Response to Glyphosate in *Alopecurus myosuroides* Populations from Lower Saxony

Wirkung von Glyphosat gegen Alopecurus myosuroides*-Populationen in Niedersachsen*

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Abstract

The broad-scale herbicide glyphosate is used worldwide in crop management system to control weeds, facilitate harvests, prepare seed beds, and desiccate cover crops. Greenhouse monitoring trials on herbicide resistance occurrence and potential spread conducted regularly in Lower Saxony, Germany, revealed a reduced glyphosate efficacy against some populations of *Alopecurus myosuroides*. This could indicate a potential shift towards a reduced sensitivity in some populations. A dose-response experiment was conducted with six *Alopecurus myosuroides* populations from various regions of Lower Saxony. A reference population, susceptible to glyphosate, was included for comparison. Plants were treated with a commercially available formulation containing the potassium salt of glyphosate. Treatments were 0, 225, 450, 900, 1800, 3600 and 7200 g glyphosate/ha. ED_{50} values were determined from the dose-response curves and corresponding resistance factors were calculated. When assessed three weeks after glyphosate treatment, single individuals from four out of six tested populations survived doses of 1800 and 3600 g glyphosate/ha without any visible damage and continued to grow. Six weeks after glyphosate application, a regrowth of several individuals from four populations, previously assessed as completely controlled by 900 g glyphosate/ha, occurred. The observations made in this study indicate that the populations tested may have a potential to evolve glyphosate resistance. Further experiments must be conducted to investigate underlying mechanisms.

Keywords: Dose-response experiment, ED_{50} values, glyphosate, resistance factors

Zusammenfassung

Das Totalherbizid Glyphosat wird weltweit im Pflanzenbau zur Unkrautbekämpfung, Ernteerleichterung, Saatbettbereitung und Abtötung von Zwischenfrüchten eingesetzt. Regelmäßig durchgeführte Gewächshaus-Monitoringversuche zum Auftreten und zur möglichen Verbreitung von Herbizidresistenzen in Niedersachsen zeigten, dass die Wirksamkeit von Glyphosat bei bestimmten Populationen von *Alopecurus myosuroides* reduziert ist. Dies könnte auf eine mögliche Toleranzverschiebung in Richtung einer reduzierten Sensitivität in manchen Populationen hinweisen. Eine Dosis-Wirkungs-Untersuchung wurde mit sechs *Alopecurus myosuroides*-Populationen aus verschiedenen Regionen Niedersachsens durchgeführt. Eine Glyphosat-sensitive Referenzpopulation wurde zum Vergleich herangezogen. Die Pflanzen wurden mit einer kommerziell erhältlichen, Glyphosat als Kaliumsalz enthaltenden, Formulierung in aufsteigenden Aufwandmengen (0, 225, 450, 900, 1800, 3600 und 7200 g Glyphosat/ha) behandelt. ED_{50}-Werte wurden aus den Dosis-Wirkungskurven ermittelt und Resistenzfaktoren berechnet. Von sechs geprüften Populationen überlebten drei Wochen nach der Glyphosatbehandlung einzelne Individuen aus vier Populationen bei Aufwandmengen von 1800 und 3600 g Glyphosat/ha ohne jeglichen sichtbaren Schaden und wuchsen weiter. Sechs Wochen nach der Glyphosatbehandlung mit 900 g Glyphosat/ha kam es zu einem Neuaustrieb verschiedener zuvor, als vollständig kontrolliert bewerteter, Individuen aus vier Populationen. Die in dieser Studie gemachten Beobachtungen deuten darauf hin, dass die getesteten Populationen ein Potenzial zur Resistenzentwicklung haben könnten. Weitere Experimente müssen durchgeführt werden, um die zugrundeliegenden Mechanismen zu untersuchen.

Stichwörter: Dosis-Wirkungsuntersuchung, ED_{50}-Werte, Glyphosat, Resistenzfaktoren

Introduction

The rise of chemical weed control began in the 40s of the 20th century. Since then, numerous herbicidal active ingredients have been discovered and developed (DUKE and POWLES, 2008; LORENTZ, 2014). The individual herbicidal actives known and largely used commercially today in herbicide formulations in their entirety have 25 different Modes of Action (MoA) (HEAP, 2019). One of these actives is glyphosate (N-(phosphonomethyl)glycine), which was introduced into the market in 1974.

It has become the most widely used active ingredient (a.i.) of herbicides worldwide. The outstanding success of glyphosate usage in practice is based on the fact that it can be used as a broad-spectrum herbicide against most weed species, shows a low toxicity to non-target organisms and is rapidly degraded in soil. Today, glyphosate is used worldwide in crop management system to control weeds, facilitate harvests, prepare seed beds, and desiccate cover crops on arable land. It is also used for weed control in orchards, vineyards, green areas such as national parks and amenity areas or along roads and railways (Duke and Powles, 2008; Powles, 2008; Powles and Yu, 2010; Lorentz, 2014). In addition, glyphosate is used extensively since the introduction of glyphosate-resistant crops in several countries as a selective in-crop herbicide for weed control (Duke and Powles, 2008; Powles, 2008; Lorentz, 2014). For a long time, it has been assumed that there is a low risk for the development of glyphosate-resistant weeds and that this phenomenon is unlikely to occur (Bradshaw et al., 1997; Baerson et al., 2002). The opposite became apparent in 1996, when the first naturally evolved resistance in a population of the weed species *Lolium rigidum* has been discovered in Australia (Powles et al., 1998). The second case of an evolved glyphosate resistance was observed in an *Eleusine indica* population from Malaysia in 1997 (Baerson et al., 2002). In 2019, resistant populations of 45 different weed species were documented (Heap, 2019) according to criteria defined by the Herbicide Resistance Action Committee (HRAC) (Heap, 2005). No glyphosate-resistant weed populations have been confirmed in Germany to date. Regularly conducted greenhouse monitoring trials on herbicide resistance occurrence and potential spread in Lower Saxony (Germany) revealed a reduced glyphosate efficacy against some populations of *Alopecurus myosuroides*. Since 2015, individual plants in bioassays occasionally survived the recommended field rate of 1800 g glyphosate/ha or even higher rates (Wolber et al., 2018). This could indicate a potential shift towards reduced glyphosate sensitivity in some populations of this species. *A. myosuroides* is one of the most important weeds on arable land in Germany (Gehring et al., 2012; Petersen et al., 2012). It is also one of the most problematic weeds with regard to herbicide resistance in Germany (Balgheim, 2009). The aim of this study was to determine if six *A. myosuroides* populations from various regions of Lower Saxony show a reduced sensitivity or are even resistant to glyphosate. A dose-response study was conducted to determine ED_{50} values and to estimate resistance factors.

Materials and Methods

Plant Material

Seeds of six *A. myosuroides* populations for which a reduced glyphosate-sensitivity was suspected were used in this study, hereinafter referred to as P8, P11, P36, P39, A1 and A2. Seeds for populations P8 and P39 were collected from herbicide field trials conducted in 2018 in spring wheat fields in the Hanover Region and the district of Aurich, respectively. Seeds for populations P11 and P36 were collected in 2018 from a winter wheat field in the Hanover Region and from an oilseed rape field near Bremervörde, respectively. The plants from which the seed were collected survived field applications of herbicides with MoA’s different to glyphosate. F1-Plants germinated from the collected seeds mentioned above were already evaluated in a greenhouse monitoring trial. Few plants of all four populations survived a glyphosate treatment of 1800 g a.i./ha (max. registered dose rate in Germany). Seed sampling from *A. myosuroides* populations A1 and A2 was conducted in 2018 from two sites in the district of Aurich. At these two sites, glyphosate had been applied as part of a field trial with different glyphosate treatments (variants). *A. myosuroides* seeds from three variants receiving different glyphosate rates were used (Tab. 1). To differentiate between the three variants, the population ID was extended by an additional number (e.g. A1.1). Population A1.1, A1.2 and A1.3 were treated three times at different dates with 1080, 1800 and 3600 g glyphosate/ha, respectively. Population A2.1, A2.2 and A2.3 were treated two times with the same glyphosate rates at different dates. It is unknown whether the plants used for seed sampling survived the glyphosate applications described in Tab. 1 or emerged at a later stage. Seeds of a glyphosate-susceptible *A. myosuroides* reference population were obtained from “Appels Wilde Samen”, hereinafter referred to as S.

Preparation, growing conditions

Before the experiment, *A. myosuroides* seeds were placed in a freezer at -18 °C for a period of five days to break dormancy. Plastic foil (PE-LD) and hereon irrigation fleece were laid out in plant trays. Pots (Jiffypot®, round, 250 ml volume, Jiffy Products International BV, Zwijndrecht, Netherlands) were filled with approximately 300 g soil per pot (strong loamy sand, pH value 6.8, organic matter 1.9 %, steamed) and placed in the plant trays.

Tab. 1 Application dates and glyphosate dose rates for variants of populations A1 and A2.

Tab. 1 *Applikationszeitpunkte und Glyphosat-Aufwandmengen für Varianten der Populationen A1 und A2.*

Population.Variant	**Glyphosate rate 1st application 27.09.2017**	**Glyphosate rate 2nd application 14.03.2018**	**Glyphosate rate 3rd application 09.05.2018**
A1.1	1080 g a.i./ha	1080 g a.i./ha	1080 g a.i./ha
A1.2	1800 g a.i./ha	1800 g a.i./ha	1800 g a.i./ha
A1.3	3600 g a.i./ha	3600 g a.i./ha	3600 g a.i./ha
A2.1	-	1080 g a.i./ha	1080 g a.i./ha
A2.2	-	1800 g a.i./ha	1800 g a.i./ha
A2.3	-	3600 g a.i./ha	3600 g a.i./ha

On the base of a previously conducted germination test, seeds were portioned and 20 seeds were sown in each pot. The seeds in the pots were covered with a thin layer (approx. 1 cm) of the soil substrate mentioned before (sieved). After subirrigation of the pots in the plant trays, they were maintained in a greenhouse. Temperatures were set to 18 °C during day and 15 °C during night. The length of the photoperiod was 16 h. High-pressure sodium lamps (8000 Lux illuminance, 180 Watt/m^2) were used as additional light to extend the natural day length and automatically switched on under low light conditions (clouds, rainfall). The moisture level of the pots was checked daily and subirrigation conducted as needed. After glyphosate application, a nutrient solution (1 g/l H_2O, Ferty®2 (15 % N, 5 % P_2O_5, 25 % K_2O, 2 % MgO, 0.02 % B, 0.03 % Cu, 0.075 % Fe, 0.05 % Mn, 0.001 % Mo, 0.01 % Zn), Planta Düngemittel GmbH, Regenstauf, Germany) was added once a week in addition to subirrigation with water.

Glyphosate Dose-Response Experiment

After plants reached the 1- to 2-leaf stage (BBCH 11-12), they were treated with glyphosate at 0, 225, 450, 900, 1800, 3600 and 7200 g a.i./ha (Roundup® PowerFlex, potassium salt, 480 g/l glyphosate, Monsanto Europe S.A./N.V, Antwerp, Belgium). Glyphosate treatments were carried out using a laboratory track sprayer calibrated to deliver 200 l/ha of spray solution at 189 kPa and 2 km/h. There were eight replicated pots for population P8, P11, P36, P39, all three variants of A1 and S per treatment. A2.1 had five, A2.2 and A2.3 had three replications per treatment due to a lack of sufficient seed material. The experiment was not repeated. After glyphosate application, the pots were checked every two to three days for the presence of post-emerged plants in order to remove them mechanically (including the root). This was done to ensure that only herbicide treated *A. myosuroides* plants were present in the pots. Three weeks after treatment, glyphosate efficacy was visually assessed in %, compared to the untreated control on a scale of 0 to 100 %. The degree of efficacy indicates visible damages in the form of chlorosis, necrosis and growth inhibition (0 % = no effect, 100 % = completely controlled plants). In pots where individual plants survived the glyphosate treatment without any plant damage, the characteristic "non-controllable plants" was also determined. This is defined as the percentage of plants in a pot which is still alive three weeks after glyphosate treatment and continues to grow. If for example out of 12 plants in the pot one (= 0.083) survives without any plant damage, 8,3 % of the plants which were initially in that pot were not damaged by glyphosate. If a surviving plant (e.g. out of 14 = 0.071) is about a third smaller compared to the untreated control, one third is subtracted from the calculated percentage (e.g. 7.1 % - 1/3 = 4.7 %). The determined percentages of non-controllable plants have been subtracted from the percentages of glyphosate efficacy. After the assessment, the pots were maintained in the greenhouse for another three weeks for further observation.

Data analysis

The data obtained from the dose-response experiment were examined with a nonlinear regression analysis. A four-parameter log-logistic model was used to create dose response curves:

$$y = c + \frac{d - c}{1 + e^{(b(\ln(x) - \ln(ED_{50})))}}$$

The parameter d represents the upper limit and c the lower limit of the dose-response curve. Parameter b describes the relative slope around e. Parameter e is the inflection point of the curve, which at the same time marks the ED_{50} value. ED_{50} is the effective dose at which 50 % glyphosate efficacy is achieved (compared to an untreated control) (KNEZEVIC et al., 2007). The log-logistic model described above was analysed in the statistical software R (v 3.6.1) with its dose–response curve package (drc). Initially all data sets were fitted to log-logistic and Weibull functions and lack-of-fit tests were carried out to determine the appropriateness of the models in order to choose one of them for analysis. The parameters for the upper and lower limits were the same for all populations. Therefore, a model reduction was conducted where c (= 0 %) and d (= 100 %) were set as common parameters for all curves. Lack-of-fit tests (F-Test) were carried out to determine whether the model reduction did contribute to a more accurate analysis. To determine significant differences in sensitivity between S and the test populations, resistance factors were calculated from estimated ED_{50} values using the EDcomp function in the drc package (RITZ and STREIBIG, 2005; KNEZEVIC et al., 2007; RITZ, 2010; RITZ et al., 2015).

Results

Glyphosate Dose-Response

Lack-of-fit tests revealed that the four-parameter log-logistic model with c and d set as common parameters adequately described the dose-response relationship at different glyphosate rates for all populations. The curves of the populations P8, P11, P36, P39 and A1.1, A1.3 showed a slight shift towards higher glyphosate rates compared to the S curve (Fig. 1-2). The curves of A1.2, A2.1, A2.2 and A2.3 were almost congruent with the S curve.

Tab. 2 ED_{50} values and resistance factors (R/S) of tested *A. myosuroides* populations calculated from dose-response experiment data assessed three weeks after glyphosate treatment, SE= Standard error, p-value (P) > 0.05 = non-significant difference between ED_{50} of the test population and ED_{50} of the S population.

Tab. 2 *ED_{50}-Werte und Resistenzfaktoren (R/S) der geprüften A. myosuroides-Populationen berechnet aus Boniturdaten aus dem Dosis-Wirkungs-Experiment ermittelt drei Wochen nach der Glyphosatbehandlung, SE= Standardfehler, p-Wert (P) > 0.05 = nicht signifikante Unterschiede zwischen der ED_{50} der Testpopulation und ED_{50} der S Referenz.*

Population	ED_{50} ±SE	ED_{50} ratio [R/S]	p-value
S	377.9 ±104.2	-	-
P8	439.9 ±68.3	1.160	0.564
P11	426.5 ±7.8	1.129	0.525
P36	448.2 ±6.5	1.189	0.406
P39	438.8 ±10.9	1.171	0.278
A1.1	421.4 ±21.1	1.128	0.511
A1.2	393.5 ±43.5	1.053	0.794
A1.3	429.0 ±10.7	1.148	0.452
A2.1	406.0 ±98.5	1.053	0.942
A2.2	409.6 ±52.9	1.063	0.934
A2.3	388.9 ±86.5	1.009	0.99

The curve shapes were in accordance to the ED_{50} values and resistance factors showed in Table 2. The ED_{50} value of S in Table 2 is the mean value from regression analyses with each tested population. The calculated resistance factors were all close to each other and not significantly higher than one. The populations tested all show a 1.009-1.189-fold tolerance in comparison to S. The lowest resistance factor was observed for population A2.3 with a 1.009-fold tolerance compared to S. Population P36 showed the largest resistance factor with a 1.189-fold tolerance. No statistically significant differences between ED_{50} values of the test populations and the S reference could be detected indicating no statistically significant reduction in sensitivity in any of the tested populations (Tab. 2).

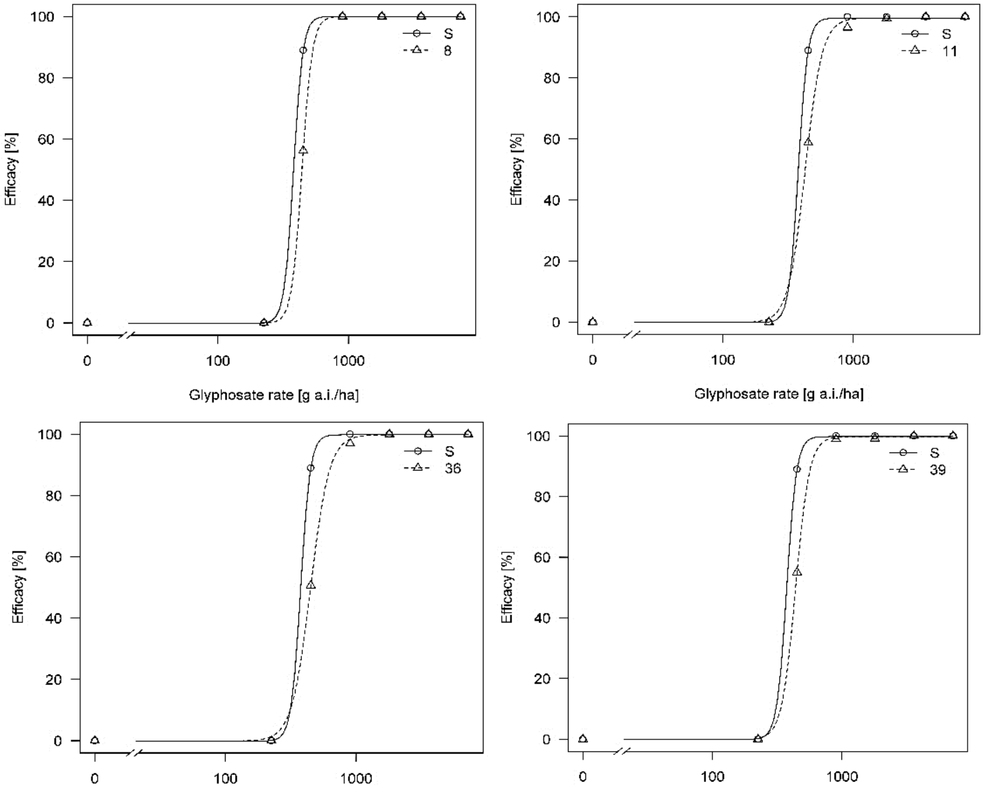

Fig. 1 Efficacy (%) of glyphosate applied at different dose rates to populations S, P8, P11, P36 and P39.

Abb. 1 *Wirkung (%) von verschiedenen Glyphosat-Dosierungen bei den Populationen S, P8, P11, P36 und P39.*

At the time of glyphosate efficacy assessment (21 days after treatment), several plants from populations P11, P36 and P39 survived the glyphosate treatment of 900 g a.i./ha without any visible damage and continued to grow. Among the populations P11, P39 and A1.3, one plant in each population survived the glyphosate treatment of 1800 g a.i./ha. Among A1.2 and A2.2, one plant in each population even survived the glyphosate treatment of 3600 g a.i./ha without any damage.

Further observation

Three weeks after assessment of glyphosate efficacy (six weeks after glyphosate treatment), the surviving plants mentioned above had developed several tillers and continued to grow vitally. Compared to the untreated control, survivors from populations P11, A1.3 (surviving 1800 g glyphosate/ha) and A1.2, A2.2 (surviving 3600 g glyphosate/ha) were delayed in development. Survivor from P39 (surviving 1800 g glyphosate/ha) developed just as fast as the untreated control.

In addition, a regrowth of various individual plants rated as completely controlled at glyphosate efficacy assessment (21 days after treatment) occurred in populations P8, P11, A1.1, A1.2, A1.3, A2.1 and A2.2. This phenomenon was observed in pots which were treated with 900 g glyphosate/ha.

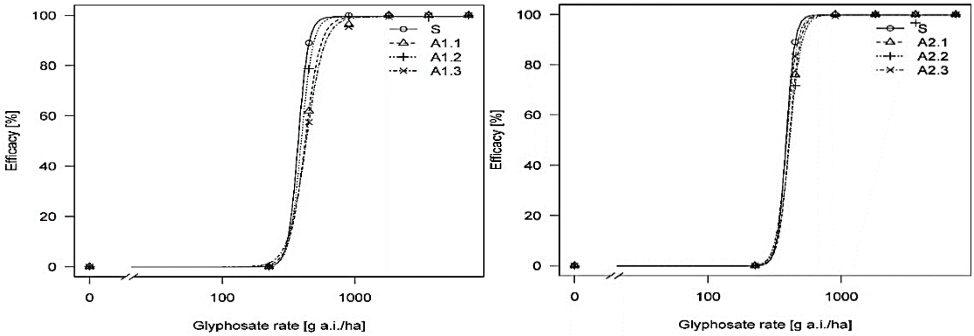

Fig. 2 Efficacy (%) of glyphosate applied at different dose rates to population S and three variants of populations A1 and A2.

Abb. 2 *Wirkung (%) von verschiedenen Glyphosat-Dosierungen bei der Population S und drei Varianten der Populationen A1 und A2.*

Discussion

The resistance factors determined for the six tested *A. myosuroides* populations were very low. In similar studies with glyphosate in different grass species, consistently higher resistance factors were determined e.g. 1.9-30 in *Lolium rigidum*, 5-10.3 in *Lolium multiflorum* or 25.1 in *Lolium perenne* (WAKELIN and PRESTON, 2006; PEREZ-JONES et al., 2007; BOSTAMAM et al., 2012; ADU-YEBOAH et al., 2014; GHANIZADEH et al., 2014; GHANIZADEH et al., 2016). When carrying out dose-response studies, seeds are often collected from plants having survived a glyphosate application and have therefore undergone a selection process (WAKELIN and PRESTON, 2006; PEREZ-JONES et al., 2007; BOSTAMAM et al., 2012; ADU-YEBOAH et al., 2014; GHANIZADEH et al., 2014; GHANIZADEH et al., 2016). In other studies, seeds from plants (*Amaranthus palmeri*, *Ambrosia trifida*) surviving glyphosate application have been collected and their progeny subjected to further glyphosate selection. The seeds of the surviving progeny were then used for glyphosate dose-response experiments (NANDULA et al., 2012; NANDULA et al., 2015). Such approaches may lead to higher resistance factors being identified. On the other hand, dose-response studies using seeds from non-preselected plants show lower glyphosate resistance factors, e.g. a resistance factor of 3 was observed for *Lolium multiflorum* (NANDULA et al., 2007), 1.1-22.7 for *Amaranthus palmeri*, and 1.3-7.5 for *Amaranthus tuberculatus* and *Amaranthus blitum* (LORENTZ, 2014). It is unknown whether the seeds for the dose-response experiment presented in this paper originate from preselected plants. In the case of low observed resistance factors, the question arises as to whether the tested plants are actually resistant to the tested herbicide. Observed differences in glyphosate efficacy could also be due to natural differences in the herbicide sensitivity of a weed species (NORDMEYER and ZWERGER, 2010). Significant interpopulation variation in sensitivity to glyphosate was detected in 40 different populations of *A. myosuroides* in the UK (DAVIES and NEVE, 2017). Determined ED_{90} values from five populations were significantly higher compared with the reference susceptible population used. The least and most sensitive populations had a ratio of 1.7 between ED_{90} values. No glyphosate resistance among the tested field-collected populations could be proven (DAVIES & NEVE, 2017). When looking at dose-response curves, ED_{50} values, resistance factors and the fact that no statistically significant differences could be detected between the ED values of the test populations and the S reference, the populations tested cannot be classified as resistant. However, in the work presented here individual plants from the populations P11, P39, A1 and A2 have survived the full (1800 g a.i./ha) or double (3600 g a.i./ha) glyphosate dose rate permitted in Germany without any visible damage and continued to grow vitally. Beyond that, three weeks after assessment of glyphosate efficacy a regrowth by various visually dead appearing plants treated with half permitted application rate (900 g a.i./ha) from P8, P11, A1 and A2 have occurred. The consideration of the observations made in this study indicates that the populations tested may have a potential for resistance development against glyphosate. Among these populations there may already be a possible tolerance shift towards a reduced sensitivity and as a result occasional plants can survive the full permitted application rate or even higher rates. It has to be noted that the observed survivors are not plants which have emerged after glyphosate application as emergence was checked in the pots on a regular basis. The occasional survivors can be considered as tolerant biotypes for which the resistance mechanism remains unknown. In the case of a repeated assessment of glyphosate efficacy at a later point of time with the inclusion of regrowed plants from populations P8, P11, A1 and A2 treated with 900 g glyphosate/ha, the resulting ED_{50} values and resistance factors might be higher. The phenomenon of a tolerance shift can be caused by repeated application of reduced herbicide rates which can lead to survivors. Selection experiments with reduced glyphosate rates against *A. myosuroides* and *Lolium rigidum* have shown that after only two to three selection cycles, a reduction in sensitivity against glyphosate can occur within a population (Busi and Powles, 2009; Davies and Neve, 2017). As a result, individual plants may survive the full recommended dose rate. The reason for this is a slow accumulation of genetically caused properties within a population that cause an increased tolerance (Busi and Powles, 2009; Davies and Neve, 2017). Selected plants with the mentioned (genetically caused) characteristics transmit those characteristics to their progeny (Busi and Powles, 2009; Davies and Neve, 2017). In addition, frequent application of glyphosate without rotation of different MoA can also lead to a selection process and survivors. In this case, tolerance-increasing characteristics would also be accumulated in a concerned population (Busi and Powles, 2009; Norsworthy et al., 2012). With regard to the facts described above and in order to preserve glyphosate as an essential part of weed management, more diverse weed management systems must be realized in the future among crop producers in Lower Saxony. It is strongly advised to apply glyphosate at the full recommended dose rates. Furthermore, a sequential application of glyphosate-containing herbicides should be avoided. Instead, the usage of herbicides with different MoA in annual rotations or tank mixtures and sequential applications should be practiced. Better, an extended crop rotation should be applied. This is advisable because risks of resistance development are greater in production systems with limited crop rotation (Norsworthy et al., 2012). The repeated use of a cultivator should be included in soil tillage after crop harvest and before new planting in order to stimulate uniform weed seed germination. In addition, the use of a plough can bury weed seeds in deeper layers and thus reduce a later emergence (Norsworthy et al., 2012; Wolber et al., 2018). The observations presented in this study suggest that further experiments must be conducted to investigate underlying mechanisms.

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