuroides,* ALOMY-NTSR1, ALOMY-NTSR2, ALOMY-TSR, were collected from different fields in Germany. The population ALOMY-S was used as a sensitive reference standard. Molecular analysis of plants of the populations ALOMY-NTSR1 and ALOMY-NTSR2 has shown that none of the known mutations in the ACCase gene (1781, 2027, 2041, 2078 and 2096) are responsible for the resistance against fenoxaprop-P-ethyl, and were therefore declared as populations with a non-target-site resistance (NTSR). In plants of the population ALOMY-TSR a mutation (codon position 1781) could be detected.

Dose response studies

The seeds of the four populations of *A. myosuroides* were pre-germinated in vermiculite. In BBCH 11-12 two plants in each case were transplanted in 8 x 8 cm paper pots (Jiffy A/S, Denmark) containing a soil mixture (50% compost, 25% clay, 25% sand). Plants were placed in a heated

greenhouse (25/15 °C, 12 h photoperiod). At 2-3 leaf-stage (BBCH 12-13) plants were treated with a precision application chamber using a flat-fan nozzle (8002 EVS, TeeJet Spraying Systems Co., Wheaton, IL, USA, pressure 300 kPa, speed 800 mm^{-1}, water amount 200 l ha^{-1}). Fenoxaprop-P-ethyl (Ralon Super Power Plus, 69 g a.i. L^{-1}, EW, Nufarm plc) was sprayed with 10 different dose rates (662.4 – 2.6 g a.i. ha^{-1}) including a control variation. Each treatment was repeated three times and placed in the greenhouse in a randomised design. Three weeks after herbicide treatment plants were cut at ground level and dried for 48 hours at 80 °C in a drying cabinet. For the dry weight determination a precision balance was used.

Degradation- and metabolism studies

For the metabolism studies a defined amount of seeds was sown in 20 x 30 cm boxes. Soil mixture, plant growth and herbicide treatment technique was the same as described for the dose-response studies. To ensure herbicide uptake, plants were watered from below. Two-leaf stage seedlings of the four *A. myosuroides* populations were treated with fenoxaprop-P-ethyl (165.6 g a.i. ha^{-1}). Control variations were sampled prior to herbicide treatment (0 hours after treatment (HAT)). Further sampling dates were 2, 8, 24, 48 and 96 HAT. The experiment had a completely randomised design with three replicates per population and sampling date. To investigate the degradation and metabolism, two grams of leaf tissue per sample were ground in liquid nitrogen using a mortar and pestle. Together with 25 ml acetonitrile the sample was homogenized for 30 s using an Ultra-turrax homogenizer, filtered and gathered in a rotary piston. Acetonitrile was removed in a vacuum rotary evaporator and a water bath (30 °C). The residue was dissolved with acetonitrile (1.5 ml). After evaporation of acetonitrile with nitrogen on a heating block (60 °C), the residue was taken up with acetonitrile/water (1:1) and centrifuged at 20,000 x g for 10 min at 4 °C. Samples were measured with a HPLC - High Performance Liquid Chromatography (Waters Corporation, USA) in combination with a diode array detector (DAD). The analysis standards of fenoxaprop-P-ethyl and fenoxaprop-P were obtained from Bayer CropScience and from Sigma-Aldrich (USA).

Statistical analyses

Statistical analyses were performed using the software R (R Development Core Team, 2011). For analysis of the dose response studies and the degradation of fenoxaprop-P-ethyl, R was supplemented with the 'drc' package (Ritz and Streibig, 2005). A nonlinear model was used as described in Knezevic *et al.* (2007) and a model lack of fit test was performed. If necessary a Box–Cox data transformation was carried out to achieve homogeneity of variance. The resistance factors (RF) were calculated as the ED50/ED90 of the resistant population divided by the ED50/ ED90 of the sensitive population.

To evaluate the effects of population and time (HAT) on fenoxaprop-P-ethyl and its metabolite fenoxaprop-P a two-factorial analysis of variance (ANOVA) was used, followed by Tukey HSD test at the 5% probability.

ANOVA requirements were checked and if necessary a transformation of data prior to ANOVA was carried out to stabilize the variance. To describe the dynamics of the metabolite fenoxaprop-P a nonlinear pharmacokinetic model was used.

Results

Dose response studies

As can be seen in the dose response curves for the populations ALOMY-S, ALOMY-NTSR1 and ALOMY-NTSR2 dry weight decreased as fenoxaprop-P-ethyl dose increased (Fig. 1). For ALOMY-TSR no curve could be fitted, because the different dosages of fenoxaprop-P-ethyl had no influence on plant development and biomass.

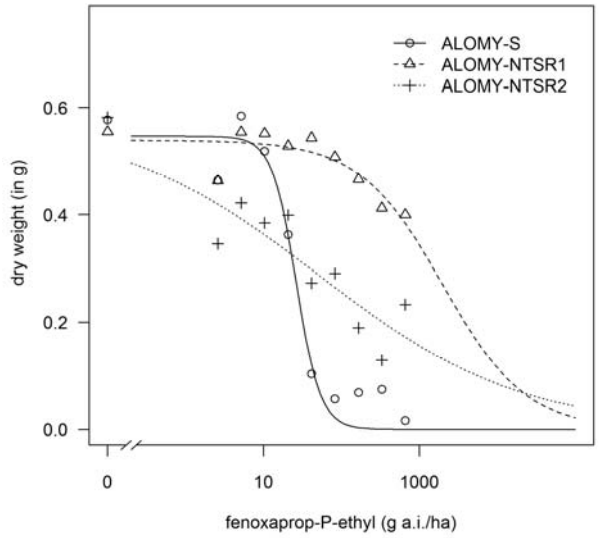

Fig. 1 Effect of fenoxaprop-P-ethyl on dry weight of the populations ALOMY-S, ALOMY-NTSR1 and ALOMY-NTSR2. Each point is the mean of three repetitions.

Abb. 1 *Wirkung von Fenoxaprop-P-ethyl auf das Trockengewicht der Populationen ALOMY-S, ALOMY-NTSR1 und ALOMY-NTSR2. Jeder Punkt stellt den Mittelwert von drei Wiederholungen dar.*

Effective dosages causing a 50 and 90%-reduction in dry weight and corresponding resistance factors calculated from regression curves are listed in Table 1. For ALOMY-S 26.31 g a.i. ha^{-1} were needed to reach ED50 and 57.76 g a.i. ha^{-1} to reach ED90. For ALOMY-NTSR1 both estimates were gained by extrapolation and account for 1996.89 g a.i. ha^{-1} for ED50 (RF 76) and 29937.01 g a.i. ha^{-1} for ED90 (RF 518). For ALOMY-NTSR2 50.81 g a.i. ha^{-1} were needed to reach ED50 resulting in a resistance factor of 2. To reach ED90 38153.02 g a.i. ha^{-1} (extrapolation) were needed, resulting in a resistance factor of 660. The test for lack of fit was non-significant ($p = 0.75$), indicating that data was well described by the model. As can be seen at the curves, the resistance of population ALOMY-NTSR2 became apparent at higher dosages of the active substance. At lower dosages plants of this population reacted more susceptible than plants of the sensitive population ALOMY-S, whereas the resistance of ALOMY-NTSR1 became apparent even at lower dosages.

Tab. 1 Fenoxaprop-P-ethyl ED50- and ED-90 values and corresponding resistance factors of the dose response studies depending on the population.

Tab. 1 *ED50- und ED90-Werte von Fenoxaprop-P-ethyl, sowie entsprechende Resistenzfaktoren der Populationen in den Dosis-Wirkungsversuchen.*

	ED50		**ED90**	
Population	**Estimate (g a.i. ha^{-1})**	**RF**	**Estimate (g a.i. ha^{-1})**	**RF**
ALOMY-S	26.31	1	57.76	1
ALOMY-NTSR1	1996.89	76	29937.01	518
ALOMY-NTSR2	50.80	2	38153.02	660

* Estimates of ED50 and ED90 were calculated from regression curves (Fig.1), RF; Resistance factor

Degradation- and metabolism studies

As can be seen in Figure 2 a degradation of fenoxaprop-P-ethyl was measurable in all populations. The test for lack of fit was non-significant (p = 0.84), indicating that the model is appropriate for data.

ANOVA showed a significant effect of the factor time (p = 0.0001). The mean content of fenoxaprop-P-ethyl was significantly different at all sampling dates. The factor population was a non-significant effect (p = 0.99). In all populations fenoxaprop-P-ethyl degraded within four days (96 HAT).

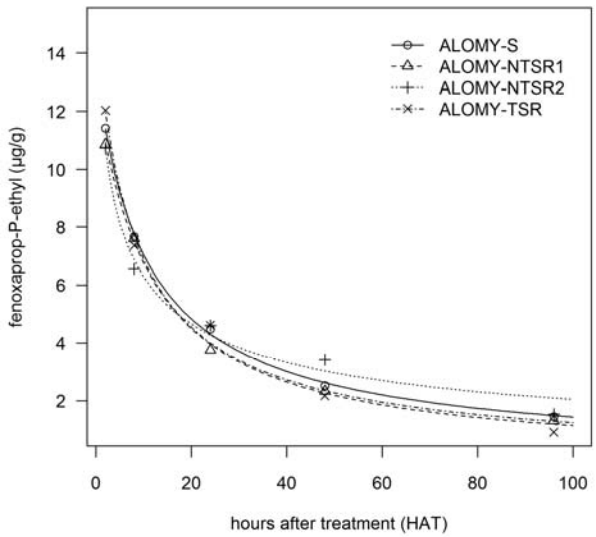

Fig. 2 Degradation of fenoxaprop-P-ethyl in ALOMY-S, ALOMY-NTSR1, ALOMY-NTSR2 and ALOMY-TSR within 96 hours.

Abb. 2 *Abbau von Fenoxaprop-P-ethyl in den Populationen ALOMY-S, ALOMY-NTSR1, ALOMY-NTSR2 und ALOMY-TSR innerhalb von 96 Stunden.*

As shown in Figure 3 metabolite fenoxaprop-P could be detected in all populations. ANOVA showed a significant interaction effect of the factors time and population (p = 0.0005). The mean content at 2 HAT was between 3.05 and 3.38 µg/g and did not significantly differ between the different populations (Tab. 2). The mean content of fenoxaprop-P increased in all populations until 8 HAT. At this time the mean content in ALOMY-TSR (7.75 µg/g) was significantly higher than in ALOMY-S, ALOMY-NTSR1 and NTSR2 (5.6 -5.95 µg/g). At 24 HAT the mean content of fenoxaprop-P was significantly higher in ALOMY-S (7.12 µg/g) and ALOMY-TSR (5.27 µg/g) than in the NTSR-populations (3.09 and 4.6 µg/g). At the two last sampling dates fenoxaprop-P further degraded without significant differences between the populations. At 96 HAT the mean content in ALOMY-S (1.62 µg/g) is around twice as high as in the resistant populations where the metabolite was almost completely degraded (0.62-0.82 µg/g).

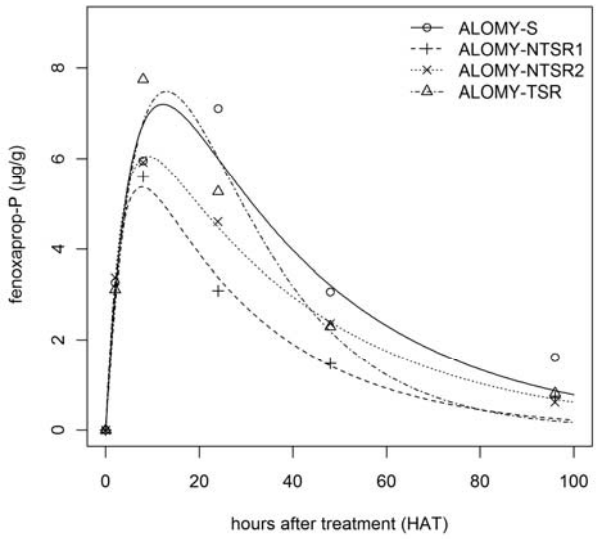

Fig. 3 Dynamics of the metabolite fenoxaprop-P in ALOMY-S, ALOMY-NTSR1, ALOMY-NTSR2 and ALOMY-TSR within 96 hours.

Abb. 3 *Dynamik des Metaboliten Fenoxaprop-P in ALOMY-S, ALOMY-NTSR1, ALOMY-NTSR2 und ALOMY-TSR innerhalb von 96 Stunden.*

Tab. 2 Fenoxaprop-P mean contents of the populations ALOMY-S, -NTSR1, -NTSR2 and –TSR prior to herbicide treatment (0 HAT) and 2, 8, 24, 48 and 96 hours after treatment.

Tab. 2 *Mittlere Gehalte des Metaboliten Fenoxaprop-P in den Populationen ALOMY-S,-NTSR1,-NTSR2 und –TSR vor Applikation (0 HAT) sowie 2, 8, 24, 48 und 96 Stunden nach Applikation.*

	Hours after treatment (HAT)					
	0	**2**	**8**	**24**	**48**	**96**
Population	**Fenoxaprop-P (µg/g)***					
ALOMY-S	0 (± 0) a	3.23 (± 0.21) a	5.95 (± 0.8) a	7.12 (± 0.8) a	3.07 (± 0.49) a	1.62 (± 0.29) a
ALOMY-NTSR1	0 (± 0) a	3.05 (± 0.35) a	5.6 (± 0.9) a	3.083 (± 0.6) b	1.48 (± 0.18) a	0.717 (± 0.2) a
ALOMY-NTSR2	0 (± 0) a	3.38 (± 0.38) a	5.92 (± 1.07) a	4.6 (± 1.08) b	3.48 (± 0.48) a	0.62 (± 0.03) a
ALOMY-TSR	0 (± 0) a	3.12 (± 0.78) a	7.75 (± 0.65) b	5.27 (± 0.7) a	2.3 (± 0.22) a	0.82 (± 0.13) a

*Means within columns (± standard deviation) followed by the same letter are not significantly different according to Tukey's HSD (p=0.05)

Discussion

When regarding the results of dose response studies it can be said that the resistant populations differ among each other when exposed to fenoxaprop-P-ethyl. For ALOMY-NTSR1 and -NTSR2 362 and 461 times the recommended dose would be necessary for the control. For ALOMY-TSR the 8 fold dose of fenoxaprop-P-ethyl didn't even influence biomass production. The mutation at position 1781 is the most common and is known as a strong ACCase mutation (POWLES and YU, 2010). The calculation of ED90-values has shown that the use of recommended dose for fenoxaprop-P-ethyl would be enough to control the sensitive population ALOMY-S sufficiently. Results have shown that it is very important to calculate ED50 and ED90 values, because in some populations, like ALOMY-NTSR2, the resistance becomes apparent only at higher dosages.

Contrary to the expectations the studies have shown that the degradation of the active substance is similar in all populations whether sensitive or resistant and whether the resistance is target-site-based or not. Fenoxaprop-P-ethyl degraded in all populations within four days. The metabolite fenoxaprop-P, known from wheat and soil could be detected in all populations two hours after herbicide treatment. This gives an indication that hydrolysis of the active ingredient has taken place (CHEN *et al.*, 2011). In ALOMY-S the formation and the degradation of the metabolite was slower and at the last sampling date, the mean content was twice as high as in the resistant populations. The dynamics of the metabolite in the NTSR-populations differed significantly from that in the ALOMY-S and ALOMY-TSR population at 24 HAT. At that time the mean content of fenoxaprop-P was significantly lower in the NTSR populations. Results indicate that the formation of the metabolite is not responsible for the differences in sensitivity towards fenoxaprop-P-ethyl, because it was formed in all populations independently whether sensitive or resistant or whether the resistance is target-site-based or non-target-site-based. It should be emphasized that for all populations the ACCase gene has been investigated for mutations at the codon positions 1781, 2027, 2041, 2078 and 2096. It cannot be excluded that in the NTSR populations a rare or an unknown mutation is present. It also cannot be excluded that in the TSR population multiple resistance mechanisms occur. It is known, that some resistant populations have an insensitive ACCase as well as an increased rate of detoxification (COCKER, 1999).

In grasses the plastid ACCase is the target for AOPP herbicides (ABBASPOOR and STREIBIG, 2005). The reason why sensitive populations die off is that the ACCase is essential for lipid biosynthesis. Among other processes the production of reactive oxygen species (ROS) leads to several damaging reactions resulting in plants death. This could be shown for ALOMY-S in the dose response studies. In populations with a target-site resistance the ACCase is altered due to a gene mutation, conferring amino acid change (POWLES and YU, 2010). This means that the active ingredient cannot be effective as mentioned for ALOMY-TSR in the dose response studies, where none of the dosages influenced plant development. In populations with a non-target-site-based herbicide resistance, different mechanisms cause that the amount of herbicide reaching the target is nonlethal (POWLES and YU, 2010). NTSR mechanisms include decreased rates of herbicide translocation, decreased herbicide penetration or increased rates of herbicide metabolism or sequestration. In these studies the focus has been on enhanced herbicide metabolism. It is conceivable that other NTSR mechanisms, like decreased translocation or increased sequestration exist in the populations. Furthermore it is conceivable that other metabolites which could not be identified in the conducted experiments due to the measurement technique occur in the NTSR populations which declare the resistance.

In the study of CUMMINS *et al.* (2009) experiments were carried out to investigate if safeners enhance detoxification pathways. In this study fenoxaprop-P-ethyl was detoxified in wheat and *A. myosuroides* by conjugation with glutathione. Through ester hydrolysis and GST-mediated cleavage, metabolites were formed. Thus, it cannot be excluded, that these metabolites also were formed in the present study.

The dynamics of the metabolite suggest that in the time between 8 and 48 HAT other metabolites were formed. In the next experiments the existence of other unknown metabolites is to clarify using a liquid chromatography with detection via tandem mass spectrometry (LC/MS-MS).

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