Sektion 1: Herbizidresistenz
Section 1: Herbicide resistance

Herbicide resistance in German and Swiss *Lolium* spp. populations – resistance factors and cross-resistance spectrum

Herbizidresistenz in Lolium *spp. aus Deutschland und der Schweiz – Resistenzfaktoren und Kreuzresistenzmuster*

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DOI 10.5073/jka.2016.452.004

Abstract

In monitoring trials investigating the occurrence and spread of herbicide resistance in German and Swiss *Lolium* populations 26 samples could be included since 2008. Biotypes which showed resistance to post-emergence herbicides were included into a detailed greenhouse trial in 2014. Based on dose-response experiments, resistance factors and cross resistance patterns for cycloxydim, flufenacet, glyphosate, iodosulfuron, meso- and iodosulfuron, pinoxaden and pyroxsulam could be determined. Resistance to ALS as well as ACCase inhibitors was found. In a few cases also resistance to flufenacet could be detected. In contrast, no resistance to glyphosate was discovered. Resistant populations were found in four German federal states (Hessen, Mecklenburg-Vorpommern, Sachsen and Schleswig-Holstein). Two populations were resistant to all cereal selective post-emergence herbicides and to flufenacet. Some populations from Switzerland indicated presence of ACCase inhibitor resistance. In the future, more problems with herbicide resistant *Lolium* species as weeds in cereals may arise due to limited amount of available selective herbicides and climatic change with more favourable conditions for *Lolium* spp. as weeds.

Keywords: ALS and ACCase inhibitors, dose-response experiments, flufenacet, glyphosate

Zusammenfassung

In Monitoringversuchen zur Verbreitung der Herbizidresistenz wurden seit 2008 26 *Lolium*-Herkünfte mit Resistenzverdacht aus Deutschland und der Schweiz untersucht. Herkünfte, die sich als resistent erwiesen, wurden in 2014 in einen weiteren Gewächshausversuch eingehender untersucht. Durch Dosis-Wirkungs-Experimente wurden Resistenzfaktoren und Kreuzresistenzmuster für die Wirkstoffe Cycloxydim, Flufenacet, Glyphosat, Iodosulfuron, Meso- + Iodosulfuron, Pinoxaden und Pyroxsulam bestimmt. Resistenzen traten bei ALS- und/oder ACCase-Hemmern auf. Bei einigen Herkünften wurde eine deutliche Resistenz gegen Flufenacet festgestellt. Alle Herkünfte erwiesen sich als sensitiv gegenüber Glyphosat. Resistente Herkünfte kamen aus Schleswig-Holstein, Mecklenburg-Vorpommern, Sachsen und Hessen. Zwei Herkünfte erwiesen sich als besonders widerstandsfähig und konnten durch alle registrierten getreideselektiven Herbizide nicht mehr kontrolliert werden. Einige Herkünfte aus der Schweiz wiesen Resistenzen gegen ACCase-Inhibitoren auf. Mit einer Ausweitung herbizidresistenter *Lolium*-Biotypen muss aufgrund des nur eingeschränkt möglichen Wirkstoffklassenwechsels, der klimatischen Bedingungen und veränderter Ackerbausysteme zukünftig gerechnet werden.

Stichwörter: ALS- and ACCase-Hemmer, Dosis-Wirkungsbeziehungen, Flufenacet, Glyphosat

Introduction

Alopecurus myosuroides and *Apera spica-venti* are the most important grass weeds in cereals of central Europe. Also, the occurrence of herbicide resistance is concentrated in these two species. In rare cases also *Lolium perenne* or *L. multiflorum* do appear as weeds in cereals. However, where *Lolium* is presented, this species often is the most important one due to its strong competitive ability. As a consequence, weed control is focused on *Lolium* spp. on these fields. However, only a few active ingredients are available for *Lolium* control. Furthermore, genetic variability in the genus *Lolium* is very high. Consequently, *Lolium* species with resistance to several herbicides can be found in many countries all over the world (HEAP, 2015).

In central Europe, *L. perenne* and *L. multiflorum* are grown as a crop for forage or seed production in rotations with other arable crops. Consequently, in these rotations *Lolium* can appear as a weed in subsequently grown cereals. However, beside this, *Lolium* as a weed can also occur in cereal rotations in absence of *Lolium* crops. Reasons for this are unknown but in some regions spread of *Lolium* weeds can be observed. Selective *Lolium* control in cereals with herbicides is possible but only a few active ingredients are available. Consequently, selection pressure can be high and development of resistance is only a question of time.

Between 2008 and 2014, *Lolium* populations suspicious for resistance were sampled in Germany and Switzerland. Seeds were used for a post-emergence herbicide resistance monitoring greenhouse trial. Populations with lower herbicide efficacy were additionally included into dose-response experiments with several herbicides to investigate the resistance factors and cross resistance profiles. Furthermore, some resistant populations were analyzed for potential target-site resistance mutations.

Materials and Methods

Herbicide resistance monitoring

Lolium spp. seeds were sampled in cereal fields were herbicide treatments resulted in inefficient control. In September of the sampling year, seeds were sown in a greenhouse and plants were sprayed with the registered doses of Axial 50, Atlantis WG, Broadway, Focus Ultra and Husar OD (Tab. 2). Growing and spraying conditions were the same as mentioned below for the dose-response trials. 21 days after treatment, herbicide efficacy was assessed in comparison to an untreated control of each population. Efficacy rates were converted into resistant classes according to Table 1.

Tab. 1 Classification of biotypes according to herbicide efficacy.

Tab. 1 *Klassifizierung der* Lolium-*Herkünfte nach Herbizidwirkung.*

Resistance class	Upper limit [%]	Lower limit [%]
0	100	85
1	< 85	70
2	< 70	55
3	< 55	40
4	< 40	25
5	< 25	0

Dose-response experiments

All seeds used in the experiments were stored at -20°C before the experiment started. The seeds of resistant populations assessed in the monitoring trials were pre-germinated by sowing them on filter paper saturated with tap water in 18 x 13 x 6 cm plastic boxes and locked with a transparent cover. Those boxes were kept for 7 days at 12-15°C until the radicle was visible and roots of 1 to 8 mm had developed (BBCH 03-05). For all further tests, pre-germinated seeds were used with clearly visible primary root (>2 mm).

As substrate, a sieved and sterilized (4 hours at 70°C) soil (sandy loam, pH-value 6.5, organic matter content ~2%) was used in Jiffy speedy pots (10 cm). A slow-release granular fertilizer (urea, 46% nitrogen) was added to the soil before planting. Five germinated seeds with a root length of 3 to 8 mm were put on the soil surface of every pot and covered with a soil layer of 4 mm. The pots were watered from below when required.

Flufenacet was applied directly after transplanting the seeds. All post-emergence herbicides were sprayed at the 1-2-leaf stage. A lab sprayer (SCHACHTNER nozzle TEE JET 9502EVS, with a water volume of 250 L ha^{-1}, speed of 2.5 km h^{-1} and a pressure of 250 kPa) was used for herbicide application. Herbicides and dose rates used are given in Table 2.

For each herbicide, rate and population, six replications were conducted. The herbicide doses were 0, 12.5, 25, 50, 100, 200, 400 and 800% of the registrated dose (Tab. 2). The trials were performed as randomized complete block design in which the different replications represented the blocks.

Data analyses were performed by a log-logistic model (STREIBIG, 1988). Non-linear regressions were calculated using the PROC NLIN procedure of the SAS system (Version 9.3). Homogeneity of variance was required due to the reciprocal standard deviation. All measurements were converted to relative values of the mean values of the untreated control. Resistance factors (RF) for each biotype and herbicide were calculated as the quotient of the ED_{50} value of the test population and the mean ED_{50} value of the three susceptible references.

Tab. 2 Herbicides, active ingredients, concentrations and registrated dose rate used in the experiments.

Tab. 2 *Eingesetzte Herbizide, Wirkstoffe, Konzentrationen und maximale Aufwandmenge.*

Herbicide	Active ingredient	Concentration	Registered rate**
Atlantis WG*	meso- & iodosulfuron	30 + 6 g/kg	400 g/ha
Axial 50	pinoxaden	50 g/L	1.2 l/ha
Broadway*	pyrox- & florasulam	68.3 + 22.8 g/kg	275 g/ha
Cadou SC***	flufenacet	500 g/L	0.5 l/ha
Focus Ultra****	cycloxydim	100 g/L	2.5 l/ha
Husar OD*	iodosulfuron	100 g/L	0.1 l/ha
Roundup Ultra Max	glyphosate	450 g/L	1.0 l/ha

*applied with additive Biopower resp. FHS, **registered dose rate in Germany (except Roundup); ***Cadou SC is not registrated in Germany for *Lolium* control (but effective); ****2.5 l/ha Focus Ultra registrated for annual grass control in oilseed rape

Target-site resistance analysis

For eight populations (Tab. 5) a target-site resistance analysis was conducted. Five single plants which survived herbicide treatment (full rate of pinoxaden, cycloxydim or meso- + iodosulfuron) were sampled 21 days after treatment and plants were dried under greenhouse conditions for five days. Samples were sent to a contract lab. In the lab, individual samples were analysed using the pyrosequencing method for presence of target-site mutations in ALS (Pro-197, Asp-376, Trp-574) resp. ACCase gene (Ile-1781, Trp-2027, Ile-2041, Asp-2078, Gly-2096) according to the findings of BECKIE et al. (2006), BECKIE and TARDIF (2012) and MENEGAT et al. (2015).

Results

Herbicide resistance monitoring

Herbicide resistance monitoring trials could confirm resistance in 18 out of 26 *Lolium* spp. populations. However, in two of these 18 populations, herbicide efficacy was only slightly reduced, perhaps indicating a beginning metabolic-based resistance (Tab. 3). Population R14-802 did not germinate well. Consequently, results should be interpreted carefully. In 14 populations, resistance was quite obvious and ACCase as well as ALS inhibitors were both reduced in efficacy. In populations from Switzerland, only ACCase inhibitors showed resistance in some populations and there was one population (R14-801) which showed only resistance to the ALS inhibitor pyroxsulam. The German populations from Fargau, Thandorf and Penig showed strong resistance to all or nearly all herbicides tested. Populations which showed resistance and provided enough seeds were included into dose-response experiments.

Tab. 3 Resistant classes of different *Lolium* populations in herbicide resistant monitoring trials 2010 – 2014 (n =26); (0 = susceptible; 5 = highly resistant – efficacy < 20%).

Tab. 3 *Resistenzklassen von* Lolium*-Proben aus Herbizidresistenz-Monitoringversuchen der Jahre 2010-2014 (n = 26); (0 = sensitiv; 5 = hoch resistent - Wirkung < 20 %).*

Year	**Lolium - sample**	**Origin (post code village)**	**coverage % untreated**	**pinoxaden**	**meso- & iodosulfuron**	**cycloxydim**	**pyrox- & florasulam**	**iodosulfuron**
2010	We10-802	23909 Römnitz (Stubbenteich)	100	1	0	1	0	1
2010	We10-803	23909 Römnitz	98	4	0	3	0	1
2011	R11-002	CH - 1896 Vouvry	90	0	0	0	-	-
2011	R11-003	CH - 7205 Zizers	90	5	0	5	0	0
2011	R11-004	CH - 1433 Champvent	30	5	0	0	-	-
2011	R11-005	CH - 1418 Vuarrens	30	3	0	0	-	-
2011	R11-006	CH - 1174 Montherod / Pizy	90	0	0	0	-	-
2011	R11-007	CH - 1565 Vallon	60	0	0	0	0	0
2011	R11-008	CH - 1299 Crans près Céligny	80	0	0	0	0	0
2011	R11-009	CH - 1286 Soral	90	0	0	0	-	-
2011	R11-010	CH - 1298 Céligny	90	0	0	0	0	0
2011	R11-011	CH - 1297 Founex	90	0	0	0	0	0
2011	R11-012	CH - 1291 Commugny	90	0	0	0	-	-
2012	R12-801	19217 Thandorf	100	5	4	5	5	-
2012	R12-802	19217 Thandorf	100	5	4	3	5	3
2012	R10-802	23909 Römnitz	100	4	4	1	5	-
2013	Lol 899	24256 Fargau	90	5	5	1	5	5
2013	R13-801	18574 Gustow	100	0	0	0	0	0
2013	R13-802	17166 Teschow	100	4	1	5	1	2
2014	R14-801	19071 Brüsewitz	100	0	0	0	3	-
2014	R14-802	64720 Michelstadt/Rehbach	5	3	-	-	4	-
2014	R14-803	09322 Penig	100	5	2	4	5	-
2014	R14-804	04552 Borna/ OT Wyhra	100	4	5	0	5	-
2014	R14-805	18519 Sundhagen-Horst	30	5	0	1	4	-
2014	R14-806	04552 Borna	100	1	0	1	2	-
2014	R14-807	17166 Dalkendorf	100	0	0	0	0	-

- not tested

Dose-response experiments

Dose-response experiments confirmed the results of the monitoring trials in most cases. However, ACCase resistance detected in the Swiss population from Zizers could not be confirmed by dose-response experiments. Furthermore, resistance to the pre-emergence active ingredient flufenacet was found in the populations ‘Fargau’ and ‘Römnitz’ (Tab. 4).

Tab. 4 Resistant factors of different *Lolium* populations for various herbicides, based on ED_{50} values – greenhouse dose-response trial. ED_{50} values of sensitive populations used for calculation of resistant factors are based on mean ED_{50} of susceptible populations "variety 1-3" (RI = 1.0).

Tab. 4 *Resistenzfaktoren verschiedener* Lolium-*Proben gegenüber verschiedenen Herbiziden – Dosis-Wirkungs-Gewächshausversuch. Als ED_{50}-Wert der sensitiven Population wurde der mittlerer ED_{50}-Wert der sensitiven Standards (variety 1, 2 und 3) verwendet (RF = 1,0).*

Post code	Sample Nr.	Village	region	species	meso- & iodosulfuron	pinoxaden	pyrox- & florasulam	flufenacet	cycloxydim	iodosulfuron	glyphosate
variety 1'	sen 1			1	0.7	1.0	2.1	0.5	0.9	1.1	0.9
variety 2'	sen 2			2	1.7	1.0	0.7	1.8	1.2	1.0	1.1
variety 3'	sen 3			1	0.6	1.0	0.3	0.7	0.9	0.9	0.9
D-24256	899	Fargau	SH	2	19.9	11.3	34.1	4.3	10.7	49.6	0.7
D-23909	R10-802	Römnitz (Stub.)	MV	1	1.8	2.0	6.7	2.1	7.4	2.4	0.9
D-23909	R10-803	Römnitz	MV	1	1.9	10.2	4.5	7.7	21.3	2.7	1.0
CH - 1298	R11-010	Céligny	GE	-	3.9	1.1	0.8	1.5	1.2	1.2	0.9
CH - 7205	R11-004	Zizers	GB	-	0.1	1.1	1.1	0.8	1.2	0.3	1.0
CH - 1299	R11-008	Crans près Cél.	GE	-	1.8	1.0	0.1	1.7	0.7	1.5	0.9
CH - 1565	R11-007	Vallon	FR	-	1.7	1.1	2.0	1.5	1.4	0.8	1.0
CH - 1297	R11-011	Founex	GE	-	0.7	1.3	1.5	2.0	1.2	1.6	0.9
D-18574	R13-801	Gustow	MV	-	2.2	1.1	2.4	1.1	0.3	1.6	0.8
D-17166	R13-802	Teschow	MV	-	3.1	5.6	2.7	1.6	13.0	12.2	1.0
D-23909	R12-802	Römnitz	MV	1	5.1	7.8	33.4	3.9	18.0	13.6	1.2
D-19071	R14-801	Brüsewitz	MV	1	1.8	0.9	2.0	0.5	1.1	1.1	1.4
D-09322	R14-803	Penig	S	1	10.6	16.8	19.5	2.0	15.7	28.8	1.2
D-04552	R14-804	Wyhra	S	1	29.5	4.9	16.0	2.3	5.2	48.4	1.2
D-04552	R14-806	Borna	S	3	19.5	2.2	2.5	1.0	7.3	2.0	1.2

- species unknown; 1 - Lolium multiflorum; 2 – L. perenne; 3 – L. temulentum

CH - Kantone: GE-Genf; GB - Graubünden; FR - Freiburg

D - Federal states: MV-Mecklenburg-Vorpommern; S - Sachsen; SH - Schleswig-Holstein

No resistance to glyphosate could be detected. The cross resistance spectrum of the populations 'Fargau', 'Römnitz', 'Teschow', 'Penig' and 'Wyhra' showed resistance to all used selective and registered ACCase and ALS inhibitors. Consequently, in three different German federal states (Schleswig-Holstein, Mecklenburg-Vorpommern and Sachsen) these multiple resistant populations occurred. Resistant factors for some of these populations and herbicides were quite high, indicating presence of target-site resistance to ACCase and/or ALS inhibitors. Dose-response curves for four populations and herbicides are shown in Figure 1.

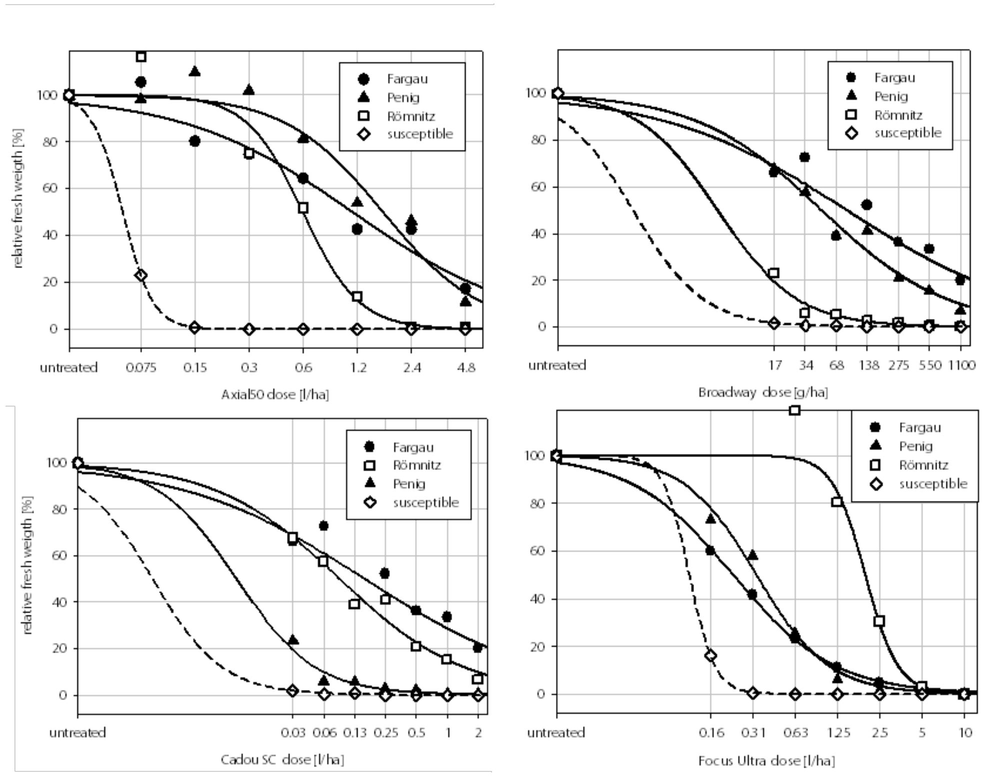

Fig. 1 Dose-response curves of four German *Lolium* spp. populations to four different herbicides (Fargau – 899; Penig - R14-803; Römnitz - We10-803; susceptible: Mean of sensitive populations).

***Abb. 1** Dosis-Wirkungskurven von vier deutschen* Lolium-*Herkünften bei vier unterschiedlichen Herbiziden.*

Genetic analysis

Analysis of potential target-site resistance in ACCase and ALS genes showed no presence of mutation in the ALS-coding region (Tab. 5). Surprisingly, population R12-801 showed no surviving plants after the meso- + iodosulfuron application which in contrast to the findings of the resistance monitoring.

However, in seven out of eight populations, an ACCase target-site resistance was detected. Mutations were mainly found at positions 2041 and 2078. In six populations, mutations at position 1781 or 2078 were present. As a consequence, resistance to all ACCase inhibitors including 'DIM'-herbicides occurred in these populations. In population Fargau (899), no target-site mutation could be detected. However, this population showed a strong resistance pattern in the glasshouse studies that may indicate effective enhanced metabolic activity. Population R14-804 showed a mutation only at position 2041 which indicates resistance to 'DEN' and 'FOP' herbicides but not to 'DIMs'. Five populations showed two different mutated positions at the ACCase gene. Additionally, mutated homozygous plants were found for position 2078 only while all other detected target-site resistances were based on heterozygous allels.

Tab. 5 Number of plants with ACCase and/or ALS target-site resistance in eight German herbicide resistant *Lolium* populations (no of plants sampled/population = 5); *wild type / heterozygous / homozygous.

Tab. 5 *Anzahl Pflanzen mit ACCase- und/oder ALS-Zielortresistenzen in acht deutschen herbizidresistenten* Lolium-*Herkünften (Anzahl beprobter Pflanzen/Population = 5).*

Population	*Pro-197*	*Asp-376*	*Trp-574*	*Ile-1781*	*Trp-2027*	*Ile-2041*	*Asp-2078*	*Gly-2096*
899	*5 / 0 / 0**	*5 / 0 / 0*	*5 / 0 / 0*	*5 / 0 / 0*	*5 / 0 / 0*	*5 / 0 / 0*	*5 / 0 / 0*	*5 / 0 / 0*
We10-802	*n.i.*	*n.i.*	*n.i.*	*5 / 0 / 0*	*5 / 0 / 0*	**3 / 2 / 0**	**0 / 0 / 5**	*5 / 0 / 0*
R12-801	*n.i.*	*n.i.*	*n.i.*	*5 / 0 / 0*	*5 / 0 / 0*	**3 / 2 / 0**	**0 / 0 / 5**	*5 / 0 / 0*
R12-802	*5 / 0 / 0*	*5 / 0 / 0*	*5 / 0 / 0*	*5 / 0 / 0*	*5 / 0 / 0*	**3 / 2 / 0**	**0 / 2 / 3**	*5 / 0 / 0*
R14-802	*5 / 0 / 0*	*5 / 0 / 0*	*5 / 0 / 0*	**3 / 2 / 0**	*5 / 0 / 0*	**1 / 4 / 0**	*5 / 0 / 0*	*5 / 0 / 0*
R14-803	*5 / 0 / 0*	*5 / 0 / 0*	*5 / 0 / 0*	*5 / 0 / 0*	*5 / 0 / 0*	**0 / 4 / 1**	**1 / 2 / 2**	*5 / 0 / 0*
R14-804	*5 / 0 / 0*	*5 / 0 / 0*	*5 / 0 / 0*	*5 / 0 / 0*	*5 / 0 / 0*	**0 / 5 / 0**	*5 / 0 / 0*	*5 / 0 / 0*
R14-806	*n.i.*	*n.i.*	*n.i.*	*5 / 0 / 0*	*5 / 0 / 0*	**3 / 2 / 0**	**0 / 0 / 5**	*5 / 0 / 0*

n.i. – not investigated

Discussion

In many regions of the world, *Lolium* spp. are of importance as weeds and control is quite complex due to occurrence of herbicide resistant populations. The situation in Germany is different. At present, *Lolium* species are relatively unimportant arable weeds. Only in some locations with high density farmers must focus their weed control on *Lolium* spp. The first *Lolium perenne* population with resistance to ACCase and ALS inhibitors was identified in Northern Germany (Schleswig-Holstein) in 2008 (KRATO et al., 2009). Since that time, resistant *Lolium* spp. populations appeared also in Mecklenburg and in 2014 in Sachsen and in Hessen. Cross resistance to ACCase and ALS inhibitors occurs quite often. At two locations in Germany, resistance to flufenacet was also found. To our knowledge that are the first flufenacet resistant *Lolium perenne* resp. *L. multiflorum* populations in Europe. Only in the US there is a report on flufenacet resistant *L. perenne* population published (LIU, 2013). Detailed investigations indicate that enhanced metabolic activities may be responsible for flufenacet resistance (DÜCKER et al., 2015).

Cross resistance to post- and pre-emergence herbicides makes it difficult to control resistant *Lolium* populations in cereals. Consequently, in principal, the resistance situation for *Lolium* does not principally differ much compared to *Alopecurus myosuroides*. However, the occurrence of *Lolium* spp. is much lower compared to *A. myosuroides* at the moment. For both species there are no glyphosate resistance cases reported in Germany so far. This indicates that potential metabolic pathways involved in flufenacet, ACCase and ALS inhibitor degradation do not influence glyphosate efficacy in *Lolium* spp.

Target-site resistance could be identified for ACCase inhibitors but not for ALS herbicides. These findings are in contrast to the high resistant factor of some populations even to ALS inhibitors (e.g. population 'Fargau"). This may indicate that resistance is caused by very effective non-target-site resistance mechanism or that another unknown ALS mutation is present in this population. The presence of mutation at ACCase inhibitor relevant positions 1781 and 2078 indicates that not only in cereals but also in dicot crops control of *Lolium* spp. might be difficult at some locations. Furthermore, presence of ACCase target-site mutations indicates potential for rapid spread of resistance to other populations.

An open question is if importance of *Lolium* species as weeds will increase in central Europe. Some indications like climatic change, conservation tillage, management of buffer strips and use of cover crop mixtures including *Lolium* seeds may lead to more and quicker spread of *Lolium* in the future. Due to high genetic diversity in the genus and limited number of different herbicide for selective *Lolium* control, fast development of resistance might be the consequence.

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