Molecular Mechanisms of Flufenacet Resistance in Grass Weeds

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1. List of abbreviations

ABC	ATP-binding cassette
ACCase	Acetyl-CoA carboxylase
ai	Active ingredient
ALS	Acetolactate synthase
Beauv.	First described by Palisot de Beauvois
BLAST	Basic local alignment tool
CDNB	1-Chloro-2,4-dinitrobenzene
CI	Confidence interval
CYP	Cytochrome P450 monooxygenase
DT_{50}	Degradation half-time in plant tissue
DTT	Dithiothreitol
$ED_{50(90)}$	Effective dose rate necessary for 50 (90)% growth reduction
EDTA	Ethylendiaminetetraacetic acid
EPSPS	5-enolpyruvylshikimate-3-phosphate synthase
GFP	Green fluorescent protein
GSH	Glutathione
GSP	Gene-specific primer
GST	Glutathione transferase
GT	Glucosyltransferase
HAT	Hours after treatment
Heynh.	First described by Gustav Heynhold
HPLC	High-performance liquid chromatography
HRAC	Herbicide resistance action committee
Huds.	First described by William Hudson
K_1	HRAC group K_1 (inhibition of microtubule assembly)
K_3	HRAC group K_3 (inhibition of the synthesis of VLCFAs)
KCS	3-ketoacyl-CoA-synthase
Lam.	First described by Jean-Baptiste Lamarck
LC-MS/MS	Liquid chromatography mass spectrometry/mass spectrometry
LOLMU	*Lolium multiflorum* Lam.
LOLRI	*Lolium rigidum* Gaud.
MoA	Mode of action
Moq.	First described by Alfred Moquin-Tandon
MWCO	Molecular weight cut off
N	HRAC group N (Inhibition of lipid synthesis (not ACCase))
NMR	Nuclear magnetic resonance
NTSR	Non-target-site resistance
PDS	Phytoene desaturase
RF	Resistance factor
RI	Resistance index
RNA-Seq	RNA sequencing
Tris	Tris(hydroxymethyl)-aminomethane
VLCFAs	Very-long-chain-fatty acids
VP	Vacuolar peptidase
WSSA	Weed Science Society of America

2. General introduction

2.1 Importance of weed control

Weeds, plants that interfere with the interests of humans (Krähmer and Baur, 2013a), have been evolving in agronomic cropping systems (Baker, 1974; Zohary *et al*., 2012) and farmers have developed methods to control them since Neolithic times (Lal *et al*., 2007). Worldwide, weeds are estimated to cause yield losses of about 34% and may lead to total yield loss (Oerke, 2006). Therefore, successful cropping systems depend on effective weed control for several reasons:

1. They compete with crops for resources *e.g.* nutrients, light and space and therefore considerably reduce the crop yields (Oerke, 2006; Zimdahl, 2007).
2. They hinder the harvest by potentially increasing the harvesting time and wear and tear on machinery (Zimdahl, 2007).
3. The moisture of the weeds can increase the water content of the harvested goods during this process.
4. Particularly, climbing weeds can foster lodging and finally lead to reduced quality and yield (Weaver and Riley, 1982; Gerowitt and Heitefuß, 1990; Nakajima *et al*. 2008).
5. Weeds can contaminate the harvested seeds and, if not cleaned out, may be sown and propagated in the field in the following year (Zimdahl, 2007). Additionally, seeds from toxic weeds *e.g. Agrostemma githago* L., *Datura stramonium* L. or *Solanum nigrum* L. are a danger to human and animal health (Kingsbury 1964; Evers and Link, 1972)

Different forms of hand-weeding and tillage systems in combination with good cultural and sanitation practices as well as crop rotation have been relied on since the beginning of agriculture and still are part of integrated weed management programs. The introduction of synthetic organic herbicides in the late 1940s has changed weed management and cropping systems fundamentally (Kudsk and Streibig, 2003). By today, more than 20 modes of action (MoAs) of commercial herbicides have been identified (Fedke and Duke, 2005; Dayan *et al*., 2015) and offer a cost and time effective alternative to manual and mechanical weeding. The effectiveness of herbicides contributes to increasing yields on limited arable land and fresh water resources (HRAC, 2018). Due to concerns about potential risks of pesticide residues for human health and the environment herbicides go through strict

registration processes, leaving a limited number of compounds available for application in practice (Kudsk and Streibig, 2003). However, the range of use is broadened by optimized formulations or mixtures, which can improve the efficacy of a given compound. The use of herbicide safeners can improve the selectivity by protecting the crop from injury (Rosinger *et al*., 2012). Yet, reliance on the same herbicide MoAs, particularly in combination with less diversified cropping systems, has affected the weed flora (Kudsk and Streibig, 2003; Busi, 2014; Krähmer, 2016a). As an example, weeds such as *Lolium* spp. (ryegrass) and *Alopecurus myosuroides* Huds. (black-grass) have adapted to the new conditions and become increasingly problematic weeds during the last decades (Krähmer, 2016a).

2.2 Herbicide resistance and herbicide detoxification

Herbicide resistance is an adaptive trait and has been defined as “the inherited ability of a plant to survive and reproduce following exposure to a dose of herbicide normally lethal to the wild type; in a plant, resistance may be naturally occurring or induced by such techniques as genetic engineering or selection of variants produced by tissue culture or mutagenesis” by the Weed Science Society of America in 1998 (WSSA, 1998). Resistance can arise in a weed population from mutations or pre-existing genes can spread under selection pressure (Maxwell and Mortimer, 1994), as demonstrated by the detection of a resistance-conferring mutation of the ACCase codon 1781 in an *A. myosuroides* herbarium sample collected in 1888 (Délye *et al*., 2013). The selection of herbicide resistance in weeds in the field depends on many factors *e.g.* ‘gene mutation, initial frequency of resistance alleles, inheritance, weed fitness in the presence and absence of herbicide, mating system, and gene flow’ (Jasieniuk *et al*., 2016), as well as the herbicide dose rate (Neve and Powles, 2005).

In the late 1980s a massive increase in frequency and diversity of herbicide resistance cases was observed in several cropping systems worldwide (Burnet *et al*., 1994a; Heap, 2018), challenging farmers, extension services, authorities and industries to find new answers and solutions for effective and sustainable weed control. While no commercially successful new herbicide modes of action (MoA) were found since that time, various mechanisms conferring resistance to herbicides have been described and make weed (resistance) management increasingly complex. Resistance mechanisms are categorized into target-site resistance and non-target site resistance (NTSR). Target-site resistance comprises target-site mutations (*e.g.* mutations of ALS and ACCase (Powles and Yu, 2010) and increased gene copy numbers of target genes *e.g.* increased EPSPS gene copy number (Gaines *et al*., 2010). NTSR mechanisms range from targeted translocation and vacuolar sequestration (Shaner, 2009; Ge *et al*. 2010) to enhanced metabolism (Délye *et*

al., 2011) as well as other mechanisms *e.g.* rapid cell death response (van Horn *et al*., 2018).

While different resistance mechanisms may occur in a single plant, NTSR may additionally cause unpredictable cross-resistance patterns across different herbicide MoAs including herbicides not yet marketed (Beckie and Tardif, 2012; Délye, 2012; Busi, 2014). Although in certain cases metabolism-based resistance was linked with single nucleotide polymorphisms (Busi *et al*., 2014; Beffa *et al*., 2016), the regulation of NTSR is generally considered a polygenic trait (Délye, 2012; Heap, 2014). Various steps are known to be involved in the detoxification of xenobiotics (see Figure I), starting with activation by hydrolysis or oxidation (phase I), followed by conjugation reactions (phase II) and compartmentation into vacuole and apoplast as well as further processing reactions (phase III) (Coleman *et al*., 1997; Yuan *et al*., 2007). Several enzyme superfamilies involved in these processes have been described. Cytochrome P450 monooxygenases (CYPs) are known to catalyze phase I reactions and their activity has frequently been linked with herbicide resistance *e.g.* in diclofop-methyl resistant *Lolium* populations (Gaines *et al*., 2014; Yu and Powles, 2014).

The activated xenobiotics can follow different pathways depending on their chemical characteristics *e.g.* electrophilic sites and may be detoxified by conjugation to glutathione (GSH) or glucose (Coleman *et al*., 1997; Yuan *et al*., 2007). Direct glutathione conjugation without previous activation by CYPs has previously been shown for several herbicides *e.g.* flufenacet, S-metolachlor, atrazine or ETPC (Lamoureux *et al*. 1970; Ezra and Stephenson, 1985; Bieseler *et al*., 1997; Dixon *et al*., 1997) generally leads to more hydrophilic and less toxic compounds (Coleman *et al*., 1997).

Plant GSTs comprise eight distinct classes including the two largest classes tau and phi, which are frequently reported in the context of detoxification of xenobiotics, as well as theta, zeta, lambda, DHAR, TCHQD and microsomal GSTs (Cummins *et al*., 2011; Cummins *et al*., 2013). Glucosyltransferase (GT) activity, however, has mainly been observed after previous modification of the respective herbicide (Tal *et al*., 1993; Gaines *et al*., 2014). After conjugation by GSTs or GTs, xenobiotics are described to be transferred into the vacuole by ATP-binding cassette (ABC) transporters (Bartholomew *et al*., 2002; Klein *et al*., 2006), where further degradation occurs *e.g.* hydrolysis by vacuolar peptidases (Wolf *et al*., 1996; Chronopoulou *et al*., 2017). The regulation and the functions of these processes as well as the role of the individual isoforms are poorly understood. However, first steps towards the understanding of the regulation of resistance genes were made by the analysis of the location of differentially overexpressed genes found in multiple-resistant *Amaranthus tuberculatus* Moq. populations. So-called ‘hotspots’ with high densities of upregulated

genes were identified, suggesting upregulation of entire chromosome sections (Tranel, 2018). Corresponding mechanisms in grass weeds have not yet been described.

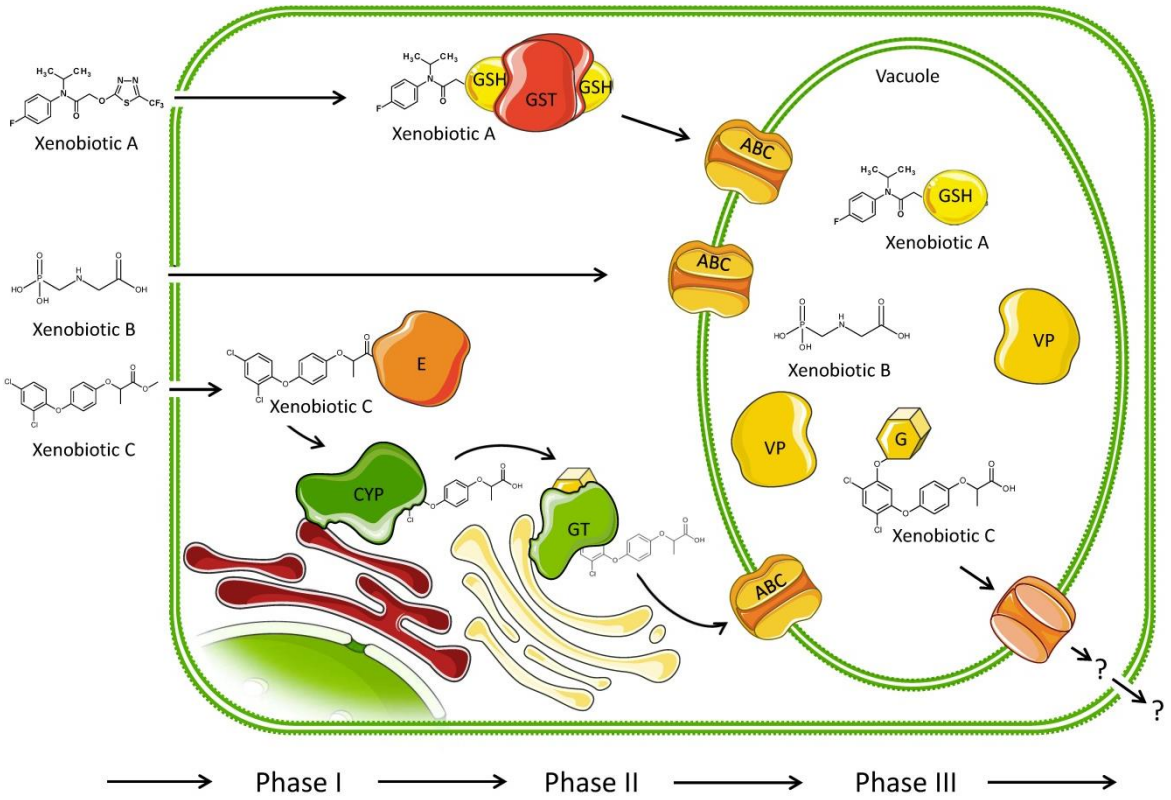

Figure I: Detoxification of xenobiotics in plants using the examples flufenacet (xenobiotic A), glyphosate (xenobiotic B) and diclofop-methyl (xenobiotic C). The pathways include hydrolysis and hydroxylation catalyzed by esterases (E) and cytochrome P450 monooxygenases (CYPs) in phase I, conjugation with glutathione (GSH) and glucose (G) catalyzed by glutathione transferases (GSTs) in phase III, transport into the vacuole *e.g.* via ABC-transporters (ABC) and further degradation by vacuolar peptidases (VPs) as well as further catabolism and compartmentation in phase III. Adapted from Coleman *et al*. (1997); Yuan *et al*. (2007); Gaines *et al*. (2014); Sammons and Gaines (2014) and Dücker *et al*., 2019b).

2.3 Resistance to inhibitors of the synthesis of VLCFAs in *Lolium* spp. and *Alopecurus myosuroides*

Lolium spp. and *A. myosuroides* are representative examples of grass weeds with the ability to accumulate resistance mechanisms. In *L. rigidum* and *L. multiflorum* Lam. populations resistant to 14 and 8 herbicide MoAs have been described, respectively, while in *A. myosuroides* resistance to 7 different herbicide MoAs was found (Heap, 2018). The term

'population', herein below defines a representative sample of a weed species collected within a field in a given year. The described resistance cases also comprise populations with resistance to herbicide MoAs to which resistance has evolved at slower rates *e.g.* the inhibition of the synthesis of very-long-chain fatty acids (VLCFAs, HRAC classification K_3) (see Figure II). Interestingly, resistance to the inhibitor of the synthesis of VLCFAs flufenacet has, so far, only been observed in multiple-resistant populations of *Lolium* spp. and *A. myosuroides* (Rauch *et al*., 2010; Hull and Moss, 2012; Rosenhauer and Petersen, 2015). However, the level of resistance differs between the species. In *Lolium* spp. field relevant levels of flufenacet resistance with high resistance factors (RFs) have been described (Rauch *et al*., 2010; Dücker *et al*., 2016; Dücker *et al*., 2019b) in the Northwest of the USA, while the reduced efficacy observed for European *A. myosuroides* field populations ranged within the so-called 'low-level resistance' according to Heap (2005) with RFs below 10 (Hull and Moss, 2012; Rosenhauer and Petersen, 2015; Dücker *et al*., 2019b). However, targeted recurrent selection of initially pendimethalin resistant *A. myosuroides* with 180 flufenacet ha^{-1} in an outdoor pot trial led to a decrease in flufenacet efficacy of 5-7% per year (Hull and Moss, 2012).

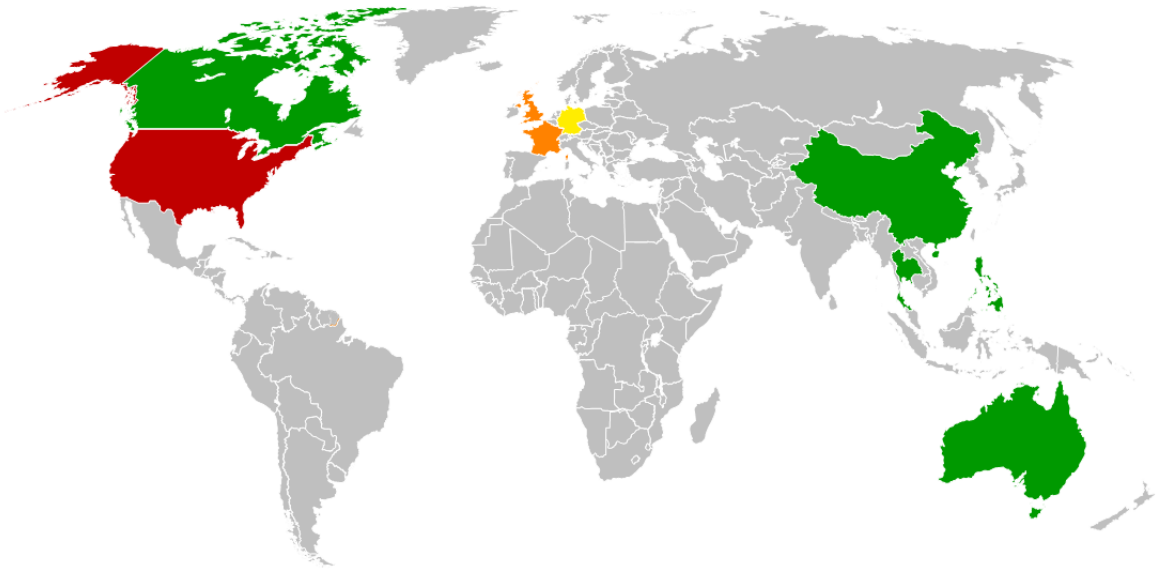

Figure II: Distribution of resistance to herbicides inhibiting the synthesis of VLCFAs. ■ Flufenacet resistant *Lolium* spp. ■ Flufenacet resistant *Lolium* spp. and reduced flufenacet efficacy on *Alopecurus myosuroides* ■ Reduced flufenacet efficacy on *Alopecurus myosuroides* ■ Resistance to other herbicides inhibiting the synthesis of VLCFAs ■ No resistance to inhibitors of the synthesis of VLCFAs reported (Busi, 2014; Heap, 2018; Dücker *et al*., 2019b).

2.4 Biology of *Lolium* spp. and *Alopecurus myosuroides*

The grass weeds *Lolium* spp. and *A. myosuroides* have similarities, which suggest that the development of flufenacet resistance in these species may be partly linked with their biology (see Table I). Both species have large genomes despite a chromosome number of 2n=14 (Bowen, 1962; Stewart *et al*., 2009; Byrne *et al*., 2015). They are wind-pollinated and obligate outbreeders due to self-incompatibility systems (Chauvel and Gasquez, 1994; Neve *et al*., 2014). These characteristics promote high genetic variability and rapid accumulation of resistance genes under selection pressure (Délye, 2012; Neve *et al*., 2014). In addition, *Lolium* spp. often occur as mixed populations, are highly inter-fertile and hybrids often reach reproduction rates similar to intra-species pollination and may even cross-pollinate with *Festuca* spp. (Charmet *et al*., 1996; Yamada *et al*., 2005). The outcrossing nature of *Lolium* spp. facilitates inter-specific flow of resistance genes. Because of the high frequency of hybrids, populations belonging to the genus *Lolium* are herein below not assigned to individual species but regarded as '*Lolium* populations'.

Table I: Comparison of biological characteristics of *Alopecurus myosuroides* and *Lolium* spp.

Biological characteristics	*Lolium* spp.	*Alopecurus myosuroides*
Probable origin	Central and Southern Europe, North-west Africa and South-west Asia (Hubbard, 1968)	Europe and the Mediterranean area (van Himme and Buckle, 1975)
Occurrence	Predominatly in warmer climates *e.g.* Mediterranean climate (Krähmer, 2016b)	Winter annual crops in temperate Europe (Naylor, 1972a; Krähmer and Baur, 2013b)
Fertilization	Obligate outcrossing (Terrell 1968; Yamada *et al*., 2005)	Obligate outcrossing (Chauvel and Gazques, 1994)
Hybridization	Hybridizes freely within the genus (Charmet *et al*., 1996)	Leads to reduced pollen fertility and can disturb meiosis (Sieber and Murray, 1981)
Chromosome number	2n =14 (Kattermann, 1930)	2n = 14 (Kattermann, 1930)
Genome size	2068 Mb (Byrne *et al*., 2015)	Expected genome size between 1200 and 4330 Mb (Bowen, 1962; Stewart *et al*., 2009)
Multiple resistance	Up to 14 MoAs (Heap, 2018)	7 MoAs (Heap, 2018)
Flufenacet resistance factor (RF)	Up to 46 (Dücker *et al*., 2016)	Up to 6 (Rosenhauer and Petersen., 2015)

Besides similar propagation characteristics, both species are claimed to be native to a large area around Europe and the Mediterranean Sea and are well-adapted to wheat-dominated cropping systems. Although *Alopecurus* as a genus is common throughout Eurasia (see Figure III), the winter annual grass weed *A. myosuroides* has become a predominant weed in winter annual cereals particularly in temperate Europe (Naylor, 1972a; Krähmer, 2016b). It is known to be competitive on moist, medium-heavy to heavy soils with high percentages of clay and silt, but also occurs on a wide range of different soil types (see Figure IV A, Ellenberg and Leuschner, 2010; Krähmer, 2016c) where it often reaches densities of several hundreds or even thousands of plants per m^2 and over 50 000 seeds per m^2 (Moss, 1983; Krähmer and Baur, 2013b). Grown as fodder crop, *Lolium* spp. have been introduced worldwide as weeds (see Figure IV B) and occur primarily in maritime climate as a predominant weed (see Figure III) where seed production rates of 31 000 to 45 000 seeds per m^2 have been reported (Rerkasem *et al.*, 1980). Yet, with about 80-200 seeds per head (Moss, 1983; Naylor, 1972b) and > 90% seed degradation in soil within four to six years (Lewis, 1958; Moss, 1985) both *A. myosuroides* and *Lolium* spp. are in the mid-range of seeds produced per plant and longevity of seeds. Also economic thresholds, more than a decade ago, were with 15.35 plants per m^2 estimated in the mid-range in comparison to other weed species (Zanin *et al*., 1993; Mennan *et al*., 2003). Particularly, if the resistance status of an individual weed population is considered, today's thresholds for *A. myosuroides* and *Lolium* spp. may differ from these numbers as reduction of the soil seedbank becomes increasingly important and crop production and tillage systems as well as herbicide ranges are typically adapted to the resistance situation (Norsworthy *et al*., 2014; Peterson *et al*., 2017).

2.5 Flufenacet, an oxyacetamide herbicide inhibiting the biosynthesis of VLCFAs

The oxyacetamide flufenacet is an herbicide which has increasingly gained importance for the control of grass weeds and small-seeded dicots, particularly in winter cereals in Europe (Menne *et al*., 2012; Krähmer *et al*., 2019). As a compound inhibiting the synthesis of VLCFAs its herbicide resistance risk is considered low (Moss *et al.,* 2019) and as such it is frequently used for the control of grass weeds already resistant to the typical herbicide MoAs used in post-emergence applications. This includes species *e.g. A. myosuroides*, *Lolium* spp. or *Apera spica-venti* L. (Bailly *et al*., 2012; Hull and Moss, 2012). In contrast to its primary use today, flufenacet was synthesized based on the structure of the paddy herbicide mefenacet in 1988 and selected based on activity and selectivity and finally launched in 1997 as a pre-emergence herbicide for the use in soybeans and corn (Bieseler *et al*., 1997; Watanabe, 2012). The activity of flufenacet has been suggested to be based on the

susceptibility of the methylene bridge of the oxyacetamide structure to a nucleophilic attack of the thiol group of the conserved cysteine in the active center of the target enzyme. The resulting reaction is suggested to lead to a covalent binding of the herbicide and the target and a split-off of the hydroxythiadiazole residue (Böger *et al*., 2000).

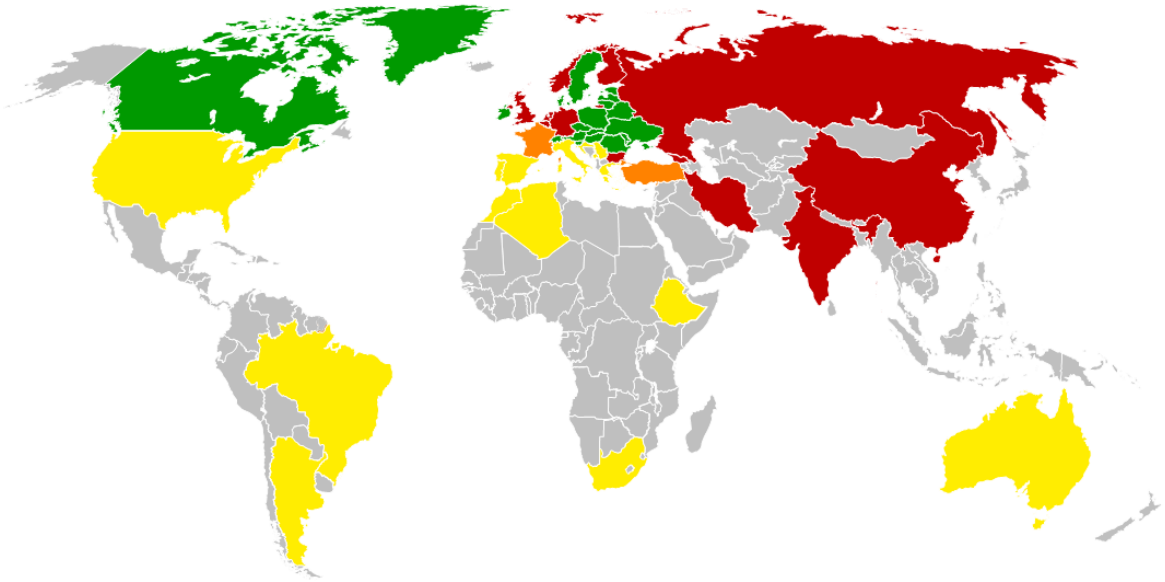

Figure III: Distribution *Alopecurus* spp. and *Lolium* spp. in cereals. ■ *Alopecurus* spp. among the three most frequent monocotyledonous weeds in cereals ■ *Alopecurus* spp. and *Lolium* spp. among the three most frequent monocotyledonous weeds in cereals ■ *Lolium* spp. among the three most frequent monocotyledonous weeds in cereals ■ Neither *Alopecurus* spp. nor *Lolium* spp. among the most frequent monocotyledonous weeds in cereals ■ No data available (modified according to Krähmer, 2016b). The data refer to at least one cereal growing region per country.

3-ketoacyl-CoA-synthases (KCSs) are a group of plant-specific enzymes catalyzing the rate-limiting condensing step of the elongation of VLCFAs in the fatty acid elongation complex (Haslam and Kunst, 2013). In *Arabidopsis thaliana* Heynh. it was shown that individual isoforms are expressed in different tissues in different growth stages (Joubès *et al*., 2008). They catalyze the elongation of substrates with different chain lengths and degrees of saturation with partly overlapping substrate spectra (Trenkamp *et al*., 2004; Haslam and Kunst, 2013). The inhibition of these functions leads to a lack of VLCFAs, necessary for various functions in the plant, including the protective function of the cuticle, the stability of highly curved membranes and processes during cell division (Schneiter *et*

al., 2004; Bach *et al*., 2011). The resulting disturbance of the cellular functions leads to reduced cell division, slower growth rates and typical organ fusions and loop formations (see Figure IV C), which can finally cause plant death, particularly during early plant development (Lechelt-Kunze *et al*., 2003; Krähmer *et al.*, 2019).

Figure IV: Heavy *Alopecurus myosuroides* infestation in a wheat field in Northern German Marshes near Cuxhaven (A). Volunteer *Lolium multiflorum* Lam. in a rye field in northern Germany (B). Characteristic flufenacet symptoms of a *Lolium rigidum* plant treated with 15 g flufenacet ha^{-1}.

2.6 Aim of the study

The extent of flufenacet resistance, particularly in European *Lolium* populations, as well as its mechanism in weeds has not yet been investigated. The aim of the study was to elucidate of the molecular mechanisms involved in flufenacet resistance of *A. myosuroides* and *Lolium* spp. For that purpose the concept of the study comprised the estimation of the level of flufenacet resistance in *A. myosuroides* and *Lolium* spp. with a limited number of field populations in a dose-response screening and the selection of suitable populations to investigate the resistance mechanism using analytical methods (HPLC, LC-MS/MS) and protein assays. Finally, candidate genes involved in flufenacet resistance in *Lolium* spp. were identified using an RNA-Seq approach (Illumina sequencing) including bioinformatic analyses and subsequent validation of recombinant candidate proteins *in vitro*.

The knowledge about the level and spread of flufenacet resistance can create awareness of evolving flufenacet resistance in *A. myosuroides* and *Lolium* spp. and thus can allow farmers to take action to prevent resistance from evolving. The knowledge about cross-resistance patterns and the molecular mechanisms of flufenacet resistance may help understanding the evolution of flufenacet resistance and finding targeted weed management programs (Beckie and Tardif, 2012). Finally, the molecular understanding of flufenacet resistance provides a basis for improvement of crop protection compounds.

3. Glutathione transferase plays a major role in flufenacet resistance of ryegrass (*Lolium* spp.) field populations

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Abstract

BACKGROUND: Herbicides inhibiting the synthesis of very long-chain fatty acids (HRAC group K_3, WSSA group 15), such as flufenacet, play an important role in weed management strategies, particularly when herbicide resistance to inhibitors with other modes of action, such as acetolactate synthase or acetyl coenzyme A carboxylase (ACCase), has already evolved. So far, only a few cases of resistance towards inhibitors of the synthesis of very long-chain fatty acids have been described. In this study, we characterized the level of flufenacet resistance in several *Lolium* spp. field populations and investigated the resistance mechanism.

RESULTS: The screening for flufenacet resistance revealed the ability of *Lolium* spp. populations from several continents to survive flufenacet treatments at and above the field rate. This study demonstrates the way in which flufenacet is detoxified in resistant weed populations. Glutathione was found to be conjugated to flufenacet in *Lolium* spp. seedlings, and there was evidence that glutathione transferase activity was enhanced in protein extracts from flufenacet-resistant seedlings. A significant correlation was found between the resistance factor obtained by biotests and the degradation half-time of flufenacet in ryegrass plants obtained by high-performance liquid chromatography (HPLC).

CONCLUSION: At present, flufenacet resistance is not widespread; however, in certain *Lolium* spp. populations resistance levels could reach agronomic relevance due to detoxification by glutathione transferases. In Europe especially, only a few herbicide modes of action are registered for the control of *Lolium* spp. and therefore it is becoming increasingly important to apply best management practices to prevent the spread of flufenacet resistance.

Keywords

Weed resistance, non-target-site herbicide resistance, enhanced metabolism, flufenacet, ryegrass, glutathione transferases

3.1 Introduction

Members of the obligate out-crossing and genetically diverse genus of ryegrasses (*Lolium* spp.) occur worldwide and are among the most noxious weeds in cereals. Besides their competitiveness under different environmental conditions, they have displayed the ability to adapt to the application of modern crop protection compounds by evolving cross-resistance to a broad range of herbicide chemistries and modes of action (MoAs) (Burnet *et al*., 1994a; Powles and Yu, 2010; Yuan *et al*., 2007).

Within the species *L. multiflorum* Lam. resistance against eight herbicide MoAs has evolved in total (Heap, 2019). Similarly, within *L. rigidum* Gaud., resistance against 14 herbicide MoAs has evolved (Burnet *et al*., 1994a; Heap, 2019). No other genus has evolved resistance against such a diversity of chemical classes. As an example, resistance to at least nine different chemical classes has been found in a single *L. rigidum* population (Burnet *et al*., 1994a). Many of these cases of resistance are based on enhanced metabolism, which can confer unpredictable cross-resistance to other herbicides on the market and even to new chemical classes which have not yet been commercialized (Yuan *et al*., 2007; Preston *et al*., 1996; Délye *et al*., 2011). As a result, more complex weed management practices need to be implemented (Moss *et al*., 2007; Norsworthy *et al*., 2012). Ensuring high efficacy of pre-emergence treatments becomes increasingly important where reliability of post-emergence treatments, *e.g.* with acetolactate synthase (ALS) and acetyl coenzyme A carboxylase (ACCase) inhibitors, respectively herbicide resistance action committee (HRAC) group B and A (WSSA (Weed Science Society of America) group 2 and 1), decreases due to the development of resistance (Bailly *et al*., 2012). With only few cases of resistance in spite of decades of intensive usage, herbicides inhibiting the synthesis of very long chain fatty acids (VLCFAs, HRAC group K_3, WSSA group 15) are increasingly used in pre-emergence applications in weed management strategies. In temperate Europe, the use of the oxyacetamide flufenacet has become a particularly valuable tool for the management of difficult-to-control grass weed populations, particularly black-grass (*Alopecurus myosuroides* Huds.) (Hull and Moss, 2012).

More recently, flufenacet has increasingly been used for the control of *Lolium* spp. populations. However, in the Mediterranean climate zone, where this genus is particularly competitive, sufficient soil moisture for optimum efficacy of the herbicide is not always present. In addition to varying levels of efficacy due to environmental conditions, the

reduced level of activity of the chloroacetamide herbicides metolachlor, alachlor and propachlor on the Australian *L. rigidum* population was described in the early 1990s (Burnet *et al*., 1994a; Burnet *et al*., 1994b). More than 10 years later, flufenacet resistance was also reported in *L. multiflorum* populations from the northwest USA (Rauch *et al*., 2010). These species represent two out of five weed species which have evolved resistance to inhibitors of the synthesis of VLCFAs, as well as to other herbicides (Heap, 2019; Busi 2014). Limited chemical options remain for controlling multiple resistant *Lolium* spp. populations due to resistance and restrictions in registration.

It is therefore important to understand the extent and development of this resistance, including the mechanism behind it, in order to adopt effective weed management strategies and prevent resistance evolution effectively (Délye *et al.*, 2011). The understanding of the molecular targets, 3-ketoacyl-CoA synthases (KCS), which catalyze the elongation of fatty acid chains (C>20), allows a first understanding of resistance to inhibitors of the synthesis of VLCFAs. They occur in different isoforms with partially overlapping substrate specificities and distinct expression patterns (Trenkamp *et al*., 2004). Target-site resistance has previously been regarded as unlikely to occur, since K_3 herbicides have been shown to inhibit several KCS isoforms in *Arabidopsis thaliana* (L.) Heynh. Consequently, concomitant mutations of several isoforms of the target enzymes would be required to cause target-site resistance (Trenkamp *et al*., 2004; Böger *et al*., 2000; Krähmer *et al*., 2019).

Besides this, the mechanism of flufenacet resistance has not yet been investigated in weeds. However, crop tolerance was found to be caused by two different metabolic detoxification pathways: detoxification by glutathione (GSH) conjugation and the formation of flufenacet oxalate via an intermediate flufenacet alcohol metabolite (Gould *et al*., 1997; Bieseler *et al*., 1997). In addition to this, metabolic resistance to the K_3 herbicide pyroxasulfone has recently been observed in an Australian *L. rigidum* population (Busi *et al*., 2018). Pyroxasulfone resistance has been shown to co-evolve with *S*-metolachlor and prosulfocarb resistance (Busi and Powles, 2016).

In the present study we will describe a new unique case of flufenacet resistance in *Lolium* spp. populations from the UK and France: Additionally, we detected flufenacet resistance in a commercially available VLR69 (Herbiseed, Twyford, UK) population originating from Australia (Burnet *et al*., 1994a) and investigated the mechanism causing the resistance observed.

3.2 Materials and methods

3.2.1 Plant cultivation and greenhouse bioassays

Between 2011 and 2016 751 *Lolium* spp. field populations from France and 94 *Lolium* spp. populations from the UK were collected within the frame of in-house complaint handling, based on ALS and ACCase herbicide failure. The seeds were stored at 5°C for several weeks, sown in pots containing sandy loam (two replicates) and covered with coarse sand. Three days after the first watering the seedlings were treated with Herold® SC (240 g flufenacet + 120 g diflufenican ha^{-1}) on a laboratory track sprayer (teejet nozzle XR8001, 300 L ha^{-1}, 2 bar) and kept in a greenhouse, with 22/16°C day/night temperatures with a 14 h photoperiod provided by Philips Master HPI-T plus 400 W/645 E40 metal halide lamps at approximately 200 µmol m^{-2} s^{-1}. Herbicide efficacy was rated 28 days after treatment. Three UK and four French field populations were selected for further studies (see Table 1).

In a first step, these populations were tested in a screening experiment for comparison with six *Lolium* spp. populations from fields in the northwest USA where reduced flufenacet efficacy was observed, as well as two multiple resistant *Lolium* spp. populations originating from Australia and several sensitive populations of different origins (see Table 1).

In order to ensure homogenous growth stage and reduce variation due to dormancy and numb seeds, pre-germinated seedlings were transplanted. The seeds of these populations were therefore grown on solidified water agar (0.7% w/v) until the primordial root emerged. Subsequently, 25 individual plants were transplanted as replicates into pots containing sandy loam with 2.2% organic matter. Each pot contained five seedlings and was subsequently covered with a thin layer of coarse sand. The pots were then treated with dose rates of 0, 1.5, 5.9, 23.75, 95, 380, 1520, 6080 and 24320 g flufenacet ha^{-1}, formulated as Cadou® SC on a laboratory track sprayer (teejet nozzle XR8001, 300 L ha^{-1}, 2 bar). After treatment, the pots were irrigated once from above and subsequently kept in a greenhouse, with 22/16°C day/night temperatures with a 14 h photoperiod provided by Philips Master HPI-T plus 400 W/645 E40 metal halide lamps at approximately 200 µmol m^{-2} s^{-1}. The foliage fresh weight of the individual plants was assessed 28 days after treatment.

3.2.2 Determination of flufenacet degradation rates in sensitive and resistant seedlings

Seedlings of the populations LOLMU-S, LOLRI-S, FRA1-S, USA1-R, VLR69-R and FRA1-R (see Table 1) were raised on solidified water agar (0.7% w/v) in a growth chamber at 22/16°C day/night conditions, with a 14 h photoperiod provided by Philips Master TL-D 58W/840 REFLEX fluorescent lamps at approximately 400 µmolm^{-2} s^{-1}. The plants were

treated as the first leaf reached a length of about 2.5 cm. Two sets of 32 seedlings per population and time point were placed in 20 mL glass vials containing 1.2 mL 0.02 M KNO_3 mineral water (Volvic, Volvic, France) with 7.5 µM ^{14}C-radiolabeled flufenacet giving a final activity of 16.7 mBq mL^{-1}. The vials were carefully shaken and incubated at 22°C under light conditions. The seedlings were harvested 1, 2, 3, 4, 8 and 16 h after treatment, washed twice in water and once in 50% acetone. Each eight seedlings were dried, pooled and subsequently frozen in 100% methanol (four pooled biological replicates per population and time point). An extract was made, as described by Collavo *et al*. (2015) with an additional extraction step, with 600 µL 90% acetonitrile and subsequent vaporization of the supernatant and resuspension in 200 µL 80% acetone. Volumes of 90 µL were injected into a HPLC system and separated with a 250 × 4.6 mm Synergi™ 4 µm Hydro-RP 80 Å, LC column (Phenomenex, Aschaffenburg, Germany) at 40°C. The gradient was performed over 50 min with mobile phases A (0.1% formic acid (w/v)) and B (0.1% formic acid (w/v) in 98% acetonitrile) at a flowrate of 0.4 mL min^{-1}. A 3.3 min equilibration period with 5% solvent B was followed by a 30 min linear gradient from 5% to 40% solvent B, and a 3.3 min linear gradient from 40% to 100% solvent B. After a 3.3 min plateau, with 100% solvent B and a 1.6 min linear gradient from 100 to 5% solvent B, the method ended with an 8.3 min equilibration period.

3.2.3 Flufenacet metabolite identification in sensitive and resistant seedlings

In order to identify flufenacet metabolites by liquid chromatography tandem mass spectrometry (LC–MS/MS), seedlings of the populations LOLMU-S, LOLRI-S, FRA1-S, USA1-R, VLR69-R and FRA1-R were grown and treated as described in section 2.2. The seedlings were kept at 12°C and were harvested 1, 2, 3, 4, and 24 h after treatment. Additionally, the populations LOLMU-S and USA1-R were treated as described in section 3.2.2, kept at 22°C and harvested 24 h after treatment. Extracts were taken as described above. LC–MS/MS analysis of these samples was performed on a Waters Q-ToF Premier mass spectrometer (Waters, Manchester, UK) connected to a Waters 2795 HPLC System (Waters, Milford, USA) via a FlowStar LB513 radioactivity detector (Berthold Technologies, Bad Wildbad, Germany) and an electrospray interface. Chromatographic separation was achieved after injection of 50 µL of each sample, as described in section 2.2, with an extension of the plateau with 100% solvent B from 3.3 to 8.3 min. Ionization was achieved by an electrospray interface operating in the positive and negative ion mode. Instrument control and data evaluation was done with MassLynx® 4.1 (Waters). Compound identities were confirmed by high-resolution mass spectrometry (MS) (determination of the elemental

composition of molecular ions and fragment ions) in the MS and MS/MS mode (product ion scan). Control measurements containing no herbicide and no plant extract were included.

3.2.4 GST activity in sensitive and flufenacet resistant seedlings

With the purpose of measuring glutathione transferase (GST) activity (workflow displayed in Figure 1), seeds of the populations LOLMU-S and USA1-R were sterilized for 20 s in 70% ethanol and subsequently for 20 min in 2.5% sodium hypochlorite. The disinfected seeds were rinsed three times with sterile tap water, and finally dried on filter paper.

The seeds were sown under sterile conditions in plant tissue culture containers (MP Biomedicals, Eschwege, Germany) containing 80 g 4 mm glass beads and 9 mL 0.02 M KNO_3 mineral water, and kept in darkness at room temperature. Etiolated seedlings of both populations were frozen in three subsets, in liquid nitrogen, when the first leaf reached a length of 4–5 cm.

The seeds were removed, and the frozen tissue was subsequently ground into a fine powder (3 × 18 g per population). The ground tissue was vortexed with 117 mL extraction buffer (100mM Tris–HCl (pH 7.5), 1 mM ethylenediamine tetraacetic acid (EDTA), 100mM NaCl, 1 mM dithiothreitol (DTT), 4% polyvinylpyrrolidone (PVP), complete protease inhibitor (Calbiochem, Darmstadt, Germany)), homogenized for 20 s using an Ultra Turrax® blender (IKA, Staufen, Germany) and filtered through two layers of Miracloth. The extract was then centrifuged at 48000 *g* for 20min at 4°C and filtered again through four layers of Miracloth. The protein content was quantified using the Bio-Rad Protein Assay (Bio-Rad, Munich, Germany).

Due to low seedling weight large amounts of limited seed material were necessary to obtain sufficient amounts of plant material. The available tissue was sufficient to identify GSH conjugates in enriched protein extracts by LC–MS/MS; however, quantification was not possible. Therefore, two complementary experiments were conducted to estimate GST activity in sensitive (LOLMU-S) and flufenacet resistant (USA1-R) *Lolium* spp. populations. In a first step the GST activity of the crude extracts obtained from LOLMU-S and USA1-R was measured in a 200 µL reaction mix containing 20 µg total protein in 100 µL extraction buffer and 100 µL sodium phosphate buffer (100mM NaH_2PO_4/Na_2HPO_4, pH 6.5) containing 1 mM glutathione (GSH) and 1 mM 1-chloro-2,4-dinitrobenzene (CDNB). Absorbance after excitation at 340 nm was measured for 8 min with a CLARIOStar®microplate reader (BMG Labtech, Ortenberg, Germany). The optical density (OD) values were corrected using blank measurements without protein.

In a second step, the obtained crude extracts were loaded on a GSTrap™FF column (5 mL, GE Healthcare, Piscataway, NJ, USA), equilibrated with 50mL sodium chloride-Tris-EDTA

(STE) buffer (100mM Tris–HCl, pH 7.5) containing 1 mM EDTA and 100mM NaCl, at a flow rate of 1 mL min^{-1}. The protein was eluted with 5 mL elution buffer (100mM Tris–HCl, pH 7.5) containing 1 mM EDTA, 100mM NaCl and 10 mM GSH, desalted on a PD10 column (5 mL, GE Healthcare, Piscataway, NJ, USA) equilibrated with 25mL sodium phosphate buffer (100mM NaH_2PO_4/Na_2HPO_4, pH7.0) containing 1 mM EDTA, 100mM NaCl and 10 mM GSH, and finally eluted with 3.5 mL sodium phosphate buffer (pH 7.0). The eluate was concentrated using Amicon Ultra®-15 Centrifugal Filters (50mL, 10 KDa MWCO, EMD Millipore, Darmstadt, Germany) at 4000 *g* for 30 min.

A negative control and each 45 µL of the enriched protein were incubated for 4 and 24 h with 5 µL 10 mM flufenacet in ethanol, 45 µL sodium phosphate buffer (100mM NaH_2PO_4/Na_2HPO_4, pH 6.5) and GSH at a final concentration of 1 mM. The reaction was stopped by adding twice the volume of acetonitrile prior to centrifugation at 17.900 *g*.

The product of the GST reaction was further characterized using HPLC-MS/MS analysis. Flufenacet and its corresponding GSH conjugate were separated on a reversed-phase column with polar endcapping (Phenomenex Synergi™ Polar-RP, 50 × 2 mm, 4 µm), using an acetonitrile gradient 15–95% in 0.1% formic acid for 10 min at 300 µLmin^{-1} flow. Mass spectra were recorded on a triple-quadrupole mass spectrometer (TSQ Quantum Access, Thermo Fisher Scientific, San Jose, CA, USA) operated in positive electrospray ionization (ESI) ionization mode. GSH conjugate was identified by a neutral loss of 129 mass units from the parent molecular ion [M+H]+, m/z=501, after fragmentation at 30% relative collision energy. The Δm of 129 mass units represents the loss of pyroglutamate from the GSH tripeptide.

3.2.5 Statistical analyses

Resistance characterization, with dose–response assays and flufenacet degradation, was analyzed using the drc package (Ritz *et al*., 2015) of the statistical R software (version 3.4.3, R Foundation for Statistical Computing). A three-parameter log-logistic model was fitted to the square-root-transformed fresh weight data of 25 individual plants per population and treatment. Effective dose rates of flufenacet (ED_{50}, ED_{90}) and standard errors were calculated for each *Lolium* spp. population. Differences between populations were displayed as resistance factors (RFs). In this case, RF is defined as the ratio of estimated ED_{50} values of an individual population and the average of ED_{50} values of susceptible reference populations.

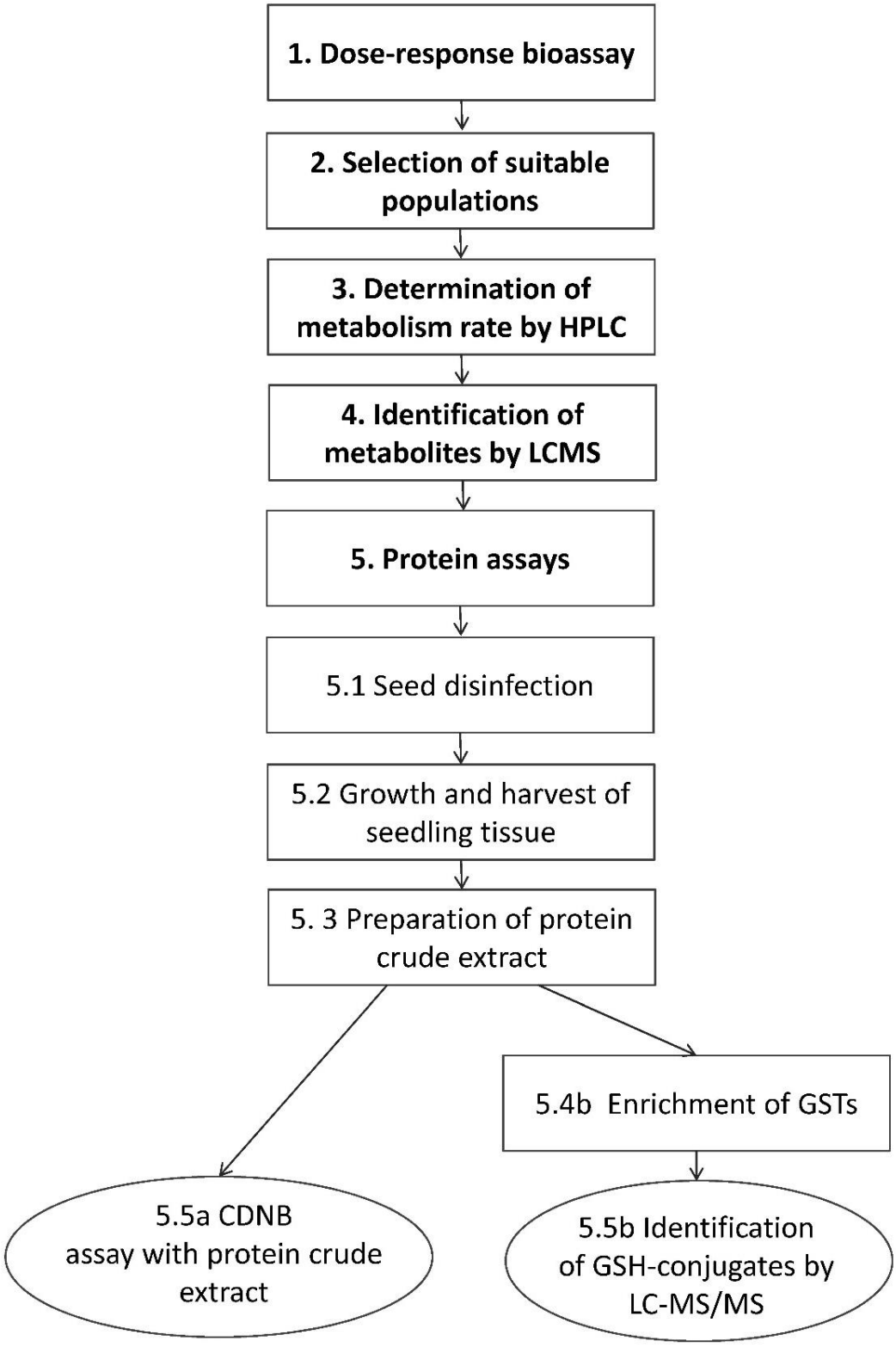

Figure 1: Experimental workflow including the characterization of GST activity in protein extracts obtained from populations LOLMU-S and USA1-R.

A three-parameter log-logistic model was fitted to the percentage of flufenacet detected by HPLC in each of four pooled samples per time point and population using the drc package (Ritz *et al*., 2015) of the statistical R software.

The time necessary for 50% degradation of the parent compound (DT_{50}) and corresponding standard errors was calculated for each population tested. Resistance indices (RIs) were calculated as the ratio of estimated DT_{50} values of an individual population, and the average of the DT_{50} values of susceptible reference populations.

The *t*-test of the statistical R software was conducted under the null hypothesis of no difference between the CDNB turnover rates in crude extracts from the sensitive population LOLMU-S versus the flufenacet resistant population USA1-R (three replicates each).

3.3 Results

3.3.1 Flufenacet resistance in different *Lolium* spp. field populations

In a first step, *Lolium* spp. field populations collected between 2011 and 2016 were sprayed with 240 g flufenacet and 120 g diflufenican ha^{-1}. Among the 94 samples from the UK, 7.4% of populations tested survived the treatment, but only 0.5% of the 751 French populations did. In a second step, flufenacet efficacy on *Lolium* spp. samples from France, the UK, Australia and the USA as well as on sensitive reference populations was assessed with full dose–response bioassays. With ED_{50} values ranging from 3.6 to 17.8, and ED_{90} values between 6.8 and 59.0 g flufenacet ha^{-1} all of the sensitive reference populations (commercially available and field samples) were well controlled with typical field rates of 240 g flufenacet ha^{-1}, depending on crop and country of origin (see Table 1). Furthermore, the suspected resistant populations FRA3-R, FRA4-R, USA2-R and USA4-R were well controlled, with estimated ED_{90} values of 110 g flufenacet ha^{-1}.

All other suspected resistant populations originating from different regions of France, the UK and the USA, as well as the Australian population VLR69, could not be controlled with the typical field rates applied in cereals, as indicated by their ED_{90} values, which exceeded 240 g flufenacet ha^{-1}. The ED_{50} values of populations GBR3-R, GBR1-R, FRA1-R and USA5-R also exceeded the field rate, which resulted in resistance factors of up to 61. The respective ED_{90} values reached levels in the range of several kilograms of flufenacet per hectare. Statistically significant differences between the populations that survived the typical field rates and the sensitive reference populations were determined by analyzing 95% confidence intervals.

Table 1. Response of 22 *Lolium* spp. populations of different origins to different dose rates of flufenacet.

Population	Origin	Sample type	ED_{50} (g ha^{-1})[a]		RF	ED_{90} (g ha^{-1})
FRA1-S[b]	Aube, France	Sensitive field sample	3.6 (0.7)	a	1	8.6 (2.5)
LOLMU-S[b]	Rhineland, Germany	Commercially available sensitive reference	4.3 (0.6)	a	1	17.5 (3.9)
USA1-S	Oregon, USA	Commercially available sensitive reference	4.7 (0.7)	ab	1	15.4 (4.0)
FRA3-S	Seine-et-Marne, France	Sensitive field sample	5. (2.7)	abc	1	7.5 (6.4)
FRA2-S	Seine-et-Marne, France	Sensitive field sample	5.1 (1.6)	ab	1	6.8 (2.0)
FRA3-R	Seine-et-Marne, France	Field sample, reduced efficacy expected	7.0 (0.6)	b	1	18.9 (4.8)
USA2-R	Washington State, USA	Field sample, reduced efficacy expected	7.3 (1.7)	abc	1	84.9 (31.9)
USA2-S	Oregon, USA	Commercially available sensitive reference	8.2 (1.0)	bc	1	37.1 (8.9)
USA3-S	Oregon, USA	Commercially available sensitive reference	12.7 (1.7)	c	2	59.0 (13.9)
FRA4-R	Marne, France	Field sample, reduced efficacy expected	13.5 (3.1)	bc	2	19.7 (44.8)
USA4-R	Washington State, USA	Field sample, reduced efficacy expected	15.9 (3.0)	c	2	88.5 (25.7)
LOLRI-S[b]	Lombardy, Italy	Commercially available sensitive reference	17.8 (2.7)	c	2	53.2 (15.3)
USA3-R	Washington State, USA	Field sample, reduced efficacy expected	55.5 (8.3)	d	7	249.7 (67.5)
GBR2-R	Essex, UK	Field sample, reduced efficacy expected	120.1 (19.6)	e	16	795.1 (258.4)
FRA2-R	Côte-d'Or, France	Field sample, reduced efficacy expected	142.7 (27.6)	ef	19	1317.8 (479.4)
USA6-R	Oregon, USA	Field sample, reduced efficacy expected	148.7 (45.2)	def	19	3558.2 (1641.4)
VLR69-R[b]	Victoria, Australia[†]	Commercially available, reduced efficacy	186.9 (36.6)	ef	24	1499.4 (530.8)
USA1-R[b]	Washington State, USA	Field sample, reduced efficacy expected	210.8 (71.3)	def	27	4079.2 (2245.6)
GBR3-R	South Yorkshire, UK	Field sample, reduced efficacy expected	253.3 (47.6)	f	33	4600.9 (1351.3)
GBR1-R	Hertfordshire, UK	Field sample, reduced efficacy expected	362.1 (94.9)	f	47	3320.6 (1670.0)
USA5-R	Washington State, USA	Field sample, reduced efficacy expected	451.9 (14.5)	f	59	4563.8 (2096.3)
FRA1-R[b]	Meurthe-et-Moselle, France	Field sample, reduced efficacy expected	465.7 (134.3)	F	61	5903.4 (2873.9)

[a] The field rates of flufenacet containing products registered for the use in cereals in Europe ranges from120 g flufenacet ha^{-1} (Bacara® Forte, Russia) via 240 g flufenacet ha^{-1} (*e.g.* Fusburi®, France; Liberator®, Spain, UK; Fence®, Germany, Poland, UK) to 254.4 g flufenacet ha^{-1} (Cadou®SC, Germany) per individual treatment. In the USA field rates up to 381 g flufenacet ha^{-1} (Axiom®, USA) are registered.

[b] Selected for further experiments.

[c] Propagated in the UK. Estimated ED_{50} and ED_{90} values expressed in g ai ha^{-1}, with standard errors in parentheses. Different letters indicate significant differences between populations, based on 95% confidence intervals. RFs represent the quotient of the respective ED_{50} value and the average ED_{50} value of the sensitive reference populations. Sensitive reference populations are indicated by the suffix '-S', and suspected resistant populations are indicated by the suffix '-R'.

3.3.2 Flufenacet degradation rates in sensitive and resistant seedlings

In order to assess differences in flufenacet degradation, three sensitive populations (LOLMU-S, LOLRI-S, FRA1-S) and three flufenacet-resistant populations (USA1-R, VLR69-R, FRA1-R) were treated with ^{14}C-radiolabelled flufenacet in a time-course experiment at 22°C. The degradation half-times (DT_{50}) revealed that the sensitive populations degraded 50% of the herbicide within 7.3 to 12.9 h while the resistant populations reached the same degradation rates after 0.1 to 0.5 h. Therefore, the resistant populations degraded flufenacet at a significantly higher rate (see Table 2 and Figure 2(A)). LOLMU-S, the most sensitive population in the bioassay, degraded the herbicide significantly more slowly than the sensitive populations LOLRI-S and FRA1-S, whereas FRA1-R, the most resistant population in the bioassay, degraded the herbicide significantly faster than the resistant populations VLR69-R and USA1-R. The RIs, calculated based on

the DT_{50} value, and the RFs, calculated based on the ED_{50} value of the respective populations, correlate. This reveals a significant linear correlation between the resistance level and the flufenacet degradation rates in the three resistant and the three sensitive populations (total of six populations; $R2$=0.89, P<0.003, see Figure 2(B)). The recovery rate was 80.0%, on average. The half-times calculated for *Lolium* spp. seedlings incubated at 12°C were 45.5, 18.5 and 46.0 h for the sensitive populations LOLMU-S, LOLRI-S and FRA1-S, respectively, and 1.3, 0.7 and 1.3 h for the flufenacet-resistant populations USA1-R, VLR69-R and FRA1-R, respectively.

Table 2: Flufenacet degradation rates in sensitive and flufenacet-resistant *Lolium* spp. populations.

Population	DT_{50}	95% CI		RI	B	D
LOLMU-S	12.90 (1.34)	10.27 - 15.54	a	1	0.94	99.29
LOLRI-S	7.02 (0.88)	5.27 - 8.76	a	2	0.66	99.09
FRA1-S	7.30 (0.84)	5.65 – 8.94	a	2	0.8	99.61
USA1-R	0.41 (0.11)	0.20 – 0.62	b	27	0.70	99.96
VLR69-R	0.48 (0.11)	0.26 – 0.71	bc	31	0.67	99.94
FRA1-R	0.09 (0.07)	-0.04 – 0.22	c	150	0.52	99.61

Degradation times (DT_{50}) estimated based on four measurements per time point (eight pooled seedlings per measurement) and seven time points per population are given with standard errors in parentheses. Significant differences between populations are indicated by different letters, based on 95% confidence intervals (CIs). RIs are calculated as the quotient of the respective DT_{50} and the DT_{50} of population LOLMU-S. Parameters *b* and *d* in the log-logistic three-parameter equation described by Ritz *et al.* (2015) are given for each population.

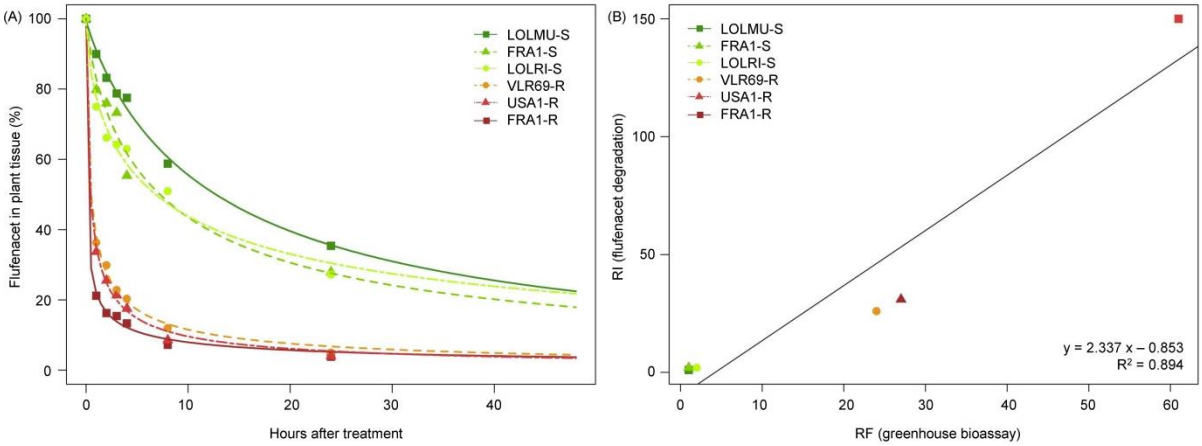

Figure 2: (A) Flufenacet degradation rates (in %) in seedling tissue of three sensitive and three resistant *Lolium* spp. populations at different time points after application. (B) Correlation between resistance factors (RFs) obtained in bioassays with flufenacet and resistance indices (RIs) calculated based on flufenacet degradation rates. Relative standard errors of the means were used to calculate the absolute errors for the RFs and RIs and are represented by the error bars.

3.3.3 Flufenacet metabolite identification in sensitive and resistant seedlings

In addition to the degradation rate, the degradation products occurring within the first 24 h after treatment (HAT) at 12°C were identified by LC–MS/MS in the populations LOLMU-S, LOLRI-S, FRA1-S, USA-R, VLR69-R and FRA1-R through a time-course experiment. Based on the metabolite structures identified and their occurrence as a function of time, a degradation pathway was constructed (see Figure 3). This pathway revealed a flufenacet GSH conjugate (M500, $C_{21}H_{29}F_1N_4O_7S_1$), likely catalyzed by GSTs, to be the first metabolite (see Figure 3). This metabolite was found at all time points in the flufenacet-resistant populations USA1-R, VLR69-R and FRA1-R. Subsequent cleavage of peptide bonds resulted in the metabolites M371 ($C_{16}H_{22}N_3O_4F_1S_1$) and M443 ($C_{19}H_{26}F_1N_3O_6S_1$) after split-off of γ-glutamyl and glycyl residues from the GSH tripeptide, until only the cysteine conjugate remained (M314, $C_{14}H_{19}F_1N_2O_3S_1$). These three metabolites were found at each time point in the resistant populations, except for population USA1-R 1 HAT. Further metabolites, typically detected during phase III metabolism (secondary conjugation), accumulated 24 HAT. Malonyl conjugation of metabolite M314 resulted in the formation of a metabolite with a molecular mass of 400 (M400, $C_{17}H_{21}F_1N_2O_6S_1$). The cysteine conjugate M314 was hydrolyzed (M315, $C_{14}H_{19}N_1O_4F_1S_1$) prior to the formation of another flufenacet malonyl conjugate (M401, $C_{17}H_{20}F_1N_1O_7S_1$), as well as the formation of a flufenacet glycosyl conjugate (M477, $C_{20}H_{28}F_1N_1O_9S_1$). While the majority of the flufenacet metabolites formed at 12°C in the sensitive reference populations were below the detection limit, a glycyl-cysteine conjugate (M443) and a cysteine conjugate (M314) as well as several phase III metabolites were formed in population LOLMU-S at 22°C 24 HAT (see Figure 4). The percentage of recovered flufenacet decreased from 92.2 to 12.6% in population LOLMU-S and from 20.8 to 0% in population USA1-R after 24 h as the incubation temperature was changed from 12°C to 22°C.

While no phase III metabolites, *e.g*. malonyl or glycosyl conjugates, were detected in either of the tested populations at 12°C, they accounted for 72.6% in population LOLMU-S and 81.8% in population USA1-R at 22°C HAT.

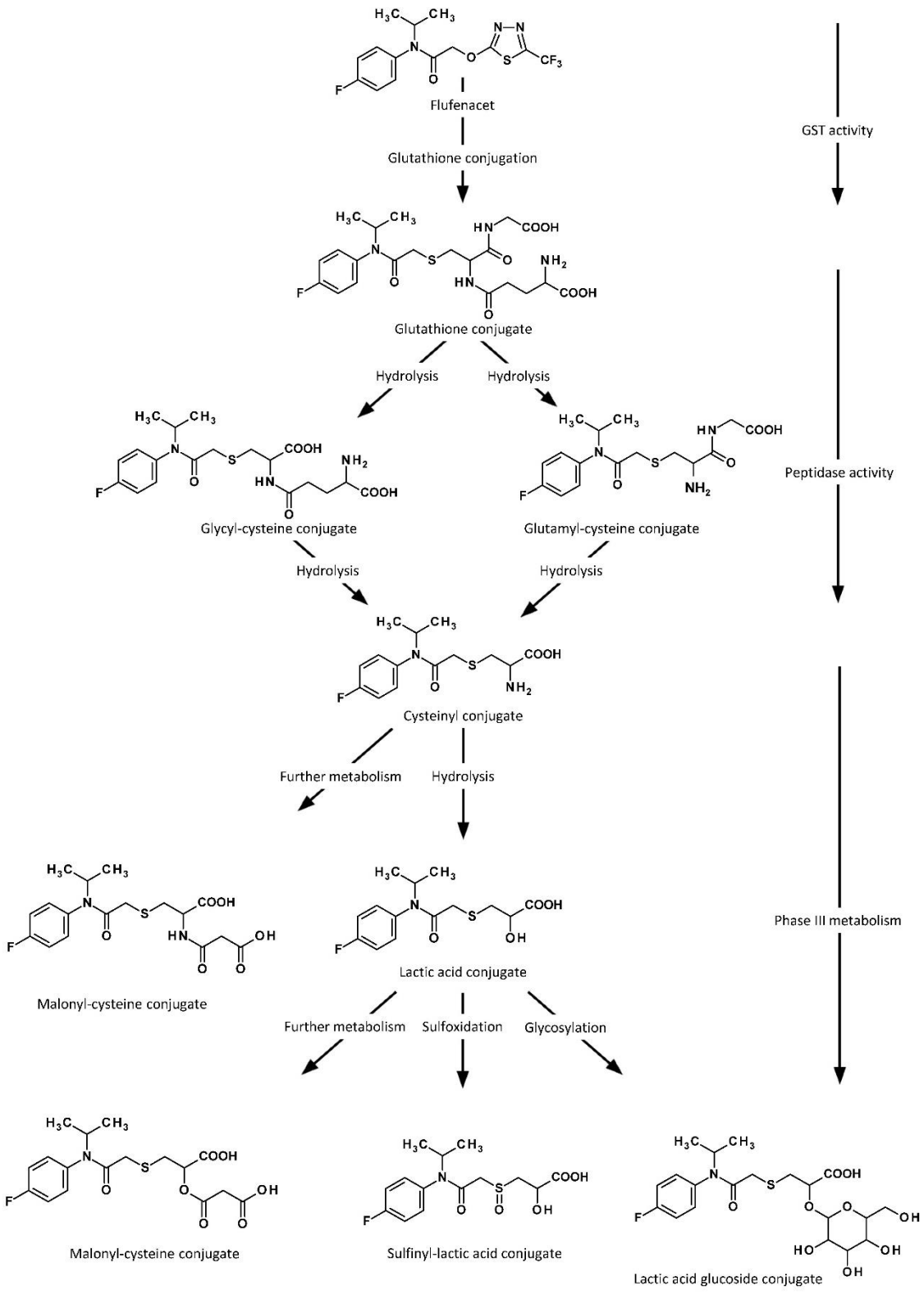

Figure 3: Flufenacet degradation pathway showing metabolites and corresponding enzymatic or chemical degradation activity. Metabolites were identified by LC-MS/MS of extracts obtained from sensitive and flufenacet resistant *Lolium* spp. seedlings treated with flufenacet.

The measurement of the total GST activity in crude extracts of the resistant population USA1-R and the sensitive population LOLMU-S revealed a significantly higher turnover of the model substrate CDNB (see Figure 5 (A)) in obtained from population USA1-R. With flufenacet used as substrate with in an enriched GST preparation of a resistant *Lolium* biotype, the corresponding flufenacet–GSH conjugate was identified by LC–MS/MS (Figure 5 (B)). Besides the expected molecular ion of the conjugate (*m*/*z*=501), collision-induced dissociation revealed the neutral loss of 129 mass units resulting in a characteristic production (*m*/*z*=372), which was explained by splitting off pyroglutamate (Figure 5 (C)).

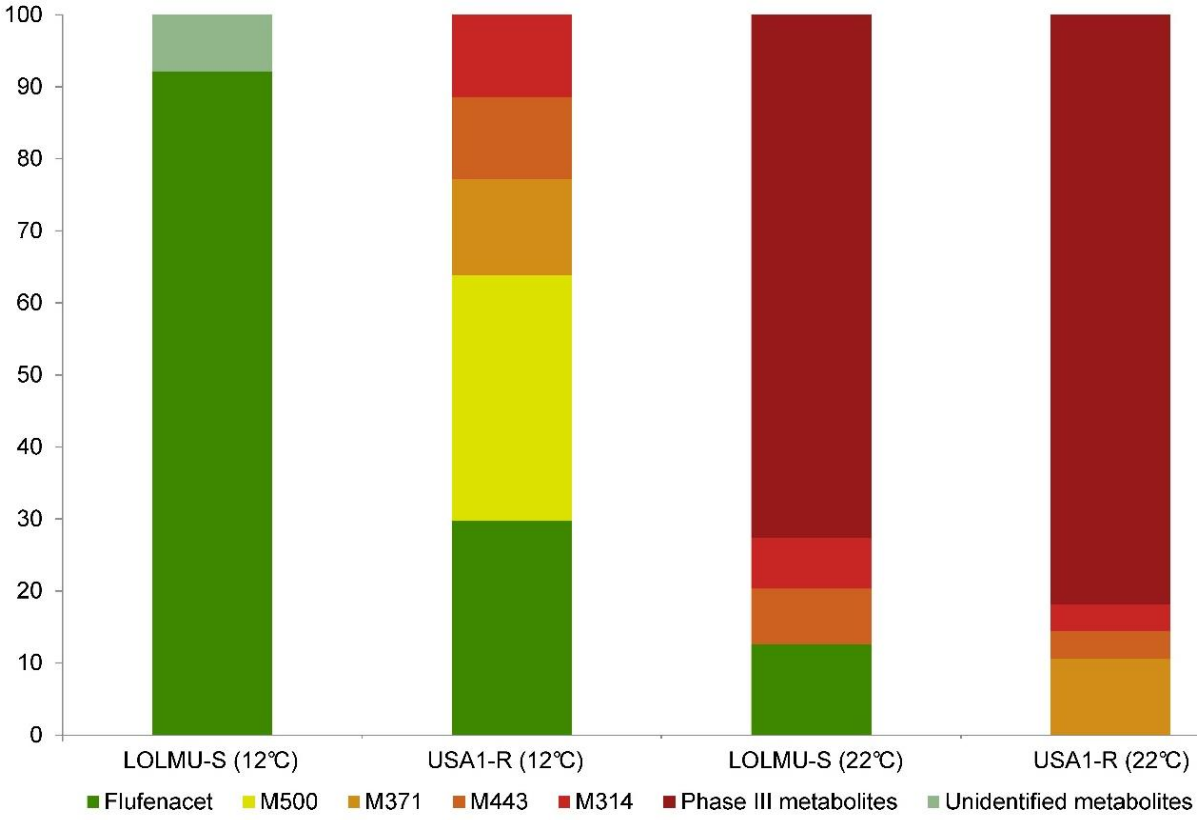

Figure 4. Percentage of identified flufenacet metabolites in populations LOLMU-S and USA1-R at 12° and 22°C 24 h after treatment.

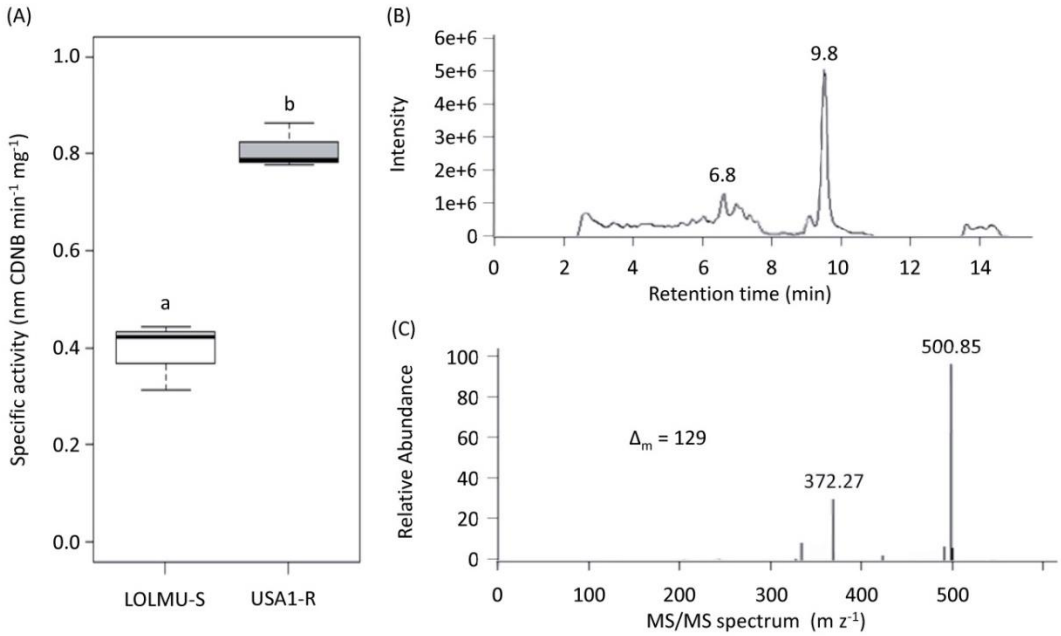

Figure 5: (A) GST activity on model substrate CDNB in crude extracts from population LOLMU-S and USA1-R. Different letters indicate significant differences between populations (*t*-test, *P*=0.0001). (B) Flufenacet and its GSH conjugate detected by LC-MS/MS at retention times of 9.8 and 6.8 min, respectively, in a sample extracted from USA1-R 24 HAT. (C) Flufenacet GSH conjugate and its fragmentation by split-off of pyroglutamate in a protein sample extracted from population USA1-R.

3.4 Discussion

This study investigated flufenacet, an inhibitor of the synthesis of VLCFAs, and its effect on *Lolium* spp. field populations from different origins and the resistance mechanism by comparing three sensitive and three flufenacet-resistant populations. These populations were selected during a screening, with eight sensitive reference populations and 14 populations in which reduced flufenacet efficacy was observed previously in the field or in greenhouse bioassays. This screening showed that one or more populations from each of the selected origins survived the treatment, with a typical field rate of flufenacet formulated as suspension concentrate. With resistance factors of up to 61 and ED_{90} values above the flufenacet field rates registered in Europe (240–250 g flufenacet ha^{-1}), a new unique resistance case is described with field relevant levels in two or more French departments, British counties and US states (see Table 1). This suggests independent development of flufenacet resistance in those regions, as previously assumed for other resistance cases, *e.g.* target-site resistance to acetolactate synthase (ALS) inhibitors, photosystem II inhibitors or glyphosate (Powles and Yu, 2010; Ge *et al*., 2012; Sammons *et al*., 2014).

Interestingly, the Australian population VLR69 was described by Burnet *et al.* (1994b) as chloroacetamide resistant, with resistance factors of up to 2.5 in 1994. The population has been propagated since, and has shown resistance to flufenacet with an RF of 24. This indicates that flufenacet resistance in this population was selected for in the field, or crossed in by the supplier. While the present study has shown that flufenacet resistance in *Lolium* spp. can reach high levels, the previous bioassays conducted with populations from France and the UK have shown that this can only be observed in a small number of fields. Only 0.5% of the populations tested originating from France and 7.4% of the populations tested originating from the UK (collected due to ACCase and/or ALS herbicide failure in the field) survived the treatment with a commercial flufenacet product containing 240 g flufenacet + 120 g diflufenican ha^{-1}. Differences in the abundance of flufenacet resistance may be caused by the later market introduction of flufenacet in France (in 2010) and the resulting delay in selection when compared to the UK, where flufenacet products have been available since 2001 and are used intensively. These results correspond to those of Rauch *et al.* (2010) who found that 7% of samples collected from 75 fields in the Palouse region (northwestern USA), the main area in which flufenacet is used, survived treatments with flufenacet + metribuzin about a decade after its market introduction. Interestingly, the majority (95%) of the samples tested by Rauch *et al.* were resistant to herbicides of at least one MoA. So far, flufenacet resistance has only been observed only in multiple resistant weed populations (Rauch *et al*., 2010; Rosenhauer und Petersen, 2015). This is indicative of non-target-site resistance and raises the question of whether flufenacet resistance has developed independently and accumulated with different MoAs, or whether flufenacet resistance is a result of cross-resistance to other herbicide(s), as demonstrated for pyroxasulfone resistance by Busi and Powles (2016). Also, in *A. myosuroides* populations reduced sensitivity to a broad range of herbicides was observed in comparison to sensitive wild-type populations (Rauch *et al*., 2010; Rosenhauer und Petersen, 2015). Even if those populations are exposed to strong selection pressure due to the intensive use of flufenacet, the level of flufenacet resistance in *A. myosuroides*, with resistance factors of up to 6 (Rosenhauer and Petersen, 2015) is tenfold lower than in the most resistant *Lolium* spp. population described in this study. This suggests that flufenacet resistance in *Lolium* spp. evolves in a different manner. The current knowledge about the target of flufenacet suggests that target-site resistance is unlikely to cause resistance to this herbicide. As an example, 21 condensing enzymes involved in the VLCFA elongation process (3-ketoacyl-CoA synthases), including several redundant isoforms, have been detected in *A. thaliana* (Joubès *et al*., 2008) and similar numbers have been described in several other plant species, *e.g. Oryza sativa* (23), *Zea mays* (27), *Brachypodium distachyon* (23) and *Glycine*

max (31) (Tresch *et al*., 2012). Several of these have been shown to be inhibited by flufenacet and other K_3 herbicides (Trenkamp *et al*., 2004). Several resistance-conferring mutations would therefore be required to cause target-site resistance (Böger *et al*., 2000). Additionally, Böger *et al.* (2000) hypothesized that target site mutations of the condensing enzymes may lead to a loss in function. As enhanced metabolism was previously identified as a driver of selectivity in crops (Bieseler *et al*., 1997), degradation rates of ^{14}C-radiolabelled flufenacet were determined in sensitive and flufenacet-resistant *Lolium* spp. populations by HPLC analysis. The analysis confirmed that, with estimated degradation half-times (DT_{50}) between 6 and 29 min at 22°C, the three flufenacet-resistant populations USA1-R, FRA1-R and VLR69-R, degraded the herbicide significantly more quickly than the sensitive reference populations LOLMU-S, LOLRI-S and FRA1-S, with degradation half-times of between 7.0 to 12.9 h. The correlation between the RIs, calculated based on flufenacet degradation half-times, and the RFs, calculated based on the response to flufenacet in bioassays (ED_{50}), was significant (see Figure 2 (B)). This indicates that enhanced metabolism is the main cause of the differences observed in the resistance levels of the populations tested. As shown before for chloroacetamides, the degradation rate in both sensitive and resistant populations was comparably high (Fuerst, 1987). For technical reasons, degradation half-times below 1 h complicated the detection of early metabolites, and the plants were therefore treated and incubated at 12°C in order to slow down the metabolism. As expected, for temperature-dependent enzymatic processes (Daniel and Danson, 2013), between 22 and 12°C degradation half-times of flufenacet increased from 7.0 to 12.9 h to 18.5 to 46.0 h in the sensitive populations, and from 0.1 to 0.5 h to 0.7 to 1.3 h in the resistant populations. This indicates that the temperature may also affect resistance in the field. Additionally, the spectrum of identified metabolites shifted from phase III metabolites, *e.g.* malonyl or glycosyl conjugates, to early metabolites, *e.g*. flufenacet GSH conjugates, when the temperature was lowered. This experimental setup allowed for the detection of an early metabolite with a molecular mass of 500 (M500), as a result of direct conjugation of GSH to flufenacet. GSH conjugation is likely the first detoxifying step in flufenacet metabolism in *Lolium* spp., as previously described for flufenacet (Bieseler *et al*., 1997) as well as for other herbicides, *e.g*. *S*-metolachlor, alachlor, atrazine or *S*-ethyl dipropylthiocarbamate (EPTC) for crops, *e.g. Z. mays* (Dixon *et al*., 1997; Carringer *et al*., 1978). The proposed reaction mechanism involved the nucleophilic attack of the GSH thiolate on the electrophilic methylene bridge of the thiadiazoyloxyacetyl side chain, followed by a split-off of the hydroxythiadiazole residue. The rapid formation of GSH conjugates suggests catalysis by GSTs, a mostly cytosolic enzyme family (Chronopoulou *et al*., 2017), causing enhanced flufenacet detoxification resulting in resistance. Similar

results have been obtained in recurrently selected pyroxasulfone resistant *L. rigidum* from Australia, in which pyroxasulfone–GSH conjugates were detected, indicating enhanced GST-catalyzed detoxification (Busi *et al*., 2018). The metabolites M371, M443 and M314 accumulating in extracts of flufenacet-resistant *Lolium* biotypes may have resulted from further cleavage of flufenacet–GSH conjugate by vacuolar peptidases (Ohkama-Ohtsu *et al*., 2007; Wolf *et al*., 1996), after vacuolar sequestration (Chronopoulou *et al*., 2017; Dixon *et al*., 2009). Additionally, several phase III metabolites were detected in the plant extracts. Metabolites which could not be derived from the GSH conjugate, as, for example, described in different crop species such as soybeans and wheat by Gould *et al.* (1997), were not found in the *Lolium* spp. samples from this study. Finally, the quantification of total GST activity using the model substrate CDNB with crude extracts from etiolated seedlings of the sensitive population LOLMU-S and the flufenacet-resistant population USA1-R indicated a significantly higher total GST activity in the resistant populations. Conjugation of flufenacet was confirmed qualitatively by mass spectrometry, using enriched fractions of total GSTs from resistant and sensitive biotypes. However, the low activities of GST fractions with flufenacet prevented exact quantification of GSH conjugate formation. In summary, the data support the hypothesis that enhanced GST activity leads to flufenacet resistance in *Lolium* spp. populations. In a second step, we are in the process of characterizing specific GST isoforms which were overrepresented in the resistant populations USA1-R, FRA1-R and VLR69-R.

3.5 Conclusions

Field relevant levels of flufenacet resistance were observed in *Lolium* spp. populations from France and the UK for the first time. Additionally, resistance was found in the Australian population VLR69 and several populations from the northwest USA. Although, or perhaps because, flufenacet resistance is not yet a widespread problem in the field, it is important to reduce selection pressure and prevent any possible loss of flufenacet efficacy. This becomes particularly important as flufenacet has, especially in Europe, become a valuable tool for the management of *Lolium* spp., as only a few other herbicide MoAs are registered for its control and some of them (*e.g.* ALS and ACCase herbicides) are affected by a moderate to severe spread of resistance. Resistance to inhibitors of the synthesis of VLCFAs has mainly been found in monotonous cropping systems (Busi, 2014). Best management practices, including wide crop rotations including spring crops and measures to reduce the weed seed bank, are therefore essential to prevent flufenacet resistance and possible cross-resistances due to metabolic resistance. The molecular and biochemical

characterization of resistance to flufenacet in different *Lolium* spp. populations will provide a deeper understanding of the evolution of metabolic flufenacet resistance.

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4. A tau class GST differentially expressed in flufenacet resistant *Lolium* spp. detoxifies flufenacet *in vitro*

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Abstract

BACKGROUND: In weeds such as *Lolium* spp. or *Alopecurus myosuroides* Huds. resistance to the pre-emergence herbicide flufenacet is predominantly caused by enhanced glutathione transferase (GST) activity. This resistance mechanism can result in survival of more than ten times the registered field rate of *Lolium* populations. Until present, neither GST isoforms nor gene regulation have been reported to be involved in flufenacet resistance in weeds. In this study, we characterized differentially expressed genes in flufenacet resistant *Lolium* populations using RNA-Seq and validated candidate GSTs.

RESULTS: A differential gene expression and gene ontology analysis revealed significant upregulation of GST and glucosyltransferase (GT) activity as well as other stress-related activity in resistant *Lolium* populations. Among four candidate genes, two heterologously expressed glutathione transferases were shown to detoxify flufenacet and one isoform additionally degraded S-metolachlor and pyroxasulfone *in vitro*. However, diflufenican, diclofop-methyl, and mesosulfuron-methyl were not detoxified.

CONCLUSION: The identification of two different GST isoforms detoxifying flufenacet at different rates suggests that flufenacet resistance is based on upregulation of at least one GST with a high affinity to flufenacet as well as a cumulative resistance in combination with GSTs with a lower substrate specificity *e.g.* GST3. Cross-resistance with *S*-metolachlor was found *in planta* and *in vitro*, however, no cross-resistance was observed with diflufenican, diclofop-methyl, and mesosulfuron-methyl. Therefore, these herbicides are likely suitable in combination with flufenacet in a resistance management program.

Keywords

Glutathione transferase, flufenacet, *Lolium* spp., herbicide resistance, RNA-Seq

4.1 Introduction

Metabolism-based resistance to herbicides is a major problem for weed control as it can result in cross-resistance to several herbicide modes of action (MoAs), including herbicides which have not yet been marketed (Beckie and Tardif, 2012; Busi *et al*., 2012). As a result, limited chemical weed control options can lead to frequent use of a limited set of chemical classes and herbicide MoAs, and thus increase the selection pressure of these herbicides on treated weed populations. As cross-resistance patterns can be complex and unpredictable, the choice of a suitable herbicide becomes increasingly difficult as resistance to different herbicide MoAs evolves (Yu and Powles, 2014).

Flufenacet is an example of a pre-emergence herbicide which is commonly used for the control of weed populations already resistant to other herbicide MoAs such as inhibitors of ACCase- or ALS. As an oxyacetamide herbicide, flufenacet inhibits the synthesis of very-long-chain fatty acids (VLCFAs) and therefore, belongs to a MoA which generally selects resistance at a comparably slow rate (Somerville *et al*., 2017; Moss *et al*., 2019). Yet, the use of flufenacet as a tool to control (multiple) resistant weed populations has not been spared from the risk of resistance evolution.

Lolium spp. have shown the potential to adapt to a broad range of herbicide chemistries and have evolved resistance to 14 different herbicide MoAs in total (Heap, 2018). In this manner, *Lolium* populations in the North West of the USA and, in some single cases, also in Europe, have evolved resistance against flufenacet at an agronomically relevant level with resistance factors up to 61 (Rauch *et al*., 2010; Dücker *et al*., 2019b). It has been shown that in *Lolium* spp. and *Alopecurus myosuroides* Huds. flufenacet was mainly detoxified by glutathione conjugation prior to hydrolysis of the peptide bonds of glutathione and subsequent glycosyl- or malonyl conjugation (Dücker *et al*., 2019b; Dücker *et al*., 2019b), similar to detoxification pathways previously described for crops *e.g.* corn (Bieseler *et al*., 1997, Gould *et al*., 1997). Detoxification by GSTs in weeds and crops has been earlier described for herbicides like atrazine (Anderson and Gronwald, 1991), fenoxaprop-P-ethyl (Tal *et al*., 1997), *S*-metolachlor (Cottingham *et al*., 1993) and pyroxasulfone (Busi *et al*., 2018).

Some GST isoforms *e.g. Am*GSTF1 or *Lm*GSTF1 have been studied as detoxifying resistance enzymes and markers (Cummins *et al*., 2013; Tétard-Jones *et al*., 2018). It was recently shown that *Am*GSTF1 was significantly upregulated in several *A. myosuroides* populations of different origins resistant to several herbicides.

Still, which GST families or isoforms are involved in flufenacet resistance in weeds, how they are regulated, and which role they play for cross-resistance has not yet been

investigated. In the present study transcriptomes of sensitive and flufenacet resistant *Lolium* populations were analyzed and candidate GSTs were identified as potentially conferring resistance to flufenacet. Finally, four recombinant candidate GSTs were produced in *E. coli*. For validation of their function, the turnover rates of these GSTs were quantified with the model substrate CDNB and several pre- and post-emergence herbicides including flufenacet as substrates.

4.2 Materials and methods

4.2.1 Plant cultivation and dose-response bioassays

The sensitive populations LOLMU-S, LOLRI-S, and FRA1-S, and the flufenacet resistant populations USA1-R, VLR69-R, and FRA1-R previously described and characterized by Dücker *et al*., (2019b) were pre-germinated on solidified water agar (0.7% w/v). As soon as the primordial root emerged, five seedlings per population and herbicide treatment were transplanted into pots containing sandy loam with 2.2% organic matter. The seedlings were subsequently covered with a thin layer of coarse sand and treated with different pre-emergence herbicides in a laboratory track sprayer (teejet nozzle XR8001, 300 L ha^{-1}, 2 bar). Flufenacet was applied as Cadou® SC at dose rates of 0, 4.7, 14, 42, 127, 380 g ai ha^{-1}; Pyroxasulfone was applied as Sakura® 850 WG at dose rates of 0, 0.5, 1.5, 4.4, 13.3, 40 g ai ha^{-1}; *S*-metolachlor was applied as Dual Gold® at dose rates of 0, 14, 44, 133, 400, 1200 g ai ha^{-1} and diflufenican was applied as Quartz at dose rates of 0, 2.3, 6.9, 21, 63, 187 g ai ha^{-1}. After treatment pots were watered once from above and subsequently kept in a glasshouse with 22/16°C day/night conditions with a 14 h photoperiod provided by Philips Master HPI-T plus 400W/645 E40 metal halide lamps at approximately 200 µmol m^{-2} s^{-1}. Foliar fresh weight of the individual plants was assessed 28 days after treatment. Dose-response data were analyzed as described by Dücker *et al*. (2019b).

4.2.2 Determination of flufenacet degradation rates in *Lolium* spp. seedlings

Seedlings of the populations LOLMU-S, LOLRI-S, USA1-R, and VLR69-R were sown in pots containing sandy loam with 2.2% organic matter and subsequently covered with coarse sand. The plants were grown under the described greenhouse conditions with a 16 h photoperiod until the plants reached the four to five tiller stage. Each 16 plants per population were treated with ^{14}C-radiolabeled diclofop-methyl and mesosulfuron-methyl and analyzed as described by Collavo *et al*. (2012). Differences in herbicide degradation were

analyzed using the t-test of the statistical software R (version 3.5.0, R Foundation for Statistical Computing).

4.2.3 Illumina sequencing of *Lolium* spp. mRNA

In order to obtain homogenously resistant progeny, flufenacet resistant individuals of the populations USA1-R, VLR69-R, and FRA1-R were treated with flufenacet formulated as Cadou® SC in a dose-response assay as described by Dücker *et al*., (2019b). Four weeks after foliage harvest, each 2x20 regrowing individuals per population, were transplanted into two 2 L pots containing sandy loam with 2.2% organic matter, enclosed with pollen-proof gauze until seed harvest after seven months. Besides the flufenacet resistant populations USA1-R, VLR69-R, and FRA1-R the sensitive populations LOLMU-S, LOLRI-S and FRA1-S (Dücker *et al*., 2019b) were chosen for an RNA-Seq experiment. The seeds of these six populations were sterilized for 20 s with 70% ethanol and for 20 min with 2.5% sodium hypochlorite and subsequently rinsed thrice with sterile tap water. Afterwards, the seeds were dried on filter paper and stored at 5°C in the dark for two weeks. The seeds were sown on 100 g 4 mm glass beads in tissue culture containers (MP Biomedicals, Eschwege, Germany) and covered with 16 mL 0.02 M KNO_3 mineral water. After five days of storage at 5°C in the dark, the containers were transferred into a growth chamber until the first leaf reached a length of about 2.5 cm. The chamber was set to 22/16°C day/night conditions with a 14 h photoperiod provided by Philips Master TL-D 58W/840 REFLEX fluorescent lamps at approximately 400 µmol m^{-2} s^{-1}. For equal treatment, each eight seedlings per population were placed in two 20 mL glass vials containing 1.2 mL mineral water (Volvic). Additionally, each eight seedlings of the populations LOLMU-S and USA1-R were placed in two 20 mL glass vials containing 1.2 mL mineral water with flufenacet formulated as Cadou® SC at a concentration of 8 µg L^{-1}. The vials were carefully shaken and incubated at 22°C under light conditions for one hour. Afterwards, the seeds were removed and single seedlings were immediately frozen individually in liquid nitrogen for RNA-Seq analysis. Also, each eight seedlings per treatment and population were pooled to one sample for sequence analyses. All samples were processed at midday between 11:00 AM and 13:00 PM to avoid differential gene expression due to circadian clock effects.

The frozen plant tissue was ground for 30 s in 2 ml reaction tubes containing each 4 tungsten carbide beads (3 mm) in a Tissue Lyser II swing mill (Qiagen, Hilden, Germany) at 30 Hz. Total RNA was extracted using the Qiagen RNeasy Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. RNA integrity (RIN scores > 7) was verified using the RNA 6000 Nano Kit (Agilent Technologies, Waldbronn, Germany) as defined in

the manufacturer's instructions. The RNA of each six individual plants per population and treatment (48 samples in total) was diluted to 20 ng μL^{-1} at a volume of 100 µL. The samples were DNase treated using the Turbo DNA free kit (Ambion, Austin, TX, USA). cDNA libraries were obtained using the Illumina TruSeq Stranded mRNA Library Prep kit (Illumina, San Diego, CA, USA)**.** The multiplexed cDNA libraries were measured with an Illumina HiSeq 2500 sequencer (Illumina, San Diego, CA, USA) with six libraries per lane as paired-end reads with a length of 125 bp in high output mode.

4.2.4 Transcriptome and gene ontology analysis

A hybrid assembly of a Roche 454-based reference transcriptome and 100 bp Illumina reads of a sensitive and a diclofop-methyl resistant *Lolium rigidum* Gaud. population described by Gaines *et al*. (2014) was performed using the Velvet-Oases assembler (Schulz *et al*., 2012) (see Table 2). The assembled 106 653 contigs were aligned against the NCBI-nr database using BLASTx (Camacho *et al*., 2009) prior to gene ontology (GO) mapping using Blast2GO PRO (Conesa *et al*., 2005) (see Table 2). The Illumina® reads described in section 2.3 were quality trimmed and mapped to the described *L. rigidum* reference transcriptome using BWA with the Maximal Exact Matches (MEM) algorithm (Li, 2013) (BWA Version 7.12) within the Genedata Expressionist Refiner Genome software (version 9.5, Genedata, Basel, Switzerland). The obtained read counts were TMM- (Trimmed Mean of M values) normalized and a differential gene expression analysis was carried out using edgeR (Robinson *et al*., 2010) within the Genedata Expressionist software (Genedata, Basel, Switzerland). Pairwise comparisons were made with the following cutoff criteria: $p \leq 0.05$ and log fold-change ≥ 2. Gene expression of the resistant populations USA1-R, VLR69-R, and FRA1-R was individually compared to the gene expression of the sensitive populations LOLMU-S, LOLRI-S, and FRA1-S as a group in order to select only gene-associated contigs differentially expressed in all three resistant populations. GO enrichment was analyzed using a multiple-testing corrected hypergeometric test of the R package GOfuncR (Grote, 2017). Multi-level pie charts of GO terms assigned to 95 significantly upregulated and 136 significantly downregulated gene-associated contigs were created using the combined GO graph function in Blast2GO Pro Version 5.0 (www.blast2go.com). Based on the differential gene expression analysis and the GO analysis, 11 candidate contigs annotated as GSTs were selected.

4.2.5 RACE PCR and candidate gene analysis

In order to verify the role of the selected candidate contigs in flufenacet resistance in *Lolium* populations, RACE PCR was conducted in order to obtain the protein coding sequences from each candidate gene. Total RNA of the untreated pooled samples of the populations

LOLMU-S, LOLRI-S, FRA1-S, USA1-R, VLR69-R, and FRA1-R was used to obtain full-length cDNA of the candidate contigs GST1 and GST2 (GST1A, GST1B), GST3 and GST4 using the Invitrogen GeneRacer™ Kit (Invitrogen, Darmstadt, Germany). Reverse transcription PCR was performed using the GeneRacer™ RNA and GeneRacer™ oligo (dT) primers and the Superscript™ III reverse transcriptase according to the manufacturer's instructions. 3'- and 5'-RACE PCR were performed using the Platinum® High Fidelity Taq DNA polymerase (Invitrogen, Darmstadt, Germany) with the full-length cDNA of the pooled sample of USA1-R as template and gene-specific primers (see Table 1) in combination with the corresponding 3'- or 5'-GeneRacer™ RACE primers according to the manufacturer's protocol. The PCR product was purified and cloned into pCR®4-TOPO® vector according to the manufacturer's specifications. After transformation into One Shot® TOP10 competent *E. coli* cells, each five colonies were picked and propagated in 3 mL of LB medium (Roth, Karlsruhe, Germany) with ampicillin as selection marker. Plasmids were purified using the QIAprep® Spin Miniprep Kit (QIAGEN, Hilden, Germany). The purified plasmids were sequenced by Eurofins Genomics using T7 primers. Based on the obtained sequences, new primers for full-length PCR amplification of the protein coding region were designed (see Table 1). These primers were used for PCR with full-length cDNA of the pooled untreated samples of the populations LOLMU-S, LOLRI-S, FRA1-S, USA1-R, VLR69-R, and FRA1-R as templates as defined in the manufacturer's instructions of the Phusion™ Hot Start High-Fidelity DNA polymerase (Thermo Fisher Scientific, Schwerte, Germany). The amplicons were cloned and transformed using the Zero Blunt® TOPO® PCR Cloning Kit (Invitrogen, Darmstadt, Germany) according to the manufacturer's specifications. Each five One Shot® TOP10 *E. coli* colonies were picked and propagated in 3 mL of LB medium (Roth, Karlsruhe, Germany) with kanamycin as selection marker. Plasmids were purified using the QIAprep® Spin Miniprep Kit (QIAGEN, Hilden, Germany) prior to sequencing by Eurofins Genomics using T7 primers. Sequence analyses were performed using MegAlign (version 6.0.1, DNAStar).

4.2.6 Production of significantly upregulated GSTs in *E. coli*

Based on the known sequences of GST1A, GST1B, GST3 and GST4 as well as green fluorescent protein (GFP) primers were designed for protein overexpression according to the manufacturer's instructions of the Champion™ pET Directional TOPO® Expression by PCR (see Table 1). Plasmids containing the sequences of interest originating from population USA1-R were selected as a template for PCR with the Phusion Hot Start High-Fidelity DNA Polymerase (Thermo Fisher Scientific, Schwerte, Germany) according to the manufacturer's protocol (see Figure A and Figure B in the annex). The PCR products were

separated on a 1% agarose gel and purified using S.N.A.P columns (Invitrogen, Darmstadt, Germany). The purified PCR products of each one sequence were cloned into pET101/D-TOPO® vector for gene expression with a C-terminal His-tag and the pET151/D-TOPO® vector for gene expression with an N-terminal His-tag according to the manufacturer's protocol of the Champion™ pET Directional TOPO® Expression Kit. Plasmids were purified using the QIAprep® Spin Miniprep Kit (QIAGEN, Hilden, Germany) prior to sequencing by Eurofins Genomics using T7 primers. Sequence analyses were performed using MegAlign (version 6.0.1, DNAStar). Two isoforms similar to the contig sequence of GST1 as well as one isoform similar to GST3 and one isoform similar to GST4 were selected for overexpression (see Figure A and Figure B). Protein overexpression in transformed One Shot® BL21 Star (DE3) *E. coli* cells was induced by the addition of isopropyl-beta-D-thiogalactopyranoside (IPTG) for a final concentration of 0.5 mM. The cells were grown for 4 hours at 37°C in 300 mL of LB medium (Roth, Karlsruhe, Germany) with ampicillin as selection marker. The cells were harvested by centrifugation at 4000 *g* for 20 min and frozen at -80°C. The His-tagged proteins were purified under native conditions using the QIAexpress® Ni-NTA Fast Start Kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. The purified proteins were desalted on PD-10 Desalting Columns (GE Healthcare, Freiburg, Germany) and eluted in 3.5 mL 100 mM phosphate buffer containing 250 µM EDTA (pH 7.0). A tenfold concentration was achieved by diafiltration at 4000 *g* for 20 min using 10 kDa MWCO Amicon Ultra®-15 centrifugal filter units (Merck Millipore, Darmstadt, Germany). Protein aliquots were stored at -80°C.

4.2.7 Protein assays with different substrates

Activity of the purified proteins was verified photometrically with the model substrate CDNB. A 200 µL reaction mix was set up in sodium phosphate buffer (100 mM NaH_2PO_4/Na_2HPO_4, pH 7.0) containing 1mM glutathione and 1 mM CDNB in duplicate with each protein at a concentration of 50 µg mL^{-1}. After excitation, absorbance was measured at 340 nm for 8 min with a CLARIOStar® microplate reader (BMG Labtech, Ortenberg, Germany). Extracts with active proteins and GFP were selected for protein assays with herbicide substrates (GST1A with N-terminal His-tag, GST1B with C-terminal His-tag, GST3 with N-terminal His-tag, and GST4 with C-terminal His-tag). ^{14}C-radiolabeled flufenacet, diflufenican, diclofop-methyl, and mesosulfuron-methyl used each at a final concentration of 50 µM were added to 100 µL sodium phosphate buffer (100 mM NaH_2PO_4/Na_2HPO_4, pH 7.0) containing 1 mM glutathione and the selected proteins at a concentration of 50 µg mL^{-1}. The reactions were set up in duplicate and were stopped after 20 min by addition of 100 µL acetonitrile and a HPLC analysis was performed as described by Collavo *et al.* (2012). The unlabeled

herbicides *S*-metolachlor and pyroxasulfone were used each at a final concentration of 500 µM, and were added to a 100 µL sodium phosphate buffer (100 mM NaH_2PO_4/Na_2HPO_4, pH 6.5) containing 1mM glutathione and the selected proteins at a concentration of 50 µg mL^{-1}. The reactions were stopped after 20 min by addition of 100 µL acetonitrile and measured by HPLC in the UV-mode at 236 nm as described by Collavo *et al.* (2012).

Table 1: List of oligonucleotides used as gene-specific primers (GSPs) for RACE PCR, as PCR primers for cloning of the full-length protein-coding sequence for sequencing and for cloning for protein overexpression.

Contig	Use	Forward-primer (5‘ – 3‘)	Reverse-primer (5‘ – 3‘)
GST1	GSP for RACE PCR	CAGGTTCTGGGCCGACTACATCGACAAG	CTTCTTGTCGATGTAGTCGGCCCAGAAC
	Protein-coding sequence	ATGGCGCCCGAGAAGAA	TCACTCGACGCCCAACTTC
		ATGGCGGGTGAGAAGAA	CTACTCGATGCCGTACTTCTTCTT
GST1A	Overexpression C-terminal	CACCATGGCGGGTGAGA	CTCGACGCCCAACTTCTT
	Overexpression N-terminal	CACCATGGCGGGTGAGA	TCACTCGACGCCCAACTTCTT
GST1B	Overexpression C-terminal	CACCATGGCGCCCGAGAA	GGACTCGATGCCGTACTTC
	Overexpression N-terminal	CACCATGGCGCCCGAGAA	TCAGGACTCGATGCCGTACT
GST2	GSP for RACE PCR	-	CACCTTSTCCGGCGAGTAGAGGCTCCTG
GST3	GSP for RACE PCR	-	GATGCCGCGCATCATTGGGTTG
	Protein-coding sequence	ATGGCGCCGGTGAAG	TCAAGCCTTGGGTGGAAC
	Overexpression C-terminal	CACCATGGCGCCGGTG	TCAAGCCTTGGGTGGAACCATG
	Overexpression N-terminal	CACCATGGCGCCGGTG	AGCCTTGGGTGGAACCATGCT
GST4	GSP for RACE PCR	-	CAGACCAAAGTCCACCGGCATGAACTC
	Protein-coding sequence	ATGGCGCCGGCGGCCGTG	TCACTGCTCTGCCTTTTTCC
		-	TCACTGCTCTGCCTTTTTCCCCAGAC
	Overexpression C-terminal	CACCATGGCGYCGGC	CTGCTCTGCCTTTTTCCCCAGAC
	Overexpression N-terminal	CACCATGGCGYCGGC	TCACTGCTCTGCCTTTTTCC
GFP	Overexpression C-terminal	CACCATGGTGAGCAAGGG	CTTGTACAGCTCGTCCATGC

4.3 Results

4.3.1 Efficacy of flufenacet and selected herbicides on sensitive and flufenacet resistant *Lolium* populations

The efficacy of flufenacet, pyroxasulfone, *S*-metolachlor and diflufenican on the sensitive populations LOLMU-S, LOLRI-S and FRA-1-S and the flufenacet resistant populations USA1-R, VLR69-R, and FRA1-R was assessed in a dose-response bioassay (see Figure 1A). With an estimated ED_{50} value of 4.2 g ai ha^{-1}, the sensitive *Lolium* populations were controlled with significantly lower amounts of flufenacet in comparison to the flufenacet

resistant populations with an estimated ED_{50} value of 174.0 g ai ha^{-1}. ED_{50} values of *S*-metolachlor of 9.1 g and 116.6 g ai ha^{-1} were estimated for the sensitive and the flufenacet resistant *Lolium* populations, respectively and differed significantly between both groups. Also, the estimated pyroxasulfone ED_{50} differed significantly between the sensitive and the flufenacet resistant *Lolium* populations with values of 1.2 and 2.8 g ai ha^{-1}, respectively. With ED_{50} values of 8.8 and 17.2 g ai ha^{-1} estimated for the sensitive and the flufenacet resistant *Lolium* populations, respectively, the differences in diflufenican efficacy were statistically insignificant. With resistance factors of 42 and 13 estimated for flufenacet and *S*-metolachlor, respectively, the differences between the sensitive and the flufenacet resistant populations were considerably higher than in the case of pyroxasulfone and diflufenican, each with an estimated resistance factor of two. Diclofop-methyl and mesosulfuron-methyl efficacy on LOLMU-S and USA1-R was measured indirectly by determination of their degradation in plant tissue in order to avoid biases due to mutations of target genes (see Figure 1B). Diclofop-methyl degradation in the sensitive populations was with 33.0% 16 hours after treatment significantly slower than in the flufenacet resistant populations with 55.1%. The mesosulfuron-methyl degradation rate of 19.6% in the sensitive population LOLMU-S did not differ significantly from the degradation rate of 20.7% in the flufenacet resistant population USA1-R 16 hours after treatment.

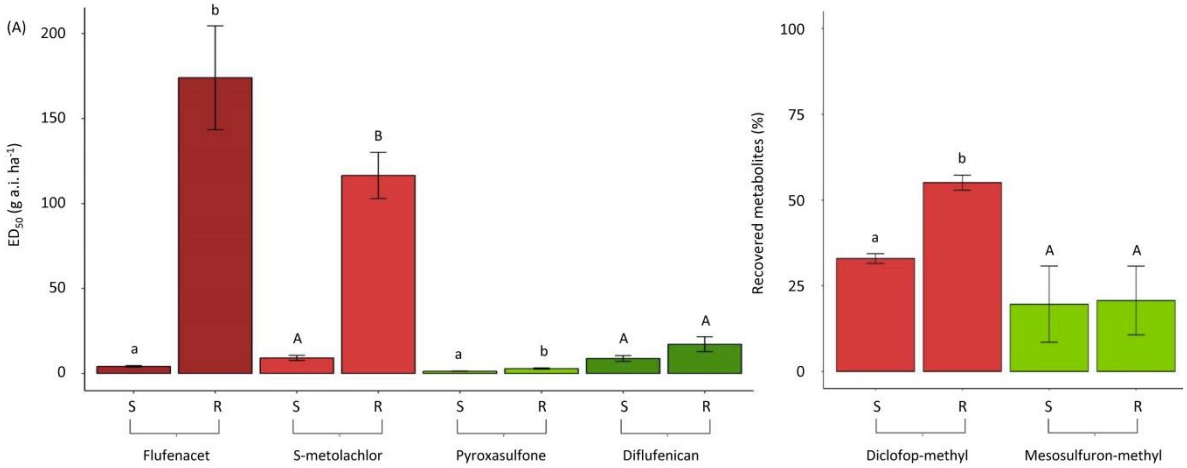

Figure 1: (A) Dose-response relationship of three sensitive (S) and three flufenacet resistant *Lolium* populations to flufenacet, *S*-metolachlor, pyroxasulfone, and diflufenican displayed as ED_{50} ± standard error. (B) Degradation rates of diclofop-methyl and mesosulfuron-methyl in the sensitive *Lolium* populations LOLMU-S and LOLRI-S (S) and the flufenacet resistant *Lolium* populations USA1-R and VLR69-R (R) 16 hours after treatment. Different letters indicate significant differences between S and R.

4.3.2 Differential gene expression and candidate gene analysis

The reads obtained from Illumina sequencing were mapped to a *L. rigidum* reference transcriptome with 106 653 contigs with a mapping percentage of 89.2 to 92.2% (see Table 2). The read counts were normalized and analyzed using edgeR. In total, 95 gene-associated contigs were found significantly upregulated in each of the three resistant populations USA1-R, VLR69-R, and FRA1-R, and 136 gene-associated contigs were found significantly downregulated in each of these three populations.

Table 2: Statistics of a hybrid assembly of a *Lolium rigidum* cDNA reference transcriptome.

Total assembled bases	53 108 293
Total assembled contigs	106653
Average contig size	498
N50	571
GC content	48.36
Contigs with GO annotation	30.6%

A multi-level analysis rating GO terms (molecular function) annotated to the differentially expressed contigs was conducted with Blast2GO Pro (see Figure 2). The annotations for 136 significantly downregulated gene-associated contigs were heterogeneous and comprised GO terms *e.g.* 'protein dimerization activity' with a score of three. This GO term was annotated to contigs which were BLAST-annotated as transcription factors. Additionally, the GO terms 'protein kinase activity', 'RNA polymerase II regulatory region sequence-specific DNA binding', and 'ADP-binding' were described the significantly downregulated contigs with a score of two (see Figure 2B). Among the GO terms annotated to the upregulated contigs the highest scores of 13 were assigned to 'quercetin 3-O-glucosyltransferase activity' and 'quercetin 7-O-glucosyltransferase activity' followed by 'oxidoreductase activity' with a score of 12.8, 'glutathione transferase activity' with a score of 11, and 'cellulose synthase activity' with a score of 5 (see Figure 2A). The GO enrichment analysis confirmed a significant upregulation of GO terms *e.g.* 'glucosyltransferase activity', 'glutathione transferase activity' as well as 'anthocyanin-containing compound and flavonoid metabolic'- and 'biosynthetic process' with FWER values < 0.001 (see Table 3). Among the 95 contigs significantly upregulated in the flufenacet resistant populations, 11 were annotated as GSTs.

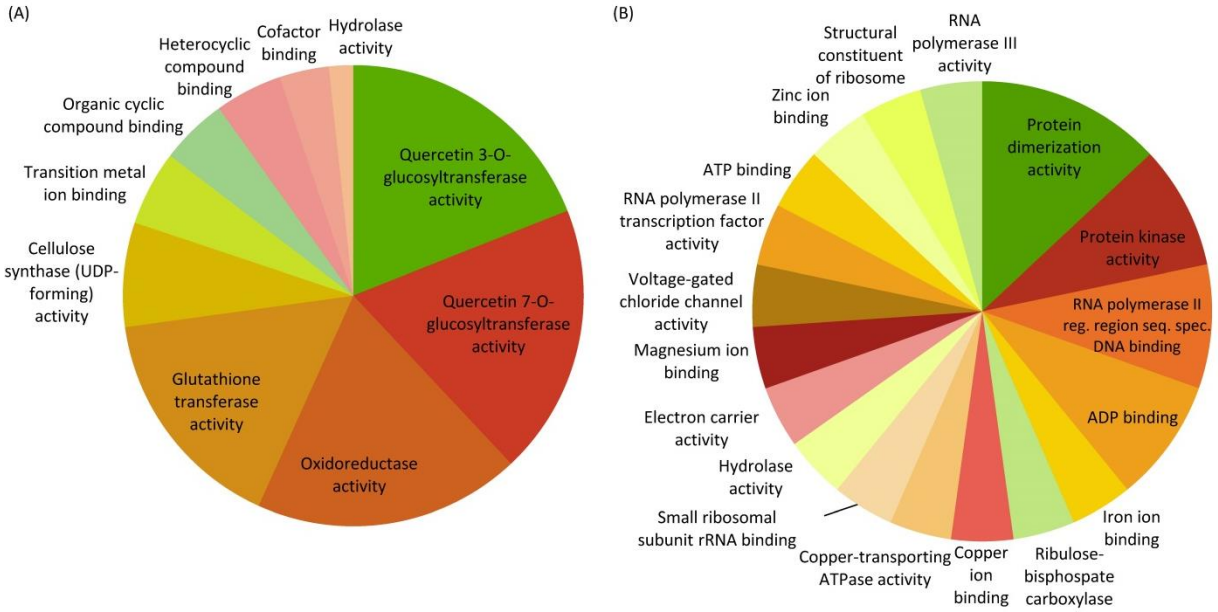

Figure 2: Multi-level pie charts of gene ontology terms assigned to 95 significantly upregulated (A) and 136 significantly downregulated (B) gene-associated contigs.

Table 3: Ontology terms significantly enriched in 95 gene-associated contigs significantly upregulated in flufenacet resistant *Lolium* populations (FWER < 0.001).

Category	Node ID	Node name	FWER
Molecular function	GO:0016758	Transferase activity, transferring hexoxyl groups	< 0.001
Biological process	GO:0009407	Toxin catabolic process	< 0.001
Molecular function	GO:0035251	UDP-glucosyltransferase activity	< 0.001
Molecular function	GO:0016757	Transferase activity, transferring glycosyl groups	< 0.001
Molecular function	GO:0046527	Glucosyltransferase activity	< 0.001
Molecular function	GO:0004364	Glutathione transferase activity	< 0.001
Biological process	GO:0006749	Glutathionemetabolic process	< 0.001
Molecular function	GO:0008194	UDP-glycosyltransferase activity	< 0.001
Biological process	GO:0009404	Toxin metabolic process	< 0.001
Molecular function	GO:0016765	Transferase activity, transferring alkyl or aryl groups	< 0.001
Biological process	GO:0009813	Flavonoid biosynthetic process	< 0.001
Biological process	GO:0009812	Flavonoid metabolic process	< 0.001
Biological process	GO:0006575	Cellular modified amino acid metabolic process	< 0.001
Biological process	GO:1900992	(-)-Secologanin metabolic process	< 0.001
Biological process	GO:1900994	(-)-Secologanin biosynthetic process	< 0.001
Molecular function	GO:0016740	Transferase activity	< 0.001
Biological process	GO:1901804	beta-glucoside metabolic process	< 0.001
Biological process	GO:1901806	beta-glucoside biosynthetic process	< 0.001
Biological process	GO:0009718	Anthocyanin-containing compound biosynthetic process	< 0.001
Biological process	GO:0098754	Detoxification	< 0.001
Biological process	GO:0046283	Anthocyanin-containing compound metabolic process	< 0.001

The analysis of their expression levels in untreated and treated samples of LOLMU-S and USA1-R revealed that all 11 significantly upregulated gene-associated contigs were additionally higher expressed in the treated plants in comparison to the untreated plants, although the differences were not significant in all cases (see Figure 3). Some contigs, *e.g.* GST1 and GST2, showed highly similar expression patterns. In comparison, the expression of actin 7 was independent from resistance status and treatment.

The alignment of the protein sequences of these contigs revealed that GST3 and GST4 belong to class phi while the other nine GSTs belong to class tau (see Figure 4). GST3 showed a high similarity to *Am*GSTF1 isolated from *Alopecurus myosuroides* and *Lr*GSTF1 isolated from *L. rigidum* and previously described by several authors (see Figure B) (Cummins *et al.,* 2013, Tétard-Jones *et al*., 2018). The analysis of the protein sequences of the individual sensitive and resistant populations revealed that various single nucleotide polymorphisms conferred amino acid substitutions (see Figure A and Figure B in the annex). Some of them were unique and only detected in resistant individuals, *e.g.* the substitution of threonine by methionine in position 144 in allele USA1-R-2 of GST1B or the substitution of lysine by threonine in position 153 of allele USA1-R2 of GST4. However, none of these amino acid substitutions was predominantly present in the resistant samples. After sequencing of RACE PCR products, the full protein-coding region of the RNA as well as the UTR regions were analyzed. The analysis revealed that the contigs GST1 and GST2 were part of the same mRNA.

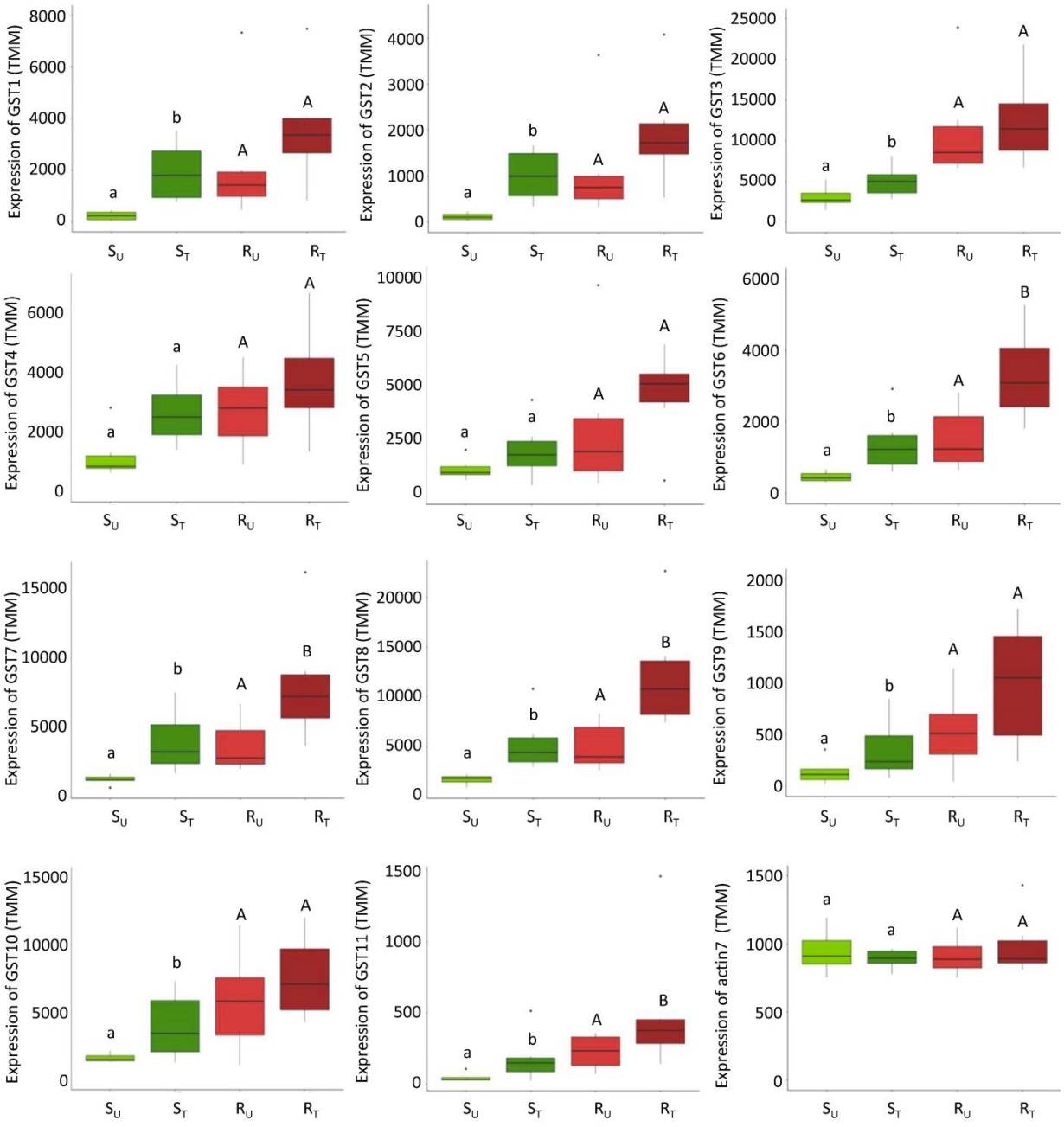

Figure 3: Expression levels of 11 significantly upregulated contigs annotated as GSTs in untreated (S_U) and treated (S_T) seedlings of the sensitive *Lolium* population LOLMU-S and untreated (R_U) and treated (R_T) seedlings of the flufenacet resistant population USA1-R, displayed in TMM (trimmed mean of M values). Significant differences between S_U and S_T are indicated by different lower-case letters, significant differences between R_U and R_T are indicated by upper case letters.

By sequencing the full protein-coding region of the RNA two similar isoforms were identified (87.2% identity between GST1A and GST1B, see Figure A). The 5' RACE sequences of GST1A clustered with the 5' UTR region present in contig GST1 while the 5'region of the other isoform clustered with a newly identified 5' UTR region (data not shown).

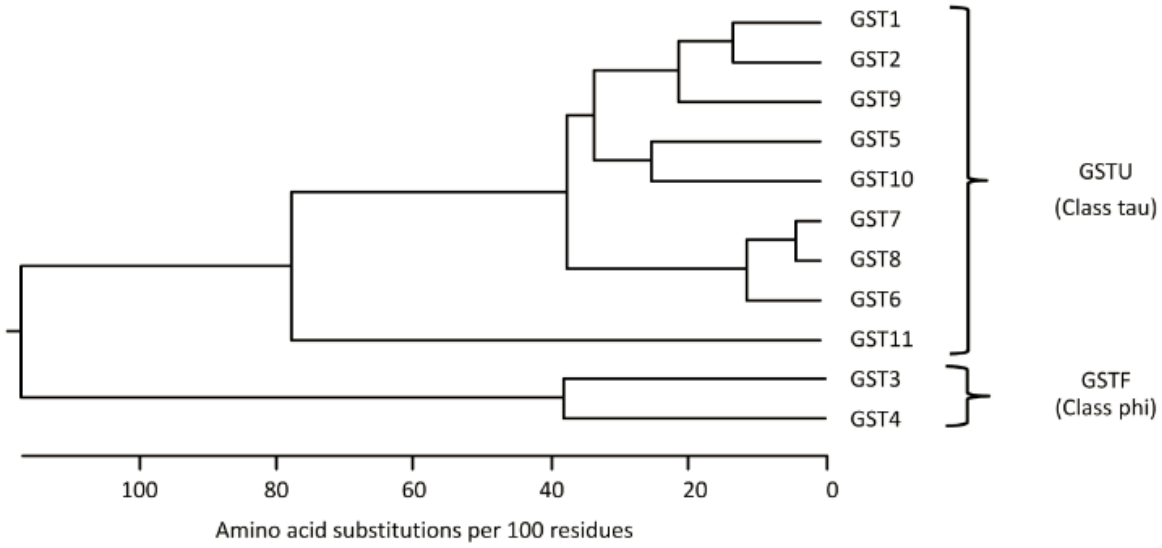

Figure 4: Cladogram displaying amino acid substitutions per 100 residues in two candidate contigs annotated as GST class phi and nine candidate contigs annotated as GST class tau.

4.3.3 Candidate gene validation with recombinant GST isoforms

Each one allele of GST1A, GST1B, GST3, and GST4 as well as GFP as a control gene were selected for overexpression in *Escherichia coli* (see Figure A and Figure B). After purification, activity of the isoforms was measured with a photometric assay using the model substrate CDNB (see Table 4). GST1A and GST3 were active with an N-terminal His-tag while GST1B and GST4 were active with C-terminal His-tag. The CDNB turnover rates of the active GSTs ranged from 2.2 to 5.1 µmol min^{-1} mg^{-1} protein while with GFP a turnover rate of 0.1 µmol min^{-1} mg^{-1} was measured. The flufenacet turnover rate of GST1A and GST3 were 44.6 and 6.1 µmol min^{-1} mg^{-1}, respectively while GST1B, GST4, and GFP degraded neither flufenacet nor any other herbicide tested. GST1A was the only isoform which degraded S-metolachlor and pyroxasulfone with turnover rates of 30.6 and 14.45 µmol min^{-1} mg^{-1}, respectively. None of the enzymes tested degraded diflufenican, diclofop-methyl, or mesosulfuron-methyl.

Table 4: Turnover rates of CDNB and selected herbicides in µmol min^{-1} mg^{-1} protein for four candidate proteins and GFP.

Protein	CDNB	Flufenacet	Metolachlor	Pyroxasulfone	Diflufenican	Diclofop-methyl	Mesosulfuron-methyl
GST1A	4.5	44.6	30.6	14.5	0.0	0.0	0.0
GST1B	5.1	0.0	0.0	0.0	0.0	0.0	0.0
GST3	2.2	6.1	0.0	0.0	0.0	0.0	0.0
GST4	3.4	0.0	0.0	0.0	0.0	0.0	0.0
GFP	0.1	0.0	0.0	0.0	0.0	0.0	0.0

4.4 Discussion

This study was designed to better understand the genes involved in resistance of *Lolium* populations to the oxyacetamide flufenacet and their impact on cross-resistance patterns. As previously shown in other studies (Dücker *et al*., 2019b; Rauch *et al*., 2010) flufenacet resistance in ryegrass can reach field relevant levels with high resistance factors. In this study, a resistance factor of 42 was calculated for the three resistant populations USA1-R, VLR69-R, and FRA1-R on average. Flufenacet resistance in these populations was previously shown to be based on enhanced GST activity. Additionally, further degradation by cleavage of the peptide bonds of the conjugated glutathione as well as subsequent conjugation to malonyl or glucose was observed (Dücker *et al*., 2019b). Target-site resistance, however, has been excluded as unlikely resistance mechanism due to the characteristics of the target of flufenacet (Böger *et al*., 2000; Trenkamp *et al*., 2004; Dücker *et al*., 2019b). While GSTs were identified as key enzymes involved in flufenacet resistance, individual isoforms of the GST superfamily, as well as the gene(s) regulating flufenacet resistance in grass weeds, have not yet been investigated. Therefore, an RNA-Seq study was conducted with three sensitive and three flufenacet resistant *Lolium* populations. Among a heterogeneous set of 136 significantly downregulated gene-associated contigs several of them were BLAST-annotated as transcription factors. Besides this, gene-associated contigs were annotated with GO terms *e.g.* ‘protein kinase activity’ or ‘RNA polymerase II regulatory region sequence-specific DNA binding’. These gene-associated contigs may potentially be involved in the upregulation of resistance-conferring gene(s) *e.g.* in the case of transcription factors acting as repressors.

In total, 95 contigs were found significantly upregulated based on a differential gene expression analysis. Nine of them were identified as GSTs belonging to the class tau and

two of them were identified as phi class GSTs. Isoform GST3 was highly similar to *Am*GSTF1 isolated from *Alopecurus myosuroides* and *Lr*GSTF1 isolated from *L. rigidum* and previously described by several authors (Cummins *et al.,* 2013, Tétard-Jones *et al*., 2018). The analysis of the expression of these 11 contigs in untreated and treated plants revealed that the corresponding genes were not only constitutively upregulated, but also induced by the herbicide treatment, although upregulation was not significant in all cases.

A GO enrichment analysis confirmed the statistical significance of the upregulation of the GO terms 'glutathione transferase', but also 'glucosyltransferase activity', 'oxidoreductase activity', and terms *e.g.* 'flavonoid biosynthetic process' or 'anthocyanin-containing compound biosynthetic process'. The distribution of the GO terms suggests constitutive overall upregulation of detoxification pathways and genes involved in oxidative stress response. While GST activity plays a key role in flufenacet detoxification, glucosyltransferase activity was upregulated at an even higher level, although glucosyltransferases were not found to be involved in the rate-liming step in flufenacet detoxification. Therefore, it is possible that an upregulation of 'hotspots' *i.e.* specific regions on a chromosome as described for *Amaranthus* spp. may play a role in this type of resistance (Tranel, 2018). Analysis of a *L. multiflorum* Lam. or *L. rigidum* Gaud. genome may provide a better understanding of the regulation of the resistance-conferring genes. The constitutive upregulation of 'flavonoid biosynthetic process' or 'anthocyanin-containing compound biosynthetic process' furthermore suggests, that the flufenacet resistant plants may also benefit from a higher protection from oxidative stress.

Based on the GO enrichment analysis, the differential gene expression analysis, and the knowledge about flufenacet detoxification in *Lolium* spp., four GST isoforms (GST1, GST2, GST3, and GST4) were selected for the validation. The sequence analysis has shown that the contigs GST1 and GST2 likely belong to the same gene (GST1A) which is consistent with the expression patterns of both contigs (see Figure 3). During sequence analyses, a similar GST with a different 5' UTR (GST1B) was identified and used for the validation trials. The analysis of the protein sequences of GST1A, GST1B, GST3, and GST4 have shown that amino acid substitution-conferring mutations were present in the analyzed alleles; however, they didn't occur in the majority of the sequenced alleles of the resistant plants and therefore are not found to cosegregate with the resistance phenotype. Finally, alleles isolated from population USA1-R were chosen for overexpression in *E. coli*.

A photometric test with the GST model substrate CDNB revealed that the recombinant proteins of GST1A and GST4 were active with C-terminal His-tag, while GST1B and GST3

were active with N-terminal His-tag. In some cases, the His-tag may interfere with the folding or block the substrate's way to the active center, which could explain why some of the proteins were inactive.

A protein assay with different substrates finally demonstrated that GST1A (class tau) was able to detoxify flufenacet with 44.6 µmol min^{-1} mg^{-1} with a tenfold higher turnover rate than the model substrate CDNB (4.5 µmol min^{-1} mg^{-1}). GST3 (class phi) detoxified flufenacet with a turnover rate of 6.1 µmol min^{-1} mg^{-1} comparably slower. Also Bieseler *et al*. have previously shown that phi class GSTs isolated from corn and *Arabidopsis thaliana* Heynh. were able to detoxify flufenacet at low rates (Bieseler *et al*., 1997). This suggests, that flufenacet resistance is based on upregulation of at least one GST with a high affinity to flufenacet as well as a cumulative resistance in combination with GSTs with a lower substrate specificity *e.g.* GST3. GST4 and, interestingly, also GST1B were not able to detoxify flufenacet despite high sequence similarity between GST1A and GST1B.

Finally, cross-resistance patterns were analyzed *in planta* and *in vitro* with the chloroacetamide *S*-metolachlor, the isoxazoline pyroxasulfone (both inhibitors of the synthesis of VLCFAs), the phytoene desaturase (PDS) inhibitor diflufenican, the ACCase inhibitor diclofop-methyl, and the ALS inhibitor mesosulfuron-methyl. Only *S*-metolachlor and pyroxasulfone, two herbicides known to be detoxified by GSTs (Cottingham *et al*., 1993; Busi *et al*., 2018), were degraded only by GST1A with turnover rates of 30.6 and 14.5 µmol min^{-1} mg^{-1}, respectively. However, these results are to be interpreted with care and structure elucidation by LC-MS/MS needs be used to further confirm these results. Diflufenican, diclofop-methyl, and mesosulfuron-methyl were not degraded by any of the tested enzymes. Although only a resistance factor of two was estimated for pyroxasulfone, the turnover rates calculated for pyroxasulfone, *S*-metolachlor, diflufenican, and flufenacet correlate generally with the resistance status assessed in greenhouse bioassays. The isoxazoline structure of pyroxasulfone differs from the chloroacetamide structure of *S*-metolachlor and the oxyacetamide structure of flufenacet. The lower turnover rate *in vitro* and the low resistance factor in the bioassay are likely linked with a lower affinity of the resistance-conferring GSTs to the pyroxasulfone. In the dose-response assay with *S*-metolachlor a resistance factor of 13 was estimated, which corresponds to the intermediate turnover rate and suggests potential cross-resistance between *S*-metolachlor and flufenacet.

The dose-response assay with diflufenican, which was not degraded by any of the candidate GSTs, has shown that no significant differences in diflufenican efficacy on sensitive and flufenacet resistant populations were present. In a similar way, a metabolism study *in planta* with mesosulfuron-methyl, which was neither degraded by the candidate GSTs, has shown that this herbicide was not degraded at a faster rate in the leaves of

flufenacet resistant *Lolium* populations. Therefore, no cross-resistance between flufenacet and diflufenican as well as mesosulfuron-methyl was found *in vitro* and *in planta*. This is in accordance with the mechanisms described for crop tolerance to diflufenican in cereals and resistance to mesosulfuron-methyl in *Lolium* spp. Crop tolerance to diflufenican was found to be correlated with reduced uptake (Haynes and Kirkwood, 1992) while mesosulfuron-methyl resistance in *Lolium* spp. was described as mediated by cytochrome P450 monooxygenases (Duhoux and Délye, 2013).

Also, diclofop-methyl resistance was described as cytochrome P450 monooxygenase-mediated (Shimabukuro *et al*., 1979; Gaines *et al*., 2014). This can explain why diclofop-methyl was degraded at a faster rate in the flufenacet resistant populations although it was not detoxified by the candidate GSTs.

Finally, this suggests that two distinct mechanisms confer multiple resistance to diclofop-methyl and flufenacet in the tested populations. Although diclofop-methyl is not suitable to control the tested *Lolium* populations due to resistance, herbicides with assigned resistance mechanisms other than enhanced GST activity (*e.g.* diflufenican and mesosulfuron-methyl) are generally suitable for a combination with flufenacet in a sustainable weed management program. Their use is unlikely to select cross-resistance. In conclusion, the knowledge about cross-resistance patterns can aid weed management decisions and the choice of suitable herbicide combinations for resistance management.

4.5 Acknowledgements

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4.6 References

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5. Enhanced metabolism causes reduced flufenacet sensitivity in black-grass (*Alopecurus myosuroides* Huds.) field populations

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Abstract

BACKGROUND: Black-grass (*Alopecurus myosuroides* Huds.) is a frequent grass weed that commonly occurs in winter wheat in temperate Europe. Evolving resistance to post-emergence herbicides, *e.g*. acetyl CoA carboxylase (ACCase) and acetolactate synthase (ALS) inhibitors requires more complex weed management strategies and ensuring good efficacy of pre-emergence treatments becomes increasingly important. Flufenacet, in particular, has become a key herbicide for the control of multiple-resistant *A. myosuroides*. However, in some of those populations, reduced flufenacet efficacy was already observed.

RESULTS: In a screening of black-grass populations from several European countries, most populations were controlled with the registered field rate of flufenacet. However, differences in the level of flufenacet sensitivity were observed and correlated with glutathione *S*-transferase-mediated enhanced flufenacet metabolism. The efficacy of the pre-emergence herbicides pendimethalin, prosulfocarb, *S*-metolachlor and pethoxamid, was also significantly decreased in populations with reduced flufenacet sensitivity. The use of flufenacet in mixtures with diflufenican, particularly in combination with flurtamone or metribuzin, however, significantly improved efficacy in less susceptible black-grass populations.

CONCLUSIONS: In several populations of different European origins, reduced efficacy of flufenacet was observed due to enhanced metabolism. Although differences between populations were relatively small, best weed management practices (*e.g.* application of full dose rates and herbicide mixtures and wide crop rotations) should be applied to reduce selection pressure and prevent flufenacet resistance from further evolving. This is particularly important as flufenacet is one of the few still-effective herbicides suitable for the

control of multiple-resistant *A. myosuroides* genotypes in Europe, whereas alternative pre-emergence herbicides were less effective against multiple-resistant *A. myosuroides* populations.

Keywords

Herbicide resistance, enhanced metabolism, flufenacet, black-grass, glutathione transferases, HRAC group K_3

5.1 Introduction

Black-grass (*Alopecurus myosuroides* Huds.) has gained importance as an agronomic weed in temperate Europe during past decades. Closer crop rotations and the resulting higher percentage of winter crops as well as evolving resistance have been mentioned in this context as driving factors (Krähmer, 2016; Moss, 2017). By 2018, resistance to seven different herbicide modes of action (MoAs) in *A. myosuroides* populations have been described and in many cases resistance to several MoAs accumulate within one population (Délye *et al*., 2011; Rosenhauer and Petersen, 2015; Hess *et al*., 2016; Heap, 2019). When resistance to inhibitors of acetyl CoA carboxylase (ACCase, HRAC group A) and acetolactate synthase (ALS, HRAC group B), in particular, has evolved within one population, only limited chemical options are available for weed control and resistance management (Moss, 2017; Peterson *et al*., 2018). As a result, the use of pre-emergence products, less affected by herbicide resistance, has become an important tool for weed control and resistance management (Beckie and Tardif, 2012; Bailly *et al*., 2012; Somerville *et al*., 2017). In particular, products containing flufenacet, an inhibitor of the synthesis of very-long-chain fatty acids (VLCFAs, HRAC group K_3) in plants, have gained significance for *A. myosuroides* control in temperate Europe (Menne *et al*., 2012). However, increasing reliance on residual herbicides leads to increased resistance selection pressure due to repetitive application of the same herbicide MoAs (Jasieniuk *et al*., 1996; Hull and Moss, 2012). Reduced flufenacet efficacy on certain multiple-resistant *A. myosuroides* populations has previously been described, with resistance factors (RFs) up to 6 (Rosenhauer and Petersen, 2015; Klingenhagen, 2012). Yet, no significant decrease in flufenacet efficacy over time could be observed during an analysis of 352 field trials. A trend of 2% reduction per year between 2001 and 2012 was observed, but other factors like soil moisture had more influence on flufenacet activity and accounted for high variability (Hull and Moss, 2012). For example, an *A. myosuroides* field population showed no shift in flufenacet resistance over 6 years despite intensive herbicide use, whereas the same population

evolved a progressive decrease in sensitivity of 5–6% per year due to recurrent selection with flufenacet in an outdoor pot trial (Hull and Moss, 2012). In a similar way, recurrent selection with the ACCase inhibitor diclofop-methyl or pyroxasulfone, an inhibitor of VLCFA synthesis, led to the evolution of non-target site resistance to these herbicides (Neve and Powles, 2005; Busi *et al*., 2012).

In the case of flufenacet, it has been suggested that target-site resistance against inhibitors of VLCFA synthesis is unlikely to evolve (Böger *et al*., 2000; Busi, 2014). These herbicides were shown to inhibit several isoforms of their molecular target, 3-ketoacyl-CoA-synthases (KCSs), enzymes involved in VLCFA elongation (Trenkamp *et al*., 2004; Tanetani *et al*., 2009). It was assumed that, due to redundancy, several mutated genes need to accumulate to effectively build up resistance, without losing their molecular function (Böger *et al*., 2000; Busi, 2014; Haslam and Kunst, 2013). Additionally, tolerance to flufenacet in crops like corn, as well as the resistance of *Lolium* spp. populations to flufenacet, was previously shown to be linked to enhanced glutathione *S*-transferase (GST) activity (Bieseler *et al*., 1997; Dücker *et al*., 2019). To date, there is little information on the extent of reduced flufenacet sensitivity in the field, and the molecular mechanisms of flufenacet resistance in *A. myosuroides* have not yet been investigated. Therefore, this study aimed to characterize the level of flufenacet sensitivity in several European *A. myosuroides* populations in a dose–response screening, and select suitable biological material to analyze the mechanisms behind reduced flufenacet sensitivity in this species. Flufenacet degradation rates and pathway(s) were analyzed using high-performance liquid chromatography (HPLC) and liquid chromatography–mass spectrometry (LC–MS), and resistance patterns were studied with several pre-emergence herbicides such as pendimethalin (inhibition of microtubule assembly, HRAC group K_1), prosulfocarb (lipid synthesis inhibition, HRAC group N) and several inhibitors of VLCFA synthesis, as well as flufenacet mixtures in greenhouse bioassays.

5.2 Materials and methods

5.2.1 *Alopecurus myosuroides* plant material

Seed samples of *A. myosuroides* were collected from 18 fields located in the Elbe marshes in Kehdingen, Germany, because of the suspected widespread occurrence of metabolism-based herbicide resistance in this area to different MoAs. The selected populations were compared with samples of different origins: five populations from Schwäbisch-Hall, Germany, an area where herbicide resistance due to target-site mutations is relatively frequent (Hess *et al*., 2016; Herrmann, 2014); five randomly selected populations from different field locations in the United Kingdom (UK), where resistance to ACCase and/or

ALS inhibitors was observed; five randomly selected populations from fields in France where resistance to ACCase and/or ALS inhibitors was observed; and ten populations from different German field locations, where flufenacet products were applied in at least six cropping seasons before seed harvest, according to field history data (populations named Selected1–Selected10). In addition, two commercially available susceptible populations (Herbiseed-S and Appel-S) obtained from Herbiseed (Twyford, UK) and Appels Wilde Samen (Darmstadt, Germany), as well as five field populations from different origins in Germany without resistance problems (populations named Field1-S–Field5-S), were used as a susceptible reference.

5.2.2 Plant cultivation and dose-response bioassays

In a screening experiment, each 35 seedlings of the 50 populations listed above were sown in each of three pots per treatment and population, containing sandy loam with 2.2% organic matter. The seeds were covered with a thin layer of coarse sand and subsequently watered to induce germination in a greenhouse under 22/16°C day/night conditions, with a 14:10 h light/dark photoperiod provided by Philips Master HPI-T plus 400 W/645 E40 metal halide lamps (Philips, Amsterdam, The Netherlands) at ~ 200 µmol m^{-2} s^{-1}. After 3 days the plants were treated with 0, 7.5, 15, 30, 60, 120, 240 and 480 g flufenacet ha^{-1} formulated as Cadou® SC on a laboratory track sprayer (TeeJet nozzle XR8001, 300 L ha^{-1}, 200 hPa; TeeJet, Wheaton, IL, USA). The plants were returned to the greenhouse and watered once from above. The foliage fresh weight of each pot was assessed 35 days after treatment. The experiment was repeated once.

Based on availability, and results obtained from the previous screening experiment, six populations were selected for further bioassays with different pre-emergence herbicides: the susceptible populations Appel-S, Herbiseed-S and Field1-S, as well as Kehdingen1, Kehdingen2 and Kehdingen3, three populations from Kehdingen, Germany with reduced flufenacet sensitivity. To ensure homogenous growth stage and reduce variation due to dormancy and numb seeds, pre-germinated seedlings were transplanted. For each bioassay, seeds of the named six populations were pre-germinated on solidified water agar (0.7% w/v) under the greenhouse conditions described above, until the primordial root emerged. Fifteen viable seedlings per treatment and population were transferred to pots containing sandy loam with 2.2% organic matter. Each pot contained five seedlings and was subsequently covered with a thin layer of coarse sand and treated with different dose rates of selected herbicides (see Table 1) using a laboratory track sprayer (TeeJet nozzle XR8001, 300 L ha^{-1}, 200 hPa). After treatment, plants were returned to the greenhouse and

the pots were watered once from above. The foliage fresh weight of each pot was assessed 35 days after treatment. The entire experiment was repeated once.

Table 1: Product name, active ingredient, registered field rate[a] and applied dose rates of herbicides applied in dose–response experiments with susceptible *Alopecurus myosuroides* populations Appel-S, Herbiseed-S Field1-S, as well as Kehdingen1, Kehdingen2 and Kehdingen3.

	Herbicide product	Active ingredient	Mode of action[b]	Field rate (g a.i. ha^{-1})[a]	Applied dose rates (g ha^{-1})					
Application of solo products	Cadou® SC (Bayer)	Flufenacet	K_3	254.4	0.0	3.1	9.4	28.3	84.8	254.4
	Stomp® Aqua (BASF)	Pendimethalin[c]	K_1	2000.0	0.0	24.7	74.1	222.2	666.7	2000.0
	Boxer® (Syngenta)	Prosulfocarb	N	2400.0	0.0	29.6	88.9	266.7	800.0	2400.0
	Sakura® 85 WG (Bayer)	Pyroxasulfone[d]	K_3	-	0.0	0.5	1.4	4.2	12.5	25.0
	Cadou® SC (Bayer)	Flufenacet	K_3	304.8	0.0	3.8	11.3	33.9	101.6	304.8
	Dual Gold® (Syngenta)	*S*-metolachlor	K_3	1200.0	0.0	14.8	44.4	133.3	400.0	1200.0
	Quantum® (Cheminova)	Pethoxamid	K_3	900.0	0.0	11.1	33.3	100.0	300.0	900.0
Application of mixtures	Malibu® (BASF)	Flufenacet	K_3	240.0	0.0	3.0	8.9	26.7	80.0	240.0
		Pendimethalin	K_1	1200.0	0.0	14.8	44.4	133.3	400.0	1200.0
	Herold®SC (Adama)	Flufenacet	K_3	240.0	0.0	3.0	8.9	26.7	80.0	240.0
		Diflufenican	F_1	120.0	0.0	1.5	4.4	13.3	40.0	120.0
	Cadou® Forte Set (Bayer)	Flufenacet	K_3	242.0	0.0	3.0	9.0	27.0	80.9	242.0
		Diflufenican	F_1	90.0	0.0	1.1	3.3	10.0	30.0	90.0
		Flurtamone	F_1	90.0	0.0	1.1	3.3	10.0	30.0	90.0
	Liberator Pro SC (Bayer)	Flufenacet	K_3	240.0	0.0	3.0	8.9	26.7	80.0	240.0
		Diflufenican	F_1	120.0	0.0	1.5	4.4	13.3	40.0	120.0
		Metribuzin	C_1	70.0	0.0	0.9	2.6	7.8	23.3	70.0
	22110H SC (Bayer)	Flufenacet	K_3	90.0	0.0	1.1	3.3	10.0	30.0	90.0
		Diflufenican	F_1	30.0	0.0	0.4	1.1	3.3	10.0	30.0
		Aclonifen	F_3	450.0	0.0	5.6	16.7	50.0	150.0	450.0

[a] Registered dose rate for corn and/or winter cereals in Germany.

[b] HRAC classification.

[c] Additional dose rate of 18 000.00 g pendimethalin ha^{-1}.

[d] Not registered in Europe.

5.2.3 Flufenacet degradation rates in seedlings of the populations Herbiseed-S and Kehdingen1

Seedlings of the populations Herbiseed-S and Kehdingen1 were raised on solidified water agar (0.7% w/v) in a growth chamber until the first leaf reached a length of ~ 2.5 cm. The chamber was set to 22/16°C day/night conditions with a 14:10 h light/dark photoperiod provided by Philips Master TL-D 58W/840 REFLEX fluorescent lamps at ~ 400 µmol $m^{-2}s^{-1}$. Some 2 × 24 seedlings per population and time point were placed in 20 mL glass vials containing 1.2 mL 0.02 M KNO_3 mineral water (Volvic, Clairvic, France) with a molarity of

7.5 µM ^{14}C-radiolabeled flufenacet giving a final radioactivity of 16.7 mBq mL^{-1}. The vials were carefully shaken and incubated at 22/16°C day/night conditions. The seedlings were harvested 6, 12, 18, 24, 48, 72 and 168 h after treatment, washed twice in water and once in 50% acetone. Eight seedlings were dried on paper, pooled and subsequently frozen in 600 µL methanol giving five pooled biological replicates per population and time point. The radiolabeled compounds were extracted as described by Collavo *et al.* (2015) with an additional extraction step with 600 µL 90% acetonitrile. The extract was resuspended in 200 µL 80% acetone. Chromatographic separation was achieved as described by Dücker *et al.* (2019). The recovery rate was 92.8% on average. The entire experiment was repeated once.

5.2.4 Metabolite identification in extracts from seedlings of the populations Herbiseed-S and Kehdingen1

To identify flufenacet metabolites in the populations Herbiseed-S and Kehdingen1, seeds were raised on solidified water agar and treated as described above. The seedlings were harvested 6, 12, 18, 24 and 72 h after treatment and extracted as described above. LC–MS analysis of the named samples was performed on a Waters Q-ToF Premier mass spectrometer (Waters, Manchester, UK) connected to Waters 2795 HPLC System (Waters, Milford, MA, USA) via a radioactivity detector FlowStar LB513 (Berthold Technologies, Bad Wildbad, Germany), and an electrospray interface operated in the positive and negative mode. Some 50 µL per sample were injected and chromatographic separation was achieved as described by Dücker *et al.* (unpublished). MassLynx® 4.1 software (Waters, Manchester, UK) was used for instrument control and data evaluation. High-resolution mass spectrometry was used for the confirmation of compound identities described by Dücker *et al.* (2019) (determination of the elemental composition of molecular ions) in the MS-mode (product ion scan).

5.2.5 Statistical analyses

Dose–response assays to characterize the resistance levels and the time course experiment to determine flufenacet degradation rates were analyzed as randomized block designs using the drc package (Ritz *et al*., 2015) of the statistical software R (version 3.4.3, R Foundation for Statistical Computing, Vienna, Austria). To determine the response of 50 *A. myosuroides* populations to different dose rates of flufenacet, only populations without significant differences (based on 95% confidence intervals) between the two experiments conducted were included in the analysis. The data of both experiments were pooled and fitted with a three-parameter log-logistic model (Ritz *et al*., 2015). Effective dose rates needed for 50% and 90% growth reduction (ED_{50}, ED_{90}) and standard errors (SE) were

calculated for each *A. myosuroides* population. Differences between estimated ED_{50} values of an individual population and the average of ED_{50} values of seven susceptible reference populations. Statistical differences between populations were determined, using 95% confidence intervals (CIs).

Effective dose rates (ED_{50}, ED_{90}) of selected pre-emergence herbicides (Table 1) were calculated for the populations Appel-S, Herbiseed-S and Field1-S as a group, and the populations Kehdingen1, Kehdingen2 and Kehdingen3 as a group. RF values were calculated as the ratio of estimated ED_{50} values of resistant and susceptible populations. Statistical differences between groups or populations were determined with 95% CIs. To determine flufenacet degradation rates in population Herbiseed-S and Kehdingen1 a three-parameter log-logistic model (Ritz *et al*., 2015) was fitted to the percentage of recovered parent compound (flufenacet) in each sample. For both populations the time required for 50% degradation of the parent compound (degradation half-time, DT_{50}) and corresponding standard errors were calculated. Resistance indices (RI) were calculated as the ratio of estimated DT_{50} values of population Kehdingen1 and the susceptible population Herbiseed-S.

5.3 Results

5.3.1 Efficacy of flufenacet on selected *Alopecurus myosuroides* field populations

The dose–response analysis of 50 *A. myosuroides* populations from different European origins revealed significantly different levels of resistance, with continuous variation in ED_{50} values ranging from 6.9 to 81.1 g flufenacet ha^{-1}. Corresponding RF values ranged from flufenacet ha^{-1} (see Figure 1). Commercially available Appel-S and Herbiseed-S were the most susceptible populations tested, with ED_{50} values of 6.9 and 9.3 g flufenacet ha^{-1}, and ED_{90} values of 36.9 and 23.5 g flufenacet ha^{-1}, respectively.

Based on 95% CIs, those two populations did not differ significantly from the five susceptible field populations from different locations in Germany without known resistance issues (Field-S). Their ED_{50} values ranged from 10.6 to 14.4 g flufenacet ha^{-1}, and their ED_{90} values from 29.1 and 45.0 g flufenacet ha^{-1}. The populations from France and the UK sampled from fields with resistance to ALS and/or ACCase resistance had ED_{50} values of 10.0 to 45.7 and 17.6 to 46.2 g flufenacet ha^{-1}, respectively. Their ED_{90} values ranged from 20.5 to 154.0 and 60.8 to 213.8 g flufenacet ha^{-1}, respectively. With RF values of 4, only populations France2, UK3 and UK4 differed significantly from all susceptible reference populations. The populations from Schwäbisch-Hall, Germany had ED_{50} values of 23.1 to 26.8 g flufenacet ha^{-1} and ED_{90} values of 57.8 to 143.7 g flufenacet ha^{-1}.

As an exception, population Schwäbisch-Hall1 with an ED_{50} value of 62.5 g flufenacet ha^{-1}

(RF=6) and an ED_{90} value of 207.1 g flufenacet ha^{-1} differed significantly from all susceptible reference populations. The populations originating from fields in Germany, treated with flufenacet in at least six different cropping seasons (Selected) had ED_{50} values of 16.1 to 81.1 g flufenacet ha^{-1} (RF=2–8) and ED_{90} values of 48.1 to 286.2 g flufenacet ha^{-1}. All these populations, except for Selected4 differed significantly from the susceptible reference populations. The populations from Kehdingen, Germany had ED_{50} values of 22.8 to 79.2 g flufenacet ha^{-1} (RF=2–8) and ED_{90} values of 106.9 to 310.9 g flufenacet ha^{-1}. All of these populations, except for Kehdingen9, differed significantly from the susceptible reference populations. In total, the ED_{90} values of six populations (Kehdingen8, Kehdingen11, Kehdingen12, Kehdingen3, Kehdingen2 and Selected5, a populations originating from Dithmarschen) exceeded the registered field rate in cereals of 240–250 g flufenacet ha^{-1}, depending on country and formulation.

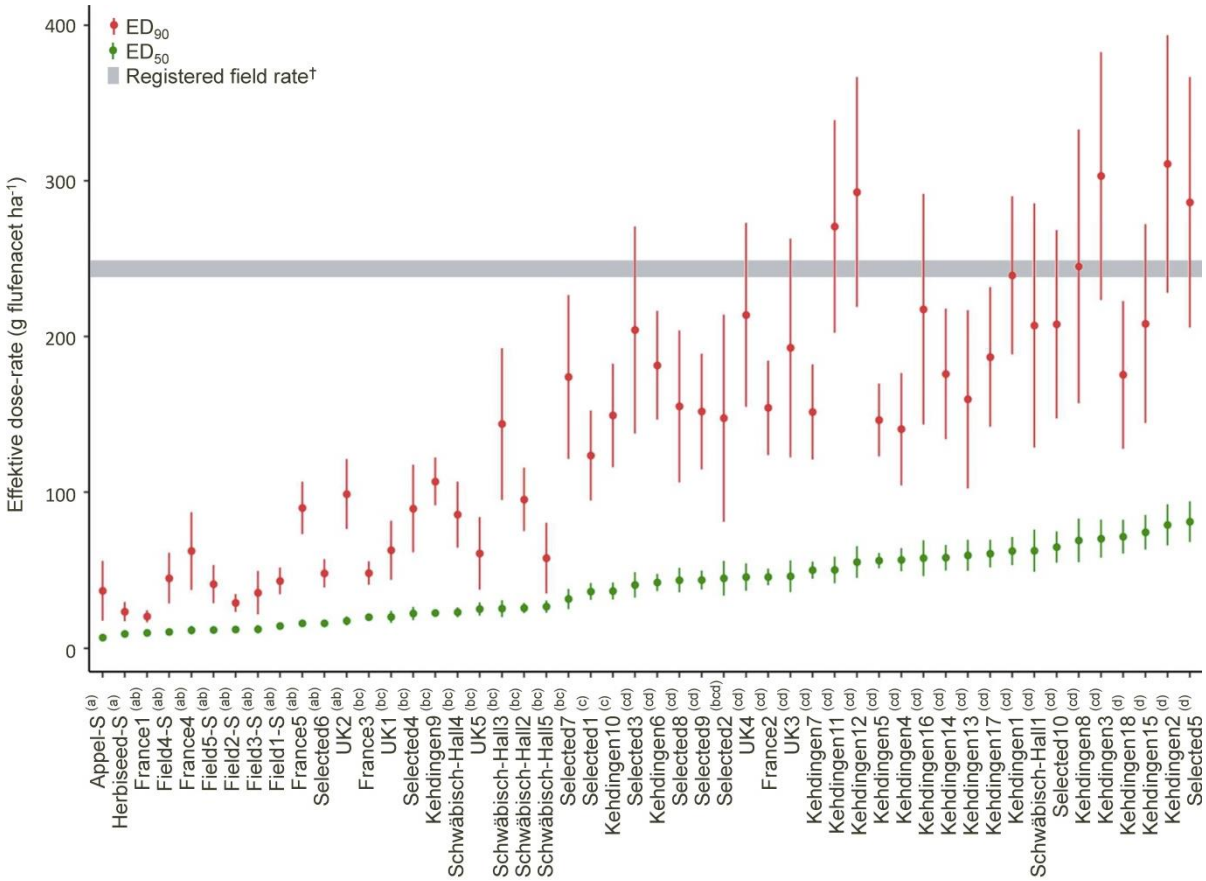

Figure 1: Estimated 50% effective dose (ED_{50}) values ± SE and 90% effective dose (ED_{90}) values ± SE from log-logistic dose–response models for 50 *Alopecurus myosuroides* populations. Different letters indicate significant differences in ED_{50} values between populations based on 95% confidence intervals. Sensitive reference populations are indicated by the suffix '-S'. †Registered flufenacet field rate in Europe in 2018 (240–254 g flufenacet ha^{-1} depending on country and formulation).

5.3.2 Dose–response relationship of susceptible reference populations and field populations from Kehdingen to selected pre-emergence herbicides

Dose–response analysis of three susceptible reference populations and three populations originating from Kehdingen has shown that the populations from Kehdingen survived significantly higher dose rates of all applied herbicides (see Figure 2). However, the extent varied from herbicide to herbicide. In one experimental setup, six herbicides were applied as solo formulations. The differences between the susceptible reference populations and the populations from Kehdingen were relatively low, displaying RF values of 2, 2 and 3 RFs for flufenacet, pyroxasulfone and pendimethalin. By contrast, higher RF values of 7, 10 and 13 were recorded for *S*-metolachlor, prosulfocarb and pethoxamid, respectively. The same trend is reflected in the ED_{90} values. However, only the ED_{90} values for flufenacet (15.2 and 55.6 g ai ha^{-1}) and pyroxasulfone (4.2 and 7.4 g ai ha^{-1}) were below the registered field rates. In the case of prosulfocarb, *S*-metolachlor, pethoxamid and pendimethalin, ED_{90} values for the control of populations from Kehdingen were above field rates registered in Europe. Similarly, the pendimethalin field rate registered in France (1000 g ai ha^{-1}) was not sufficient for controlling the susceptible reference populations (ED_{90}=1337.7 g ai ha^{-1}) under greenhouse test conditions.

However, flufenacet alone and all flufenacet mixtures controlled the populations from Kehdingen by 90%, at less than the field rate. The fresh weight of the susceptible reference populations was controlled by 90% at 5–13% of the registered field rate. The ED_{90} values calculated for the control of the populations from Kehdingen differed to a greater extent from herbicide to herbicide.

With flufenacet and flufenacet + pendimethalin, 50% and 67% of the registered field rate were needed to control 90% of the fresh weight of populations from Kehdingen. With mixtures containing inhibitors of photosynthesis and pigment synthesis, significantly lower percentages of the registered field rate were needed to control the populations from Kehdingen. Therefore, 28.2% and 14.5% of the field rates registered for flufenacet + diflufenican and flufenacet + diflufenican + flurtamone were needed for 90% control, respectively. The amount of formulated product needed for 90% control was again significantly decreased in the case of flufenacet + diflufenican + metribuzin and flufenacet + diflufenican + aclonifen with ED_{90} values of 9.9% and 7.6% of the proposed field rates. The ED_{50} values corresponded with the ED_{90} values and resulted in RF values of 4 (flufenacet + pendimethalin), 3 (flufenacet, flufenacet + diflufenican, flufenacet + diflufenican + flurtamone) and 2 (flufenacet + diflufenican + metribuzin, flufenacet + diflufenican + aclonifen). See Table 1 for applied doses and registered field rates.

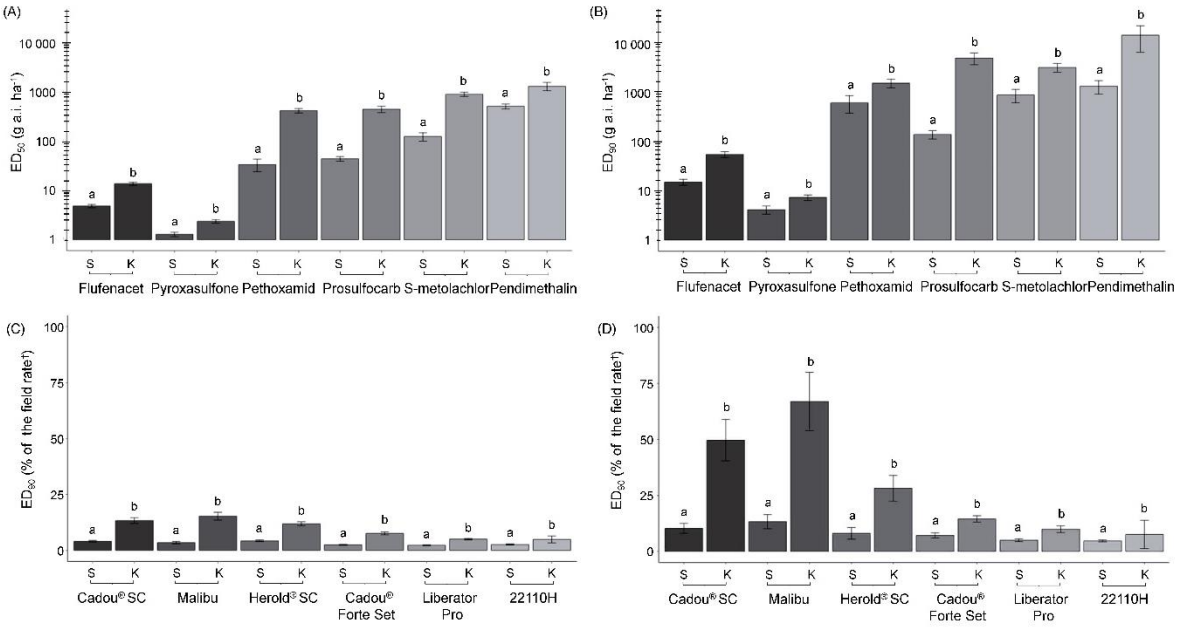

Figure 2: Dose–response relationship of three sensitive *Alopecurus myosuroides* reference populations (S) and three *Alopecurus myosuroides* populations from Kehdingen (K) to selected pre-emergence herbicides displayed as (A) 50% effective dose (ED_{50}) and (B) 90% effective dose (ED_{90}) values ± SE. Response of three sensitive *Alopecurus myosuroides* reference populations (S) and three *Alopecurus myosuroides* populations from Kehdingen (K) to selected flufenacet-based herbicide mixtures, displayed as (C) ED_{50} and (D) ED_{90} values ± SE. Significant differences between the sensitive reference populations (S) and the populations from Kehdingen (K), based on 95% confidence intervals are indicated by different letters. The active ingredients applied in mixtures include: flufenacet (FFA), pendimethalin (PDM), diflufenican (DFF), flurtamone (FLT), metribuzin (MRB) and aclonifen (ACL).

5.3.3 Flufenacet degradation rates in seedlings of the populations Herbiseed-S and Kehdingen1

Analysis of flufenacet degradation in the susceptible reference population Herbiseed-S and population Kehdingen1 revealed that Herbiseed-S degraded flufenacet with an estimated DT_{50} of 127.8 h, which is significantly slower than Kehdingen1 with a DT_{50} of 41.7 (see Figure 3). A detoxification pathway was created (see Figure 4) based on molecular masses identified by LC–MS in extracts from flufenacet treated seedlings of the populations

Herbiseed-S and Kehdingen1. A flufenacet glutathione conjugate with a molecular mass of 500 Da (M500, $C_{21}H_{29}F_1N_4O_7S_1$), likely formed by GST activity, was identified as the first occurring flufenacet metabolite. Hydrolysis of the peptide bonds resulted in the formation of a glutamyl–cysteine conjugate (M371, $C_{16}H_{22}N_3O_4F_1S_1$) followed by a cysteine conjugate (M314, $C_{14}H_{19}F_1N_2O_3S_1$). Additionally, two metabolites typical for phase III metabolism were identified: A malonyl-cysteine conjugate with a molecular mass of 400 Da (M400, $C_{17}H_{21}F_1N_2O_6S_1$) as well as a flufenacet glycosyl conjugate (M477, $C_{20}H_{28}F_1N_1O_9S_1$), likely formed from the cysteine conjugate M314 after hydrolysis of the peptide group of the cysteinyl. The same metabolites were found in populations Herbiseed-S and Kehdingen1.

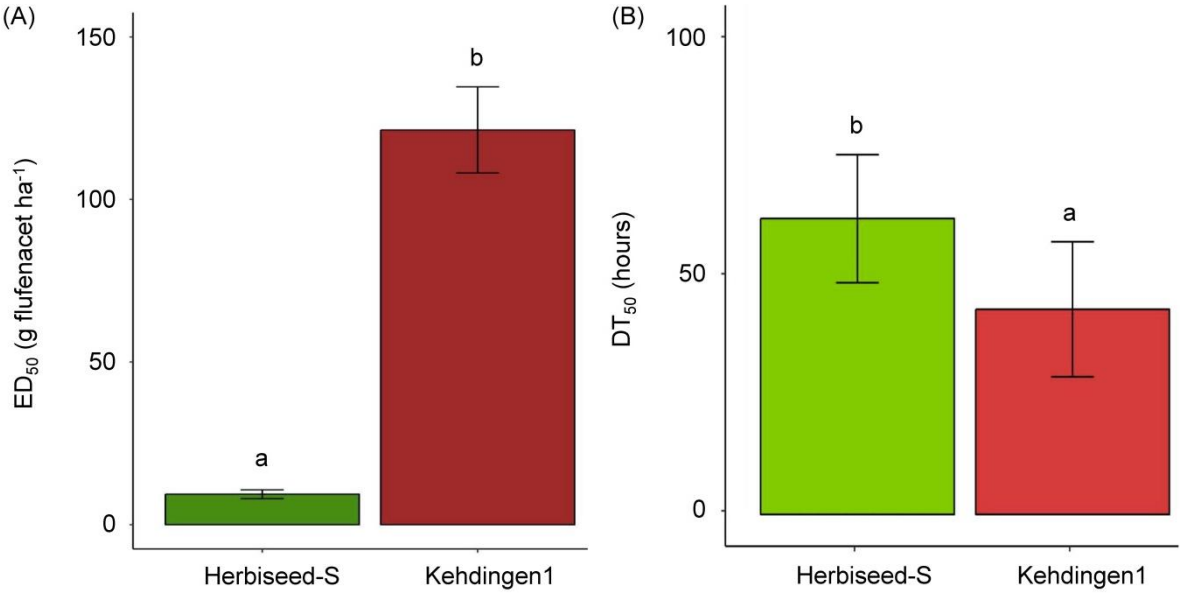

Figure 3: (A) Estimated 50% effective dose (ED_{50}) values of the sensitive population Herbiseed-S and population Kehdingen1 ± SE. Different letters indicate significant differences between populations based on 95% confidence intervals. (B) Estimated degradation half-time (DT_{50}) of the sensitive population Herbiseed-S and population Kehdingen1 ± SE. Different letters indicate significant differences between populations based on 95% confidence intervals.

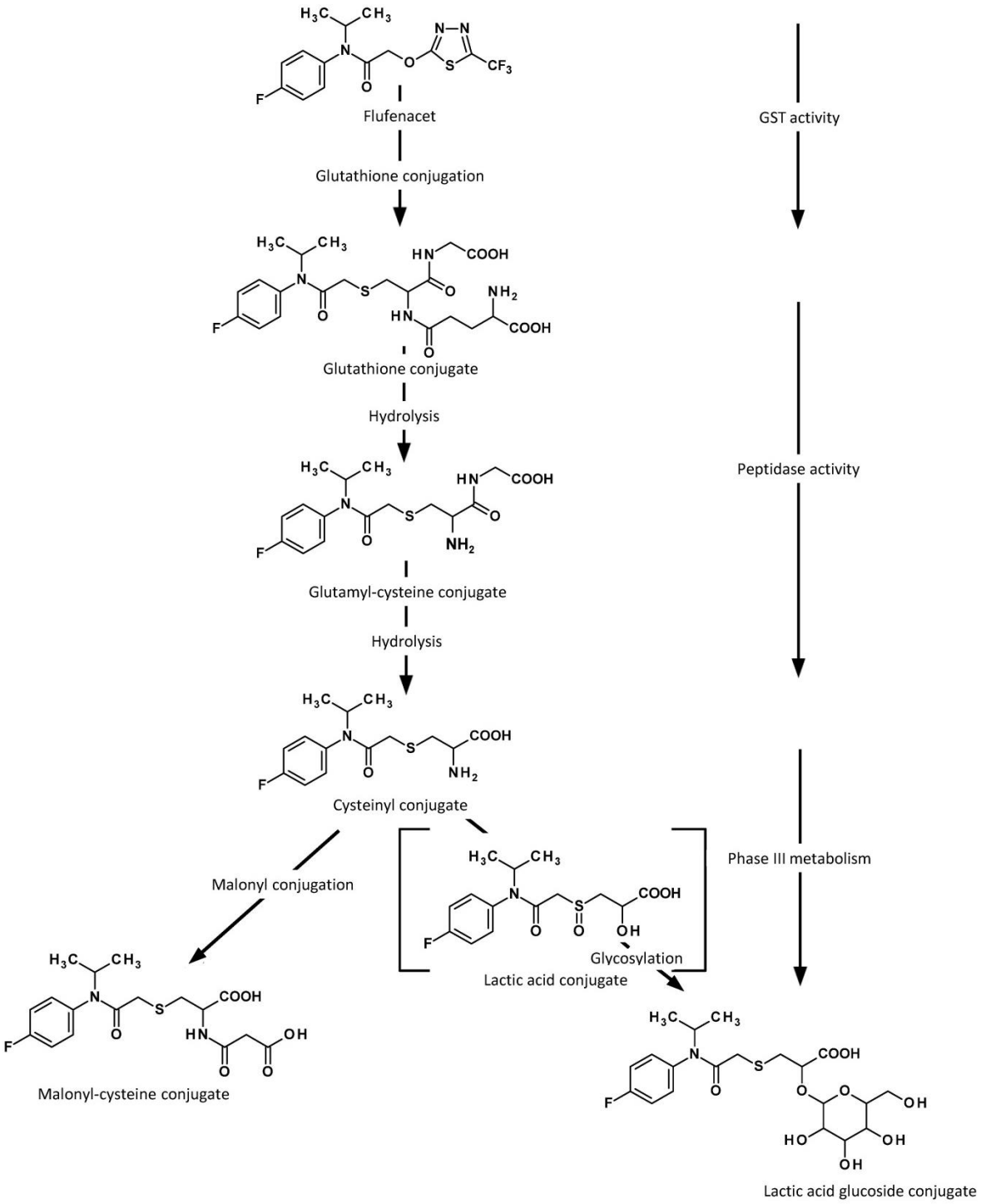

Figure 4: Flufenacet detoxification pathway showing metabolites and corresponding enzymatic or chemical degradation activity. Metabolites were identified by LC–MS analysis of extracts obtained from the sensitive population Herbiseed-S and Kehdingen1. Mass spectra of intermediate metabolites indicated by brackets were not detected by LC–MS.

5.4 Discussion

The efficacy of flufenacet on 50 *A. myosuroides* populations of different origins was analyzed in a dose–response bioassay. Widespread occurrence of significantly reduced flufenacet efficacy on field populations, from origins with resistance to ACCase and/or ALS-herbicides was observed. Continuous variation in the levels of resistance was found, which is typical for polygenic traits (Mather and Jinks, 1982). Differences between populations, however, were found within the range of 'low-level resistance', *i.e*. with resistance factors < 10.28 In total, six of the tested populations survived treatments with the flufenacet field rate registered for use in cereals in Europe (240–254 g flufenacet ha^{-1}, depending on product and country). Those populations were collected in the northern German marshes in Kehdingen south of the Elbe estuary and Dithmarschen north of the estuary. Moderately reduced efficacy with > 90% fresh weight control with the registered field rate was widespread among *A. myosuroides* populations from fields where reduced efficacy of inhibitors of ALS and/or ACCase was already observed. Moderately reduced efficacy was found in populations originating from France (Seine-Maritime), the UK (Wiltshire, Essex) and various German marsh regions and the island of Rügen, which have been selected with flufenacet in six or more cropping seasons in the past years. Significantly lower field rates were needed for control of field populations with origins without known resistance problems. This suggests that, as described previously for other resistance cases, *e.g.* ALS resistance in *A. myosuroides*, (Légère *et al*., 2000; Herrmann *et al*., 2016), management practices may have affected the level of flufenacet efficacy on those populations and possibly led to increased ED_{90} values up to 310.9 g flufenacet ha^{-1}.

A shift in efficacy in that range may not lead to yield reduction under field conditions in competition with the crop. However, successful use of pre-emergence herbicides depends strongly on environmental factors, *e.g.* weed densities, soil conditions or precipitation (Menne *et al*., 2012; Hull and Moss, 2012). Under unfavorable environmental conditions, a shift in flufenacet efficacy as observed for some populations from the northern German marshes may become field relevant.

Also, a methodological change from treating 35 seedlings per pot 3 days after watering to transplantation of five pre-germinated seedlings at the same growth stage and subsequent treatment on the same day decreased the amount of herbicide needed for 50% fresh weight reduction considerably. The ED_{50} values for the populations Kehdingen1, Kehdingen2 and Kehdingen3 decreased from ~ 70 to ~ 13 g flufenacet ha^{-1} on average, and the ED_{50} values for the susceptible populations Appel-S, Herbiseed-S and Field1-S decreased from ~ 10 to ~ 5 g flufenacet ha^{-1} on average. This difference is expected because lower plant densities (five plants per pot) are associated with higher herbicide efficacy (Menne *et al*., 2012). In

addition, transplantation of pre-germinated seedlings ensures that all treated plants are at the same susceptible growth stage.

Therefore, dose–response assays comparing flufenacet efficacy, and the efficacy of prosulfocarb (N), pendimethalin (K_1), pethoxamid (K_3) and *S*-metolachlor (K_3) on susceptible populations and populations with reduced flufenacet sensitivity from the Elbe marshes in Kehdingen were conducted with transplanted seedlings at homogenous growth stages. The obtained results generally go along with previous publications on herbicide efficacy (Rosenhauer and Petersen, 2015; Klingenhagen, 2012).

Commercial products containing pendimethalin, prosulfocarb, *S*-metolachlor and pethoxamid achieved < 90% growth reduction (ED_{90}), with the typical field rates registered in Europe when applied on seedlings originating from Kehdingen, Germany. With RF values of 3, 10, 7 and 13 respectively, significantly higher amounts of active ingredient were needed to control the populations from Kehdingen, in comparison with the susceptible reference populations. With RF values of 3 and 2, significant differences were also observed for flufenacet and pyroxasulfone, respectively. Yet, with ED_{90} values of 55.6 and 7.4 g ai ha^{-1}, comparably low amounts of herbicide were needed for a 90% reduction in fresh weight. In conclusion, among the tested herbicides available in Europe, flufenacet was most effective on the multiple resistant populations from Kehdingen despite a shift in resistance. Pyroxasulfone was comparably effective on populations with a shift in flufenacet efficacy, but is not registered in Europe. Thus, there are at present no more effective alternatives to the pre-emergence application of flufenacet available for the control of multiple resistant *A. myosuroides* populations in wheat.

To ensure successful chemical control of these populations with flufenacet, it is essential to apply this herbicide in mixture with other suitable herbicides. This is particularly necessary because the application of herbicide mixtures can delay the development of resistance (Norsworthy *et al*., 2012). Dose–response assays with flufenacet-based products, in particular, have shown that mixtures with diflufenican can considerably improve the efficacy on susceptible, as well as the described multiple-resistant *A. myosuroides* populations from Kehdingen. In particular, three-way mixtures (flufenacet + diflufenican + flurtamone, flufenacet + diflufenican + metribuzin and flufenacet + diflufenican + aclonifen) reduced the herbicide rate needed to reduce the growth of populations from Kehdingen by 90%. The respective ED_{90} values were reduced from 49.7% of the registered field rate (flufenacet only), to 14.5, 9.9 and 7.6% of the registered field rate. Also 28.2% of the registered field rate of the combination of flufenacet + diflufenican caused a 90% reduction in growth, whereas application the field rate of a combination of flufenacet + pendimethalin did not improve the efficacy in comparison with flufenacet alone (see Table 1 for applied doses and

registered field rates).

The improved efficacy observed with combinations of flufenacet and diflufenican may be explained in part by different resistance mechanisms. Diflufenican tolerance of wheat and barley was shown to be caused by reduced uptake (Haynes and Kirkwood, 1992). By contrast, metabolism-based flufenacet resistance due to GST activity was previously described as a detoxification mechanism for *Lolium* spp. and as a cause of crop tolerance, *e.g.* in wheat or corn (Bieseler *et al*., 1997; Dücker *et al*., 2019; Dücker *et al.,* 2016).

A similar mechanism was detected in the *A. myosuroides* population Kehdingen1, which survived significantly higher flufenacet rates in dose–response bioassays. With a DT_{50} of 43 h, it degraded flufenacet significantly faster than the susceptible reference population Herbiseed-S with a DT_{50} of 121 h. Metabolites detected in extracts from seedlings of these populations suggest that flufenacet was detoxified via the same pathway in both populations. As described for *Lolium* spp., flufenacet was detoxified by conjugation to glutathione (Dücker *et al*., 2019). Subsequent cleavage of glycyl known to be catalyzed by vacuolar carboxypeptidases (Wolf *et al*., 1996) likely resulted in the formation of a glutamyl–cysteine conjugate after vacuolar sequestration. Additional metabolites belonging to phase III metabolism, *e.g.* malonyl–cysteine conjugate and a lactic acid glucoside conjugate were detected in the plant extract. However, their formation plays an unlikely role in flufenacet resistance, as glutathione conjugation is likely the first detoxifying step prior to vacuolar sequestration. Metabolites belonging to the oxalate pathway described by Gould *et al.* (1997) were not detected. Finally, the mechanism of flufenacet resistance in *A. myosuroides* was described for the first time as GST-mediated metabolic resistance.

Because metabolism-based herbicide resistance can potentially confer cross-resistance to compounds not yet marketed (Beckie and Tardif, 2012), it is crucial to not only rely on chemical solutions. The integration of suitable measures, *e.g.* wide crop rotations, delayed sowing, and preparation of stale seedbeds in combination with tillage, or use of non-selective herbicides can contribute to sustainable management of herbicide resistance and decrease the soil seedbank (Herrmann *et al*., 2016; Norsworthy *et al.,* 2012; Beckie, 2011; Lutman *et al*., 2013; Henne *et al*., 2018).

5.5. Conclusions

Frequent use of herbicides can lead to reduced sensitivity to herbicides of different MoAs and chemical classes. In temperate Europe, flufenacet is an herbicide commonly used to control grass weeds that have already evolved resistance to typical post-emergence herbicides such as inhibitors of ACCase or ALS. This study demonstrates that the efficacy

of flufenacet on various *A. myosuroides* populations of different origins was reduced, although the majority of the tested populations were controlled with the registered field rate. The level of resistance correlated with enhanced flufenacet degradation. A detoxification pathway was constructed based on metabolite masses identified by LC–MS analyses and confirmed enhanced GST activity as a cause of the observed shift in efficacy. Despite an enhanced degradation rate, flufenacet controlled those populations more effectively than alternative pre-emergence herbicides such as pendimethalin, prosulfocarb and other inhibitors of VLCFA synthesis. When flufenacet was applied in mixtures with diflufenican, the control of susceptible and multiple resistant *A. myosuroides* populations improved considerably. To preserve the effective use of flufenacet as an efficient tool to control one of the most noxious grass-weeds in Europe, the application of full dose rates and herbicide mixtures is strongly recommended to slow the evolution of metabolism-based resistance (Neve and Powles, 2005; Norsworthy *et al*., 2012; Lagator *et al*., 2013). In addition, it is crucial that best management practices such as wide crop rotations, including spring crops as well as other measures reducing the weed seed bank (Norsworthy *et al*., 2012, Beckie *et al*., 2011; Lutman *et al*., 2013) are used in combination with chemical weed control and are adapted to the individual field conditions to prevent flufenacet resistance from evolving.

Comparisons of expression levels of GST-encoding genes between flufenacet resistant *A. myosuroides* (Dücker *et al.*, unpublished) and *Lolium* spp. (Dücker *et al*., 2019) will allow studying the pathways involved in flufenacet detoxification in detail. This will offer tools to study the evolution of weed resistance (Ravet *et al*., 2018), which appears to be faster in *Lolium* spp. than in *A. myosuroides*.

5.6 Acknowledgements

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6. General discussion

Flufenacet is an oxyacetamide herbicide inhibiting the synthesis of very-long-chain fatty acids (VLCFAs) used in pre-emergence and early post-emergence applications. It has become a key herbicide for weed resistance management and the control of (multiple-resistant) grass weeds and small-seeded dicotyledonous weeds (Krähmer *et al*., 2019; Menne *et al*., 2012), particularly in winter cereals in Europe. So far, only few weeds have evolved resistance against its herbicide mode of action; however two grass weed species, *Alopecurus myosuroides* and *Lolium* spp. have been described as resistant to flufenacet (Rauch *et al*., 2010, Rosenhauer and Petersen, 2015; Heap, 2018). To this point, the distribution of flufenacet resistance, particularly in *Lolium* spp. in Europe, as well as the resistance mechanism in weeds have not yet been investigated. The present study aims at estimating the distribution and characterizing the level of resistance of a limited number of *A. myosuroides* and *Lolium* spp. field populations. Based on this characterization, suitable sensitive and resistant populations were selected to study cross-resistance to alternative pre-emergence herbicides and to elucidate the molecular mechanisms underlying the observed resistance shift using analytical techniques, approaches of molecular biology and biochemistry as well as bioinformatic tools.

The analysis of 50 *A. myosuroides* populations from different regions in Germany, France and the United Kingdom and 22 *Lolium* populations from the USA, France, the United Kingdom as well as population VLR69 originating from Australia has showed great differences between the levels of resistance in these two species. Resistance factors of up to 7 and effective dose rates reducing the fresh weight by 90% (ED_{90}) ranging between 20.5 and 310.9 g flufenacet ha^{-1} were recorded for *A. myosuroides*. Similar resistance levels were previously described by other authors (Rosenhauer and Petersen, 2015; Klingenhagen, 2012). The level of resistance is situated within the range of 'low-level resistance' as defined by Heap (2005). Yet, six populations from the Northern German marsh regions survived the treatment with the flufenacet field rate registered for the use in cereals in Europe (ED_{90} values > 240-254 g flufenacet ha^{-1}). In competition with the crop this may not lead to yield reduction. However, the efficacy of pre-emergence herbicides depends strongly on environmental factors like precipitation, soil conditions or weed densities (Hull and Moss, 2012; Menne *et al*., 2012). Depending on these conditions, a shift in flufenacet sensitivity in a population to an ED_{90} value of 310.9 g flufenacet ha^{-1} may become relevant in the field. Field trials may finally clarify the field relevance of this shift in sensitivity. Moderately reduced sensitivity to flufenacet was widespread among *A. myosuroides* populations from fields where reduced efficacy of the commonly used

inhibitors of ALS- and/or ACCase was already observed. Field populations from locations without known resistance problems were controlled with significantly lower flufenacet dose rates. This suggests that management practices have affected the level of flufenacet efficacy on those populations as described previously for other resistance cases *e.g.* ALS-resistance in *A. myosuroides* (Légère *et al*., 2000; Herrmann *et al*., 2016).

While relatively small differences were found between *A. myosuroides* populations, clearly relevant levels of flufenacet resistance were observed in *Lolium* populations with resistance factors up to 61 and ED_{90} values ranging from 8.6 to 5903.4 g flufenacet ha^{-1}. High levels of resistance were found in populations from the US and in the commercially available population VLR69 (VLR69-R) originating from Australia (Burnet *et al*., 1994a, Burnet *et al*., 1994b). For the first time, levels of flufenacet resistance relevant in the field were described in *Lolium* field populations from the United Kingdom and France. Despite this high level of resistance, a previous screening of hundreds of *Lolium* spp. field populations from different locations has shown that populations surviving the field rate of flufenacet still rare (< 7.5%, Collavo, unpublished). This corresponds with findings from the Palouse region in the US where also 7% of the tested *Lolium* populations showed reduced levels of flufenacet efficacy (Rauch *et al*., 2010). However, flufenacet was only introduced as an herbicide to the French market in 2010 and a flufenacet resistant field population with a resistance factor of 61 was collected in 2015. This can be explained by three different scenarios: Strong metabolism-based flufenacet resistance may have evolved within 5 years; flufenacet resistance pre-existed in the field *e.g.* due to cross-resistance or flufenacet resistance was introduced *e.g.* by seed purchase from abroad. In any case, this shows that strong flufenacet resistance in *Lolium* spp. can potentially spread, particularly under selection pressure.

Furthermore, not only the level of flufenacet resistance but also the resistance patterns differed between *A. myosuroides* and *Lolium* populations (see Table II). On average, a resistance factor of 3 was estimated for three multiple-resistant *A. myosuroides* populations from the Northern German marsh region Kehdingen. In a similar setup with three flufenacet resistant *Lolium* populations from different locations, resistance was relatively specific to flufenacet with an average resistance factor of 42. For the inhibitor of the synthesis of VLCFAs *S*-metolachlor a resistance factor of 7 was calculated for the tested *A. myosuroides* populations and the comparison of sensitive and flufenacet resistant *Lolium* populations resulted in a resistance factor of 13. The application of pyroxasulfone, another inhibitor of the synthesis of VLCFAs, which is not registered in Europe also resulted in a resistance factor of 2, both in resistant *Lolium* spp. and *A. myosuroides* populations. The level of flufenacet resistance in combination with the recorded differences in cross-resistance patterns indicate that either flufenacet resistance is not linked with cross-resistance to

S-metolachlor or that the resistance mechanisms differ at the genetic or biochemical level in *A. myosuroides* and *Lolium* spp. and therefore result in different resistance patterns.

Table II: Dose-response of sensitive and resistant *Alopecurus myosuroides* and *Lolium* populations to different pre-emergence herbicides inhibiting the synthesis of VLCFAs. Estimated effective dose rates reducing the fresh weight by 50% (ED_{50}) are displayed with standard error in parentheses. Resistance factors are calculated as the quotient of the average ED_{50} of the respective populations and the average ED_{50} value of the three respective sensitive populations.

Parameter	Herbicide	*Alopecurus myosuroides*		*Lolium* spp.	
		Sensitive reference	Reduced flufenacet sensitivity	Sensitive reference	Flufenacet resistant
Ø ED_{50} in g ai ha^{-1}	Flufenacet	4.89 (0.37)	13.96 (1.06)	4.15 (0.45)	174.00 (30.47)
	S-metolachlor	128.22 (25.03)	927.60 (95.54)	9.09 (1.52)	116.57 (13.59)
	Pyroxasulfone	1.29 (0.14)	2.38 (0.19)	1.23 (0.12)	2.84 (0.36)
Ø Resistance factor	Flufenacet	1	3	1	42
	S-metolachlor	1	7	1	13
	Pyroxasulfone	1	2	1	2
Ø Resistance index†	Flufenacet	1‡	3‡	2	69
Detoxifying enzyme	Flufenacet	GST activity	GST activity	GST activity	GST activity

†calculated as the quotient of the estimated degradation half-time of the respective sensitive and the resistant weed population.

‡ determined with the sensitive population Herbiseed-S and the resistant population Kehdingen1.

Cross-resistances occur as side effects of non-target-site resistance to a pesticide or a chemical class (Yu and Powles, 2014). While the exact resistance mechanism to flufenacet in weed species has not been clarified, it was suggested by Böger *et al.* (2000) that target-site mutations are an improbable cause of resistance due to characteristics of the primary target of flufenacet. In *A. thaliana*, 21 condensing enzymes (KCSs), among them several redundant isoforms, have been identified (Haslam and Kunst, 2013; Trenkamp *et al*., 2004). As flufenacet and other herbicides of the same MoA were shown to inhibit several KCSs (Trenkamp *et al*., 2004), several resistance-conferring mutations would be necessary to effectively build up target-site resistance. Further evidence towards metabolism-based resistance was given from the study of flufenacet degradation in crops like corn and wheat. Bieseler *et al*. (1997) have shown that crop tolerance is caused by enhanced metabolism due to GST activity. In the present study time-course experiments with ^{14}C-radiolabelled flufenacet confirmed that resistance in *A. myosuroides* as well as in *Lolium* spp. was caused by enhanced metabolism. In both cases the determined degradation half-life corresponded

with the resistance levels determined in greenhouse bioassays (see Table I). With a similar experimental design, LC-MS/MS analyses were used to identify metabolites produced during flufenacet detoxification. In both sensitive and resistant *A. myosuroides* and *Lolium* populations the metabolites belonging to the same pathway were detected. A glutathione conjugate was identified as the first metabolite, while the other identified metabolites were downstream products of the initial glutathione conjugate. Metabolites belonging to the oxalate pathway described by Gould *et al.* (1997) were not found. Therefore, enhanced glutathione transferase activity was identified as a key mechanism of resistance in both *A. myosuroides* and *Lolium* populations. A photometric activity test with the GST model substrate CDNB confirmed enhanced GST activity in crude extracts from the flufenacet resistant population USA1-R. The formation of a flufenacet-GSH conjugate was detected with a corresponding ionized mass and a specific fragmentation (split off of pyro-glutamate) by LC-MS/MS.

In order to identify individual GST isoform(s) involved in flufenacet resistance and better understand the resistance-related gene regulation an RNA-Seq experiment with Illumina reads was conducted. The experimental design included each six individuals of 3 sensitive and 3 resistant *Lolium* populations untreated or treated with flufenacet. By differential gene expression analysis, 95 gene-associated contigs were found significantly upregulated and nine of them were identified as tau class GSTs while two of them were identified as phi class GSTs. GST3 had a high sequence similarity with *Am*GSTF1 and *Lr*GSTF1, previously found upregulated in weed populations resistant to several other herbicides (Cummins *et al*., 2013; Tétard-Jones *et al*., 2018). A GO enrichment analysis confirmed that the term 'glutathione transferase activity', besides 'glucosyltransferase activity' and 'oxidoreductase activity' was a significantly upregulated. As a large number of glucosyltransferases were found significantly upregulated but not involved in the rate-liming step in flufenacet detoxification, it is possible that an upregulation of 'hotspots' *i.e.* a specific regions on a chromosome containing a set of upregulated genes, as described for *Amaranthus* spp. may play a role in this type of resistance (Tranel, 2018). High quality reference genomes will be essential to study resistance-related gene regulation in more detail. Besides that, 136 contigs were identified as significantly downregulated. Particularly the gene-associated contigs annotated as transcription factors may play a role in the regulation of the resistance-conferring genes (such as genes coding for GSTs) as they may act as repressors.

The analyses of the sequences of two tau class GSTs and two phi class GSTs revealed various amino acid substitutions, however none of them co-segregated clearly with the resistance phenotype. A protein assay finally demonstrated, that the recombinant protein GST1A (class tau) was able to detoxify flufenacet with 44.55 µmol min^{-1} mg^{-1} with a tenfold

higher turnover rate than the model substrate CDNB (4.5 µmol min^{-1} mg^{-1}). GST3 (class phi) detoxified flufenacet with a turnover rate of 6.1 µmol min^{-1} mg^{-1} considerably slower. Also Bieseler *et al*. (1997) have previously shown that phi class GSTs isolated from corn and *A. thaliana* were able to detoxify flufenacet at low rates. In conclusion, the protein assays suggest, that flufenacet resistance is based on upregulation of the expression of at least one GST (GST1A) with a high affinity to flufenacet in combination the cumulative effect of upregulated GSTs with low specificity to flufenacet as a substrate (in the case of GST3). GST4 and, interestingly, also GST1B, were not able to detoxify flufenacet despite high sequence similarity between GST1A and GST1B.

In the case of the protein GST1A, additional cross-resistance to *S*-metolachlor and pyroxasulfone was detected, although with a lower turnover rate in comparison with flufenacet. As expected and based on the dose-response assay with diflufenican and the degradation test with mesosulfuron-methyl, none of the recombinant proteins were able to detoxify either of these herbicides. Similarly, crop tolerance to diflufenican and mesosulfuron-methyl were not found to be GST-mediated by other authors, but caused by reduced uptake and cytochrome P450 monooxygenase (CYP) activity, respectively (Haynes and Kirkwood, 1992; Duhoux and Délye, 2013). Therefore, both herbicides are well-suited for a combination with flufenacet as most probably mechanisms of resistance to these herbicides are due to different pathways and/or enzyme families and selection with these herbicides is unlikely to cause cross-resistance with flufenacet. Diclofop-methyl, however was detoxified at a faster rate in the leaves of flufenacet resistant *Lolium* populations. Resistance to this herbicide was previously described as CYP-mediated (Gaines *et al*., 2014) and none of the recombinant candidate GSTs tested in this study were able to detoxify it. Therefore, multiple resistance to diclofop-methyl and flufenacet are likely based on different resistance mechanisms.

The overexpression of different gene families and isoforms involved in resistance to different chemistries increases the complexity to develop simple resistance diagnostics. With GST1A the present study provides a novel marker for flufenacet resistance in addition to *Am*GSTF1 and *Lr*GSTF1, two general markers for metabolism-based resistance described by Cummins *et al*. (2013) and Stafford (2018).

Finally, the improved understanding of the molecular mechanisms behind flufenacet resistance provides a basis for improvement of crop protection compounds. Information about cross-resistance patterns allows for a better comprehension of the selection of metabolism-based flufenacet resistance (Beckie and Tardif, 2012). This knowledge can become particularly useful for herbicide research as no new herbicide MoAs have been introduced to the marked during the past 30 years (Duke, 2012; Gould *et al*., 2018).

In conclusion, best management practices should be implemented in weed control programs in order to keep flufenacet resistance in *A. myosuroides* and *Lolium* populations from evolving. This includes resistance management oriented use of herbicide chemistries *e.g*. the application of full dose rates of flufenacet in mixtures (*e.g.* with diflufenican) (Beckie and Tardif, 2012). Additionally, as stressed throughout the previous chapters, non-chemical control becomes increasingly important and includes measures *e.g.* wide crop rotations including spring crops as well as other measures reducing the weed seed bank which have been extensively reviewed in literature (Beckie and Tardif, 2012; Norsworthy *et al*., 2012; Beckie and Harker, 2017; Henne *et al*., 2018). And yet, the evolution of resistance and occurrence of new resistance cases continues. Considering herbicide resistance as a 'wicked' problem has recently lead to the development of integrated approaches including socio-economic aspects for the implementation of measures preventing herbicide resistance from further evolving (Shaw, 2016). This approach may finally help bringing new scientific insights into the field.

7. Summary

This study aimed to elucidate the resistance mechanisms behind flufenacet resistance in *Alopecurus myosuroides* and *Lolium* spp. field populations. In a first step field populations of both species were screened in greenhouse bioassays and suitable biological material was selected for the investigation of the further studies using analytical and biochemical techniques as well as an RNA-Seq approach.

In a screening with 50 *A. myosuroides* populations shifts in efficacy with resistance factors up to 7 were estimated and six populations from the Northern German Marshes were controlled by less than 90% with the field rate registered in Europe. The efficacy of several pre-emergence herbicides of different modes of action on sensitive populations and Northern German *A. myosuroides* populations with shift in flufenacet sensitivity was explored. While none of the herbicides registered in Europe were more effective on those populations than flufenacet, it was shown that particularly three-way-mixtures including flufenacet and the PDS inhibitor diflufenican increased the control of those populations significantly. The observed shift in flufenacet efficacy in *A. myosuroides* populations was comparably low, whereas resistance factors up to 61 were observed in a screening with 22 *Lolium* spp. field populations. For the first time, field relevant levels of flufenacet resistance were described in *Lolium* populations from France and the United Kingdom, but also in populations from the USA and the commercially available population VLR69 originating from Australia.

The level of resistance correlated in case of both, *A. myosuroides* and *Lolium* populations with flufenacet degradation rates determined by HPLC. Similar detoxification pathways with glutathione conjugation as a first rate-limiting step were elaborated for both species based on metabolites identified by LC-MS/MS. The pathways suggest enhanced glutathione transferase activity as a main driver of the resistance observed in both species tested.

The large differences in flufenacet resistance observed in *Lolium* population allowed the selection of biological material for an RNA-Seq study. By differential gene expression analysis of the transcriptomes of three sensitive and three flufenacet resistant *Lolium* populations, 11 differentially upregulated GSTs were identified. These findings were validated with four recombinant GST isoforms *in vitro*. The ability to detoxify flufenacet was confirmed with one tau class GST showing a high flufenacet turnover rate and one phi class GST with high sequence similarity to *Lr*GSTF1 and a lower flufenacet turnover rate. These results suggest that flufenacet resistance in *Lolium* populations is caused by upregulation of at least one GST with high substrate-specificity to flufenacet in combination with a cumulative effect with at least one other GST with lower substrate-specificity to flufenacet.

Finally, none of the recombinant enzymes were able to degrade diflufenican and the ALS inhibitor mesosulfuron-methyl, suggesting that these herbicides are suitable for a combination with flufenacet in resistance management program, as no cross-resistance between these herbicides is expected.

8. Outlook

This study revealed the molecular mechanisms of flufenacet resistance in grass weeds as metabolism-based resistance due to enhanced GST activity. The regulation of these genes, however, remains speculative. Therefore, the assembly of a high-quality *Lolium* spp. genome and the analyses of promoters, repressors or 'hotspots', as well as analyses of DNA methylation and histone structure and modifications can be considered as the next steps to obtain a deeper understanding the regulation of metabolism-based flufenacet resistance in grass weeds. The study of different amino acid substitutions of the candidate GSTs as well as protein-ligand modeling - and if necessary protein crystallization and NMR – can improve the understanding of substrate-specificity of plant GSTs and support the improvement of chemistry inhibiting the synthesis of VLCFAs.

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10. Annex

Table A: Dose-response of three sensitive *Alopecurus myosuroides* populations and three *Alopecurus myosuroides* populations from Kehdingen to selected pre-emergence herbicides, described by the effective dose rates ED_{50} and ED_{90} with standard errors in parentheses, resistance factors (RFs), parameters *b* and *d* of the log-logistic three-parameter model as described by Ritz *et al.* (2015) and 90% confidence intervals (CI).

Population	Herbicide	Field Rate (g ha^{-1})	ED_{50}	RF	*b*	*D*	ED_{90}	95% CI
Sensitive	Cadou® SC	flufenacet (240-254)†	4.89 (0.37)	1	1.94	0.60	15.16 (2.12)	4.17 - 5.61
Kehdingen			13.96 (1.06)	3	1.59	0.66	55.61 (7.59)	11.88 - 16.03
Sensitive	Dual Gold®	*S*-metolachlor (1200)‡	128.22 (25.03)	1	1.14	0.56	886.47 (263.95)	79.11 - 177.33
Kehdingen			927.60 (95.54)	7	1.76	0.68	3232.62 (652.33)	740.12 - 1115.08
Sensitive	Quantum®	pethoxamid (1200)‡	34.27 (9.74)	1	0.76	0.55	621.45 (239.51)	15.16 - 53.39
Kehdingen			430.65 (43.69)	13	1.72	0.65	1547.24 (313.09)	344.16 - 516.39
Sensitive	Stomp®	pendimethalin (1000-2002)†	530.40 (60.50)	1	2.38	0.49	1337.72 (412.41)	411.69 - 649.11
Kehdingen			1350.19 (257.75)	3	0.92	0.62	14604.80 (8013.25)	844.45 - 1855.92
Sensitive	Boxer®	prosulfocarb (4000)†	44.94 (4.68)	1	1.92	0.60	141.23 (27.57)	35.75 - 54.12
Kehdingen			462.94 (67.64)	10	0.92	0.70	5006.51 (1350.64)	330.22 - 595.67
Sensitive	Sakura® 85WG	pyroxasulfone (120)§	1.29 (0.14)	1	1.88	0.59	4.16 (0.79)	1.02 - 1.56
Kehdingen			2.38 (0.19)	2	1.95	0.74	7.36 (0.97)	2.00 - 2.76

†Field rate registered in cereals in the countries of seed origin (Germany, France, and United Kingdom).
‡Field rate registered in corn in the countries of seed origin (Germany, France, and United Kingdom).
§ Pyroxasulfone is not registered in Europe, therefore field rates registered in Australia and North America are displayed.

Table B: Dose-response of three sensitive *Alopecurus myosuroides* populations and three *Alopecurus myosuroides* populations from Kehdingen with reduced flufenacet sensitivity to selected flufenacet based herbicides. The populations are described by the effective dose rates ED_{50} and ED_{90} with standard errors in parentheses, resistance factors (RFs) and parameters *b* and *d* of the log-logistic three-parameter model as described by Ritz *et al.* (2015) and 90% confidence intervals (CI).

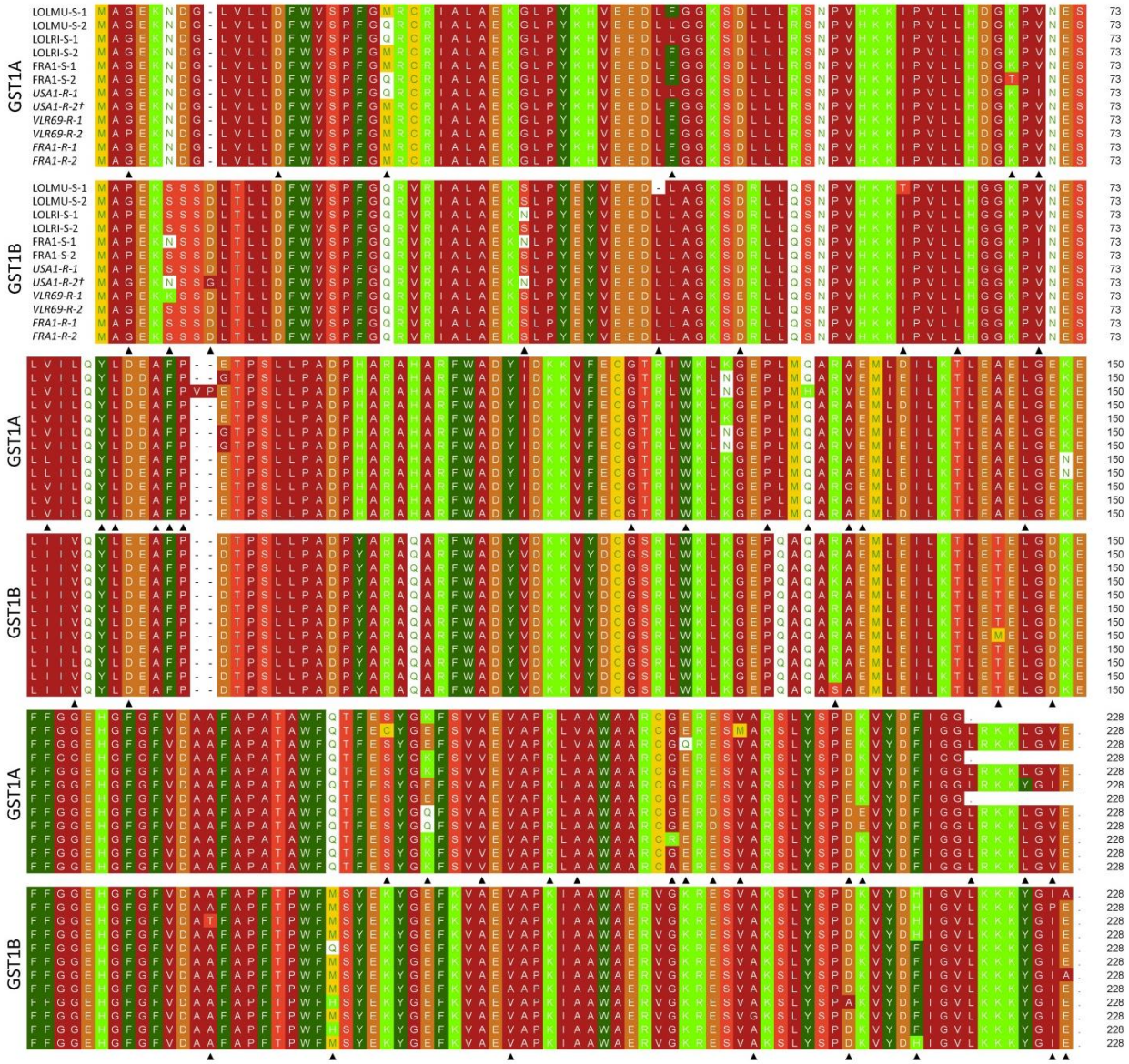
Population	Herbicide	Field Rate (g ha^{-1})	ED_{50}	RF	*b*	*d*	ED_{90}	95% CI
Sensitive	Cadou® SC	flufenacet (240-254)†	4.05 (0.36)	1	2.35	0.65	10.29 (2.28)	3.34 - 4.75
Kehdingen			13.34 (1.30)	3	1.67	0.63	49.68 (9.23)	10.78 - 15.90
Sensitive	Cadou® Forte Set	flufenacet (242) diflufenican (90) flurtamone (90)	2.46 (0.27)	1	2.06	0.52	7.15 (1.16)	1.93 - 3.00
Kehdingen			7.58 (0.66)	3	3.38	0.61	14.52 (1.42)	6.29 - 8.87
Sensitive	Liberator Pro	flufenacet (240) diflufenican (120) metribuzin (70)	2.26 (0.22)	1	2.77	0.53	4.99 (0.64)	1.83 - 2.69
Kehdingen			5.00 (0.34)	2	3.21	0.64	9.92 (1.53)	4.34 - 5.67
Sensitive	Malibu®	flufenacet (240) pendimethalin (1200)	3.44 (0.54)	1	1.62	0.51	13.28 (3.19)	2.37 - 4.50
Kehdingen			15.28 (1.72)	4	1.49	0.66	66.98 (13.04)	11.91 - 18.66
Sensitive	Herold® SC	flufenacet (240) diflufenican (120)	4.26 (0.39)	1	3.45	0.57	8.05 (2.59)	3.50 - 5.02
Kehdingen			11.88 (0.88)	3	2.54	0.63	28.22 (5.76)	10.16 - 13.60
Sensitive	22110H	flufenacet (90) diflufenican (30) aclonifen (450)	2.58 (0.27)	1	3.67	0.54	4.69 (0.48)	2.06 - 3.10
Kehdingen			4.85 (1.55)	2	4.92	0.67	7.58 (6.35)	1.81 -7.89

†Field rate registered in cereals in the countries of seed origin (Germany, France, United Kingdom)

Table C: Flufenacet degradation in seedling tissue of three sensitive *Alopecurus myosuroides* populations and three *Alopecurus myosuroides* populations with reduced flufenacet sensitivity originating from Kehdingen. Degradatin rates are described by th degradation half-time s (DT_{50}) with standard errors in parentheses, 90% confidence intervals (CI) and parameters *b* and *d* of the log-logistic three-parameter model as described by Ritz *et al.* (2015).

Population	DT_{50}	95% CI	*B*	*d*
Herbiseed-S	121.38 (13.96)	93.46 – 149.30	2.56	87.06
Kehdingen1	43.24 (13.15)	16.94 – 69.54	0.51	99.04

Figure A: Alignment of two *Lolium* spp. tau class GST isoforms (GST1A and GST1B). Each



two alleles of the sensitive populations LOLMU-S, LOLRI-S, FRA1-S and the flufenacet resistant populations USA1-R, VLR69-R, and FRA1-R were aligned per isoform. Black arrows indicate amino acid substitutions detected the populations tested in this study. Different colors indicate aliphatic (■), aromatic (■), acidic (■), basic (■), hydroxylic (■), sulfur-containing (■) and amidic (Q, N) amino acids.

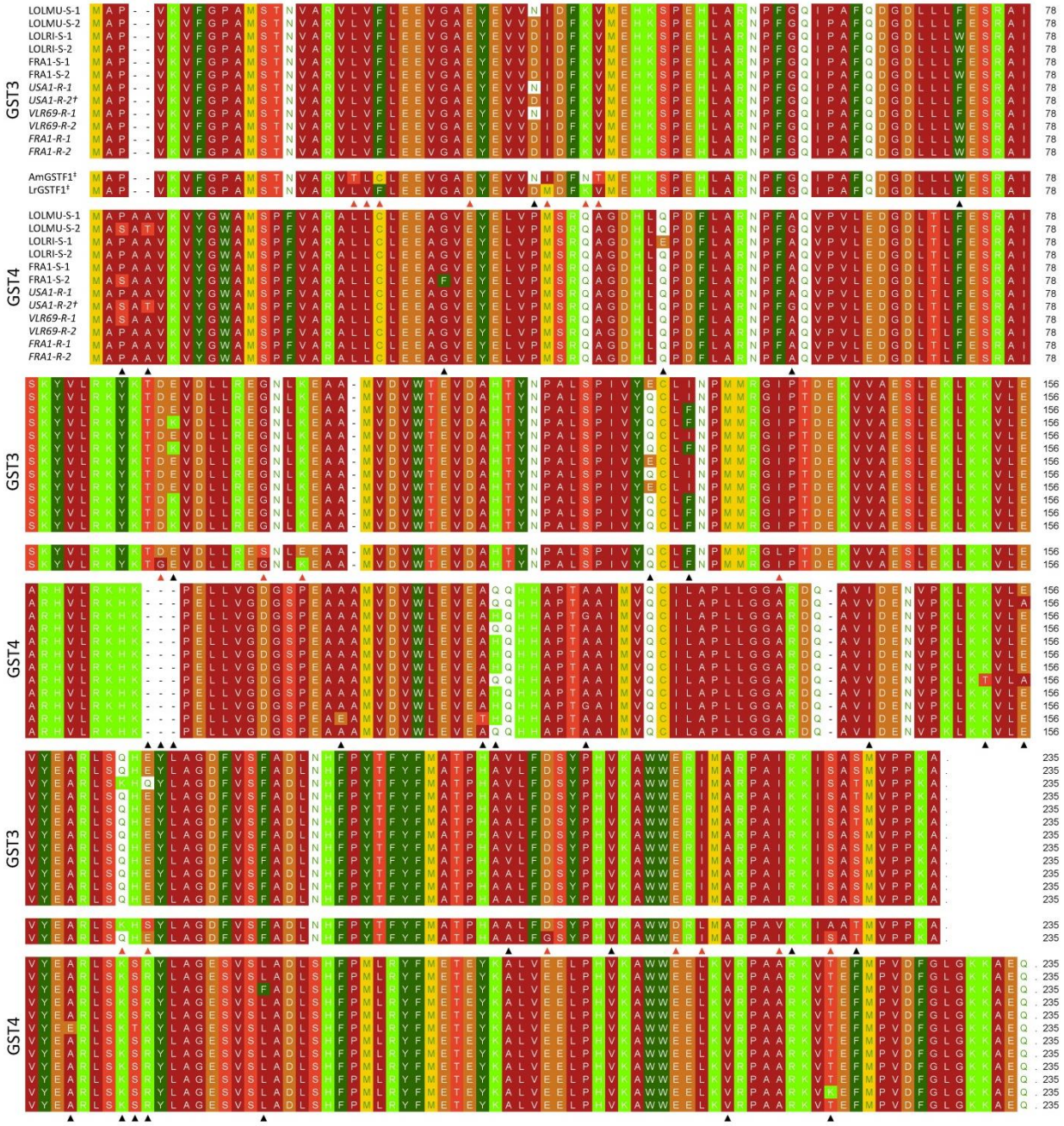


Figure B: Alignment of two phi class GST isoforms (GST3 and GST4) significantly upregulated in flufenacet resistant *Lolium* populations. Each two alleles of the sensitive populations LOLMU-S, LOLRI-S, FRA1-S and the flufenacet resistant populations USA1-R, VLR69-R, and FRA1-R were aligned per isoform. Isoform GST3 was additionally aligned with *Am*GSTF1 and *Lr*GSTF1 described by Cummins *et al*. (2013) ‡. Black arrows indicate amino acid substitutions detected the populations tested in this study. Orange arrows indicate additional amino acid substitutions detected in the orthologues described by Cummins *et al*. (2013). Different colors indicate aliphatic (■), aromatic (■), acidic (■), basic (■), hydroxylic (■), sulfur-containing (■) and amidic (Q, N) amino acids.

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12. *Curriculum vitae*

REBECKA DÜCKER

PERSONAL DATA

Born: 26.10.1988/Bremervörde

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LANGUAGES:

German:
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SOFTWARE SKILLS:

MS Office:
R:
Linux:
Sigma Plot:
Spotfire:
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PROFESSIONAL EXPERIENCE

06/2015 – 02/2019 **PhD Candidate** at Georg-August University Göttingen/Bayer AG in Frankfurt - Tasks: Greenhouse bioassays, identification of herbicide resistance mechanisms using analytical, molecular and bioformatic methods

11/2013 – 05/2015 **Laboratory assistant** at Bayer AG in Frankfurt- Tasks: Herbicide detoxification tests using HPLC

SELECTED INTERNSHIPS

08/2012-10/2013 Eurofins Agroscience Services Indaiatuba, Brazil
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07/2010-09/2012 Georg-August University Göttingen
01/2007-04/2010 Dairy farm Schröder in Kuhstedt-Siedlung
07/2009-10/2009 Raiffeisen trading cooperative Kirchwisted
01/2007-07/2010 Dairy- and holiday farm Dücker, Volkmarst

PUBLICATIONS

Submitted Krähmer *et al.* Inhibitors of the Synthesis of Very Long-Chain Fatty Acids (VLCFAs). *Modern Crop Protection Compounds.*

2016 Dücker *et al*. Discovering the mechanism of enhanced metabolism in flufenacet resistant grass weeds. *Julius-Kühn-Archiv*, 35 (452), 35-41.

2012 Breitsameter *et al.* Impact of sward properties on the predictability of forage quality and yield in grassland using remote sensing. *Proc. 24th Gen. Meet. Europ. Grassland Fed.*, 346-348.

SECONDARY AND HIGHER EDUCATION

10/2011 – 11/2014 **Georg-August University Göttingen** M. Sc. agr., Agricultural crop sciences. Master's thesis: „Investigations in flufenacet resistance mechanisms in *Alopecurus myosuroides* and *Lolium* spp.", Final mark: 1.4

10/2008 - 09/2011 **Georg-August University Göttingen** B. Sc. agr., Agricultural crop sciences. Bachelor's thesis: „Influence of Plant Species and Sward structure on the Assessment of forage quality and yield using a portable spectrometer", Final mark: 1.8

2001-2005/2006-2008 **Gymnasium Bremervörde,** Abitur mark: 1.9

08/2005-06/2006 **Presentation College Tuam, Ireland**

13. Declarations

Hiermit erkläre ich, dass diese Arbeit weder in gleicher noch in ähnlicher Form bereits anderen Prüfungsbehörden vorgelegen hat.

Weiter erkläre ich, dass ich mich an keiner anderen Hochschule um einen Doktorgrad beworben habe.

Göttingen, den .....

.....

(Unterschrift)

Hiermit erkläre ich eidesstattlich, dass diese Dissertation selbständig und ohne unerlaubte Hilfe angefertigt wurde.

Göttingen, den .....

.....

(Unterschrift)