Status and development of ACCase and ALS inhibitor resistant black-grass (*Alopecurus myosuroides* Huds.) in neighboring fields in Germany

Status und Entwicklung von ACCase- und ALS-Inhibitor resistenten Ackerfuchsschwanz (Alopecurus myosuroides Huds.) in benachbarten Feldern in Deutschland

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Summary

Neighboring fields in three local areas of southern Germany have been investigated for the infestation level and herbicide resistance structure of black-grass (*Alopecurus myosuroides* Huds.). One field within a local area, each with confirmed resistance, served as starting point to survey the surrounding fields. Eighty percent of the fields had very few black-grass ears prior to harvest, with mainly ears from single plants spread over the field. Infestation in the other fields was in large patches or widespread, yet in most situations it did not significantly impact on yield level. Resistance to ACCase-inhibiting herbicides was found on all tested fields in each region. Plants with a target-site mutation to ACCase inhibitors were found in all samples; in addition, most plants also exhibited non-target-site resistance. All five mutations conferring ACCase resistance were found. The diversity of the mutations between areas suggests that resistance evolved independently in most fields. At two locations, each one with confirmed ALS resistance, additional fields with reduced ALS efficacy were detected. At one location only the mutation P197T was found, at the second the W574L mutation was also found. Target-site resistance appears to be the major mechanism for these early cases of ALS inhibitor resistance. Understanding the resistance development in individual fields and the spatial dynamics requires investigation over several years. The example of ACCase resistance to black-grass demonstrates how a specific mode of action can be rendered ineffective over a whole region. It provides a possible model for ALS-inhibiting herbicides. In the current situation, farmers are able to manage the black-grass infestation quite well. Resistance in a field seems to develop first in patches with high population densities. Resistance management should therefore focus on the management of the seed bank of each field, using all measures to keep the population pressure low.

Keywords: ACCase inhibitors, ALS inhibitors, blackgrass, Germany, non-target-site resistance, target-site resistance

Zusammenfassung

Benachbarte Felder aus drei Gebieten in Süddeutschland wurden auf Befall und Struktur von Herbizidresistenzen bei Ackerfuchsschwanz (*Alopecurus myosuroides* Huds.) untersucht. Jeweils ein Feld mit bestätigter Resistenz diente als Ausgangspunkt für die Untersuchung der umliegenden Felder. Auf 80 Prozent der Felder fanden sich nur sehr wenige Ähren von über das Feld verstreuten Einzelpflanzen. Der Befall auf den anderen Feldern war in größeren Flecken oder über das ganze Feld verteilt, meist ohne geschätzten Einfluss auf den Ertrag. Resistenz zu ACCase-Inhibitoren wurde auf jedem der Felder in jeder Region gefunden. In allen Proben wurden Pflanzen mit Target-Site Mutationen und solche mit Nicht-Target-Site Resistenzen gefunden. Alle fünf bekannten ACCase-Mutationen wurden gefunden. Die große Diversität der Mutationen lässt vermuten, dass sich die Resistenz unabhängig auf den einzelnen Feldern entwickelt hat. In zwei Gebieten gab es von Anfang an jeweils ein Feld mit bestätigter Resistenz gegen ALS-Inhibitoren. Hier wurde auf weiteren Feldern eine reduzierte Herbizidwirkung nachgewiesen. In einer Region wurde die Mutation P197T gefunden, zusätzlich die Mutation W574L auf den Feldern des zweiten Gebiets. Target-site Resistenz scheint die Hauptursache für diese frühe Phase der ALS-Resistenzentwicklung zu sein. Um die Resistenzentwicklung auf einzelnen Feldern und die Ausbreitung über ein ganzes Gebiet zu verstehen, bedarf es Daten mehrerer Jahre. Das Beispiel der Resistenz gegen ACCase Inhibitoren von Ackerfuchsschwanz demonstriert wie ein bestimmter Wirkmechanismus über ein weites Gebiet wirkungslos werden kann. Es ist ein mögliches Model für ALS-inhibierende Herbizide. Gegenwärtig wird der Ackerfuchsschwanz in den untersuchten Gebieten von den Landwirten sehr gut bekämpft. Die Resistenzen scheinen sich zuerst auf Feldstellen mit hohen Populationsdichten zu entwickeln. Resistenzmanagement sollte daher an erster Stelle alle möglichen Maßnahmen umfassen, die den Samenvorrat im Boden niedrig halten.

Stichwörter: ACCase-Inhibitoren, Ackerfuchsschwanz, ALS-Inhibitoren, Deutschland, Non-Target-Site Resistenz, Target-Site Resistenz

1. Introduction

In this study we evaluated the herbicide resistance structure of different *Alopecurus myosuroides* biotypes in three small areas of about 3 km² in southern Germany. Starting with a field of known resistance history, contiguous fields were investigated. The following questions were to be answered: What is the overall infestation level of *A. myosuroides* of these areas and individual fields? Does resistance occur and which kind? How is resistance developing and spreading?

The answers to these questions are of great importance for the development of optimal resistance avoidance/delaying strategies and for safeguarding high yields despite the risks posed by herbicide resistance. Country-wide resistance surveys have been conducted in several regions of Europe for *A. myosuroides* resistance, in Germany (ARLT, 1998; BÜNTE and NIEMANN, 2004; PETERSEN, 2011), in France (DÉLYE et al., 2007, 2010a, 2010b; MENCHARI et al., 2006; CHAUVEL et al., 2006; PETIT et al., 2010) and in the UK (MOSS and PERRYMAN, 2007; MARSHALL and MOSS, 2008). These investigations were made on a country-wide or regional basis with sampling of individual fields, mostly separated by a large distance. Most of the seed samples were taken from fields with suspected herbicide resistance, which likely led to an overestimation of the abundance of herbicide resistance. Other investigations concentrated on the dynamics of resistance development on a defined, single field (BALGHEIM et al., 2008; CAVAN et al., 1998; CHAUVEL et al., 2006). Our approach appears to be unique as it specializes on neighboring fields in small local areas managed by only a few farmers. This paper provides a first status report of monitoring results over two years. It is however clear that these results should be considered to be preliminary, as the dynamics of resistance development can only be judged after several seasons.

2. Materials and methods

Three starting fields were selected from the 2008 and 2009 herbicide resistance monitoring program of Bayer CropScience. The distances between the starting fields are 9, 10 and 14 km. The *A. myosuroides* populations from all three fields (the fields and the surrounding area will be designated as regions H, M, Z) proved to be resistant to herbicides inhibiting the acetyl-coenzyme A carboxylase (ACCase). Fields M and Z are in addition resistant to acetolactate synthase (ALS) inhibiting herbicides. In 2010, *A. myosuroides* seeds were sampled from a limited number of fields surrounding fields H and Z. The sampling procedure was extended to more fields from different farmers in 2011 in all three regions. Fields were searched for black-grass ears by walking along two of the field borders and three sets of tractor tracks throughout the whole field. Seeds from only one ear per plant were collected as uniformly as possible over the field with up to a total of 200 ears collected per field. The infestation level was scored for ear abundance using six categories (CHANCELLOR and FROUD-WILLIAMS, 1984). The following scale was used: 0 = no ears found, 1 = traces of ears from a few solitary, scattered plants in the field or along field borders, 2 = occasional small patches, 3 = large patches, 4 = widespread throughout the field, 5 = a dense and serious infestation. Resistance analysis based on greenhouse bioassays were carried out following the method described by MENNE and HOGREFE (2012). Fenoxaprop-P-ethyl at 41 to 166 g ha^{-1} (Ralon Super®) and the combination mesosulfuron-methyl + iodosulfuron-methyl at 15+3 to 60+11 g ha^{-1} (Atlantis WG®) were used as representative herbicides for the ACCase and ALS inhibiting herbicides. PCR and pyrosequencing procedures were used for the target-site resistance analysis are described by BEFFA et al. (2012).

3. Results

3.1 Infestation level with ears of *A. myosuroides* Huds.

The infestation level as measured by a count of ears of *A. myosuroides* was very low in about two-thirds of the fields (Fig. 1). "Traces" means a few ears were dispersed over the field, sometimes only along the field border. In a few cases small spots had been missed by herbicide application; these were also counted under this category. Four fields from a total of 104 were classified with a dense and serious infestation. The infestation levels were comparable over the three regions. Within a region the infestation level was without clear clustering (Fig. 4). Only region Z had more fields with higher ear density in the centre.

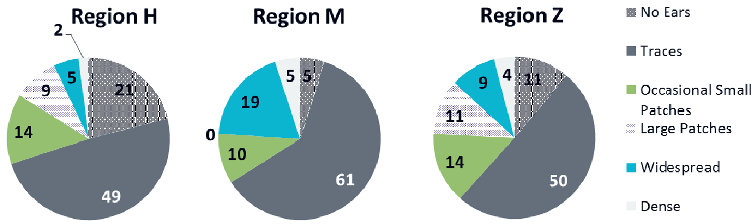

Fig. 1 The occurrence of six infestation levels of ears of *A. myosuroides* in fields from three regions. Results from regions H and Z are from 2010 and 2011, region M are only from 2011. Numbers represent % of fields in each category.

Abb. 1 *Das Auftreten von sechs Befallsstufen von Ähren von A. myosuroides auf Feldern von drei Regionen. Ergebnisse für Regionen H und Z aus 2010 und 2011, Region M nur aus 2011. Zahlen sind % Anteil der Felder in den Kategorien.*

3.2 Herbicide resistance level of plants from the collected seeds

All populations tested by bioassay had ACCase resistance (Fig. 2). In 80 % of the cases the resistance was strongly expressed with not a single sensitive plant remaining. Individual plants without confirmed target site resistance (TSR) also survived the herbicide treatments, expressing a high degree of non-target-site resistance. The degree of ACCase resistance was similar in all three regions. The resistance to ALS inhibitors was different between the areas. In region H, one population reacted with slightly reduced efficacy to ALS chemistry and the resistance level was classified as intermediate. In region Z, 9 from 39 populations expressed intermediate or full resistance to ALS inhibitors. Half of the tested populations from region M proved to be resistant to ALS herbicides. Most plants without ALS TSR-conferring mutations were sensitive, indicating the absence of non-target-site resistance mechanisms.

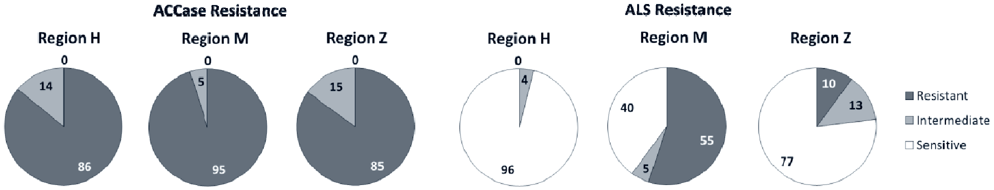

Fig. 2 The occurrence of three herbicide resistance classes of A. m*yosuroides* in fields from three regions. Results mainly from 2011, regions H and Z also results from 2010. Numbers represent % of fields in each category. Resistant < 50 %, intermediate 50 – 80 %, sensitive > 80 % efficacy.

Abb. 2 *Das Auftreten von drei Resistenzstufen von* A. myosuroides *auf Feldern von drei Regionen. Ergebnisse sind hauptsächlich von 2011, für Regionen H und Z auch aus 2010. Zahlen sind % Anteil der Felder in den Kategorien. Resistent < 50 % , intermediär 50 – 80 %, sensitiv > 80 % Wirkung.*

3.3 Occurrence of target site mutations conferring resistance

3.3.1 ACCase

All five target-site mutations conferring resistance to inhibitors of ACCase (I1781L, W2027C, I2041V, D2078G, G2096A) were found in at least one plant in at least one field population in all three regions (Fig. 3).

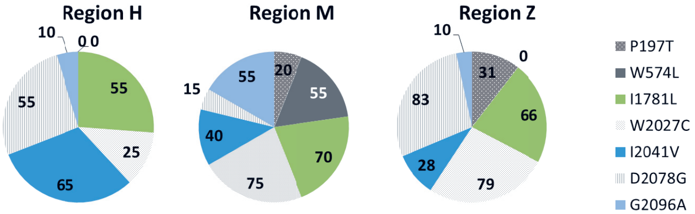

Fig. 3 The occurrence of ACCase and ALS TSR-conferring mutations in fields from three regions. Each field population had to have one plant with the specific mutation. Results are mainly from seeds collected 2011. Numbers represent % of fields in each category. Due to overlap of mutations in populations total may exceed 100 %

Abb. 3 *Das Auftreten von ACCase und ALS target-site Resistenz verursachenden Mutationen in drei Regionen. Jede Feldpopulation musste mindestens eine Pflanze mit der jeweiligen spezifischen Mutation haben. Die Ergebnisse sind hauptsächlich von Samenproben aus 2011. Zahlen sind % Anteil der Felder in den Kategorien. Wegen gleichzeitigem Vorkommen von zwei oder mehr Mutationen in den Populationen, kann die Summe 100 % überschreiten.*

The I1781L mutation was detected in over half of the fields in each of the three regions, showing the most widespread distribution. The frequency of the four other ACCase mutations differed between the regions. The W2027C mutation was found in close to 80 % of the investigated fields in region M and Z but was found in only 25 % of populations from region H. The I2041V mutation was more frequent in region H (65 %) and less frequent in the two other areas (40 and 28 %). In region Z, the D2078G mutation was found in 83 % of all fields, but was found in only 15 % of the populations in region M. The G2096A mutation was in contrast more frequent in region M and was only found in 2 out of 20 fields in the areas H and Z.

3.3.2 ALS

Both ALS resistance conferring mutations (P197T, W574L) were detected in region M. In 11 out of 20 fields at least one plant had the W574L mutation and in 6 out of 20 fields at least one plant had the P197T mutation. In region Z, only the P197T mutation was found, and this in only nine out of 29 fields. No ALS mutations were found in region H. Spatial distribution of mutation types and frequencies were different between fields within a region and between regions (Fig. 4).

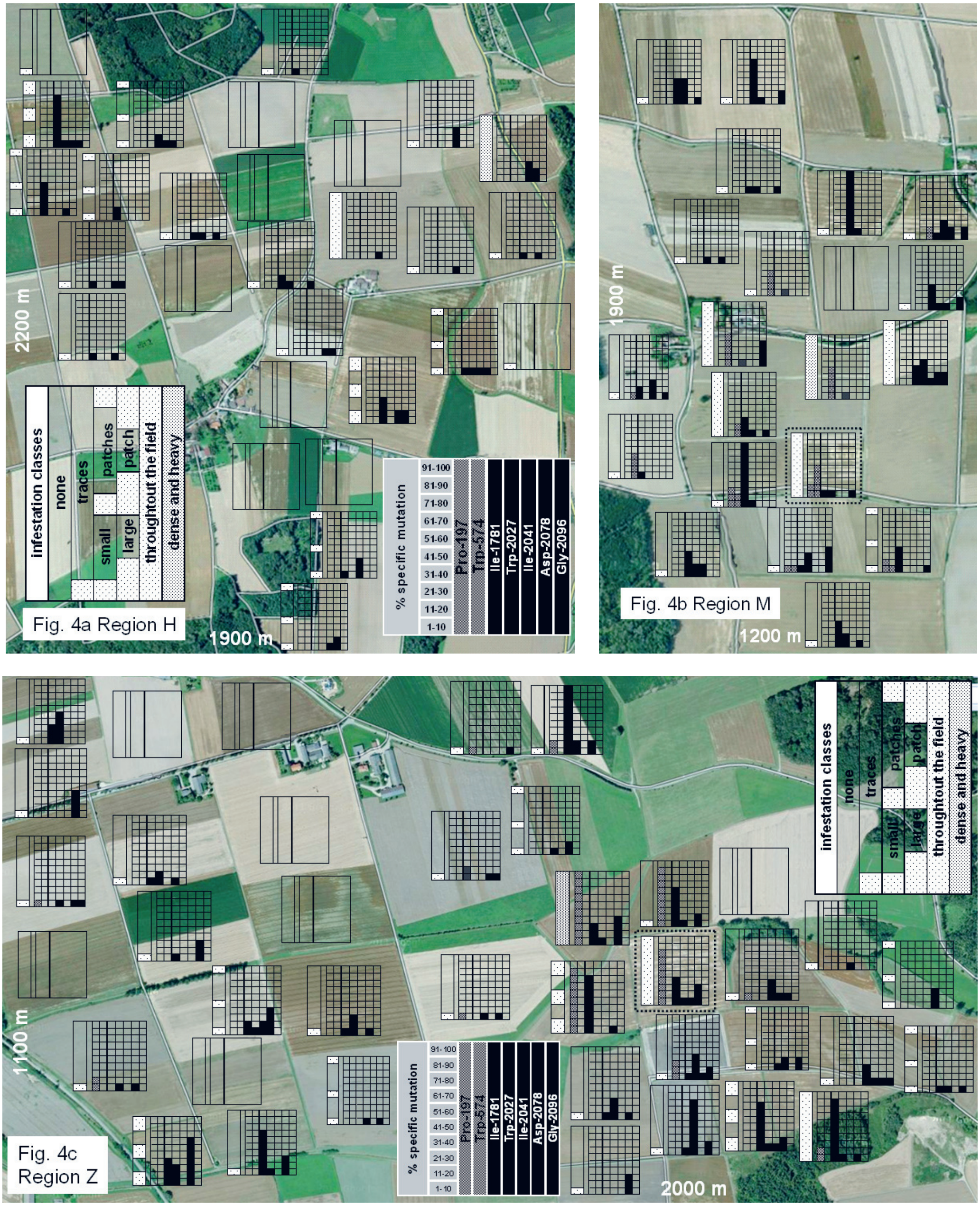

Fig. 4 Spatial distribution of ear densities using six scoring classes and frequencies of the two ALS and five ACCase mutations.

Abb. 4 *Verteilung der sechs Befallsstärken mit Ähren und die Häufigkeit des Auftretens der zwei ALS- und fünf ACCase-Mutationen.*

4. Discussion

4.1 Severity of infestation with *A. myosuroides*

In half of the fields no ears were found or the infestation level was very low. We must assume that category 0 (no ears found) has a high ratio of false negatives because ears may have been missed during counting. We defined occasional small patches as clusters of plants on a few square meters up to about 100 m^2, but covering less than 1 % of the total field area. Large patches represented a significant part of a field often up to 10 – 30 % of the area. The next highest degree of infestation, severity widespread throughout the field, does not allow any differentiation into single patches. Underestimation of infestation level could also be the case with a dense and serious infestation (category 5). The interpretation and final classification was left to the judgment of the recorder. The ratings in these studies were made just prior to harvest and represent ears from plants which have either survived herbicide treatments or have not been sprayed. An estimation of the actual infestation level in autumn or spring has not been made. We can assume that all fields in the survey areas were infested with *A. myosuroides* at different levels, but were well controlled by the farmer at harvest time. These findings on three small areas correspond well with the overall situation in Germany, where infestation levels of *A. myosuroides* in most fields are kept far below any yield loss threshold with the current tools. Ratings of the actual infestation level at harvest will allow a better estimation of the actual severity and potential yield impact of herbicide resistance beyond the pure confirmation of the presence of resistance. Farmers manage black-grass infestations on the majority of their fields well considering the current status of infestation level and type of herbicide resistance.

4.2 Herbicide resistance level

All tested field populations were resistant to ACCase inhibiting herbicides (Fig. 2). Survey results from France (DÉLYE et al., 2007; DÉLYE et al., 2010b) and England (MOSS and PERRYMAN, 2007) confirm these results with 80 to 100 % of the collected biotypes expressing ACCase inhibitor resistance. The prevalence of ACCase inhibitor resistance in Germany, based only on samples of suspected resistance, was lower in the past. ARLT (1998) found 39 % highly resistant populations from the years 1994 to 1997. DÉLYE et al. (2010b) reported 62 % resistance for the years 2001 to 2005. With a sampling focus on northwest Germany, BÜNTE and NIEMANN (2004) reported 84 % resistant samples for 2001. Latest survey results from 2008 to 2010 for the whole of Germany detected in 80 % of 236 samples either reduced activity or in most cases strong ACCase inhibitor resistance (PETERSEN, 2011). If we extrapolate our finding that each field with high, proven ACCase resistance is surrounded in a radius of several hundred meters with fields which also show ACCase inhibitor resistance, we must conclude, that ACCase resistance is widespread in Germany. The resistance of *A. myosuroides* to ACCase inhibiting herbicides therefore reflects a mature and well established situation, on a field scale as well as on a wider regional scale. Infrequent use of ACCase inhibiting herbicides, once about every 3-4 years mainly for the control of volunteer cereals in oilseed rape, will likely maintain the selection pressure for resistance. *A. myosuroides* resistance to ALS inhibiting herbicides has been confirmed in about 6 % of 236 populations with suspected resistance in Germany (PETERSEN, 2011). MARSHALL and MOSS (2008) detected ALS inhibitor resistance in England in eight out of 43 samples collected 2004 to 2006 from fields where plants had survived an ALS inhibitor treatment. In region H, one field with intermediate ALS resistance was detected. The infestation level was low and no factors which may explain this low level of resistance could be identified. The field monitoring in the regions M and Z each started from a specific field with confirmed ALS inhibitor resistance. In both areas an irregular layer of contiguous fields with ALS resistant biotypes was found (Fig. 4). This situation provides an opportunity for a retrospective controlled case study. Comparing fields with and without ALS inhibitor resistance can help us to identify possible risk factors. The main working hypothesis is based on different *A. myosuroides* infestation levels right from the beginning of the selection process.

4.3 Structure of the occurrence of TSR mutations

Each of the five tested ACCase resistance conferring mutations was found in at least one plant on at least one field in each area (Fig. 3). This is a far higher occurrence of ACCase mutations as was found in samples from Germany 2001 – 2005 (DÉLYE et al., 2010b), in which 19 % of 75 populations had the

G2096A mutation, and 12 % the G2078C mutation; the three other mutations were found with less than 10 % occurrence. Our results from the three small geographical areas in Germany are more comparable with the results from France and specifically the UK, having a similar or higher occurrence of the specific mutations. The risk factors that promote the development of herbicide resistance in *A. myosuroides* in these three regions are similar to those in greater parts of the UK, but not to those in large regions of Germany. They include close to 100 % early autumn-sown winter crops in the rotation, little use of the moldboard plough, clay rich soils and consequently high infestation levels with black-grass. Although all five investigated mutations are present in each of the regions, mutation frequencies between the regions and between single fields are very different (Fig. 3 and 4). This is similar to results from sample locations which are greater distances apart, allowing no pollen and seed exchange and where multiple, independent appearances of mutant ACCase alleles must have occurred (MENCHARI et al., 2006). This seems to be true also for the two ALS resistance conferring mutations (P197T, W574L). The P197T mutation was observed only in areas M and Z, but the W574 mutation was observed only in fields in area M. The observed pattern of mutations between fields within an area suggest that they were independently selected in each field in regions M and Z, but this is conjecture and must be investigated. The actual spatial distribution from field to field could also have resulted by spread of pollen or seed from fields with common borders, or from fields in the center of a given area (Fig. 4). The biology of *A. myosuroides* as an obligate out-crossing species supports this hypothesis. Support for the proliferation of resistance by gene flow over several kilometers could not be found with this survey by comparing fields from three distinct areas about 10 km apart and also between fields which are only a few hundred meters apart. These findings contradict the results from DÉLYE et al. (2010a) with I1781L mutations spreading over several kilometers. They are in accordance with those of CAVAN et al., (1998), who also found no evidence of the spread of resistance between patches within one field.

4.4 Emergence and spread of resistance

Analyzing emergence and spread of resistance requires data from several years. The majority of our data are only from 2011 with some fields also surveyed in 2010. This will not suffice to allow a clear description of the population and extrapolation of the resistance dynamics over subsequent years and a larger geography. The occurrence of resistance with two different herbicidal modes of action in different stages of development supports the following conclusion. Resistance to ACCase inhibitors is prevalent over all three areas. There is a high frequency of TSR to ACCase inhibitors (Fig. 3 and 4). Plants without TSR did in most cases also survive applications of ACCase herbicides, suggesting that non-TSR to ACCase was present. The highly diverse patterns and frequencies of the five resistance conferring ACCase mutations from field to field and between the regions suggest that resistance evolved independently in each field, but this remains to be proven. In the next few years, we do not expect a change in the basic ACCase resistance structure on these fields, assuming only occasional use of ACCase inhibiting herbicides and no fitness penalty associated with any of the five mutations. The specific mutations should approach the distribution predicted by the Hardy-Weinberg-equilibrium, which was observed with some of the populations having the I1781L mutation. Resistance to ALS inhibiting herbicides is in an initial stage of development. Besides the fields with known TSR resistance from previous years, we have identified a few more fields with single plants expressing resistance. As is the case with ACCase inhibitor resistance, due to the distribution pattern we assume independent evolution for each field. Even on adjacent fields, we do not see the spread of a particular type of resistance profile from one field to the other. Size, shape, and position of the single patches in these fields suggest strongly the independent evolution within a field and not the spreading of seed from neighboring fields through an infection via pollen.

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