BLACK-GRASS (*ALOPECURUS MYOSUROIDES* HUDS.)
HEREDITY OF TARGET-SITE RESISTANCE TO ALS INHIBITING HERBICIDE

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SUMMARY

Heredity assessment of target-site resistance to ALS inhibitors in Black-grass (*Alopecurus myosuroides* HUDS.)

Target-site resistance heredity in black-grass was assessed by observing the percentage of resistant individuals engendered by crossings. After germination, plants were genotyped as homo- or heterozygote for the W574 mutation on the ALS gene, which induces herbicide resistance. Individuals were then transplanted outdoor and crossed between one another and with other individuals issued from a population known as susceptible. To avoid an external pollen contamination, plants were enclosed within pollen-proof clothing during flowering time. Allogamy rate was also tested, by isolating heads one by one. Seeds issued from these crossings were collected and tested in glasshouses. Their DNA was also analysed.

Key words: Heredity, acetolactate synthase, mutation, crossings, allogamy

INTRODUCTION

One Belgian population (Quévy) of the grass weed *Alopecurus myosuroides* was identified where high proportions of individuals showed resistance to the acetolactate synthase (ALS)-inhibiting herbicides, mesosulfuron-methyl + iodosulfuron-methyl sodium mixture (commercial name : ATLANTIS WG, BayerCropScience). Screening with sulfometuron, followed by DNA analyses of the AcetoLactate Synthase (ALS) gene of resistant and susceptible individuals, showed a single point mutation at the position of the 574th codon (referred later as W574), conferring a predicted tryptophan to leucine substitution, known to endow resistance to this herbicide mode of action. This mutation modifies the structure of the enzyme targeted by the herbicide (in this case the ALS) and consequently this herbicide can not bind to the enzyme and cannot exert its action. Target-Site Resistance (TSR) must be confronted to Non-Target-Site Resistance (NTSR), which mostly implies enhanced metabolism of the plant for example, which will limit the herbicide efficacy and the duration of its action.

TSR is considered to be monogenic whereas NTSR is used to be controlled by several genes, (Petit *et al.*, 2010) thus much more difficult to trace within the offspring. That is why seeds from Quevy population were used in several experiments, including crossings with susceptible plants, implemented to follow the mutation heredity through generations and its dispersal abilities within a larger susceptible population.

MATERIAL AND METHOD

Seeds that were analysed in these experiments were either collected in farmer's fields, where chemical weeding was not efficient, or issued from crossings we implemented.

CROSSINGS

A sample of seeds were harvested in one Belgian field (in Quevy) known to present the single point mutation W574 of the ALS gene. Seeds from this population were grown in glasshouses, and then transferred in outdoor controlled conditions. After having been genotyped by DNA analyses (dCAPS, Délye & Boucansaud, 2008), plants were paired and transplanted into a parterre, then enclosed with a pollen-proof cloth placed on a metal frame (see Photo 1). This physical barrier was kept until the end of flowering time, and seeds were collected before shedding. Different couples or groups of plants were constituted, with replicates (See Table I). The progeny of these crossings was collected as a pool (named later in this paper as "F1 population"). One of each replicate was tested with different methods to detect the proportion of resistant individuals engendered, in a way to assess the heritability of target-site resistance traits

Table I : Crossings implemented and tests carried out on their progeny. (SS: homozygote susceptible – RS: heterozygote resistant – RR: homozygote resistant)

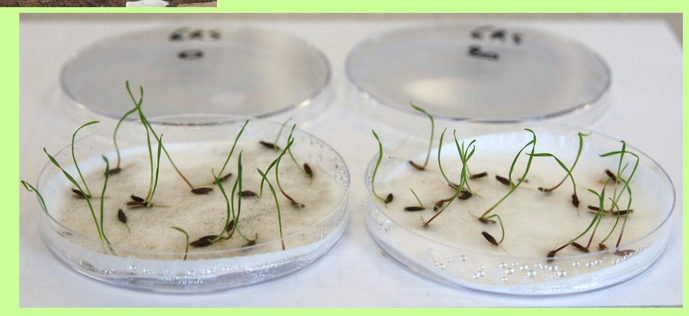
Crossings	Description	Replicates	Petri Dish Test	Glasshouse Test	dCAPS Test
RRxSS	Classic F1 Homozygotes crossing	4	✓	✓	
2RR x 4SS	Crossing with different pollen proportion. Simulating mutant introduction (two homozygotes resistant in a susceptible population)	4		✓	✓
2RS x 2SS	Crossing with different pollen proportion. Simulating mutant introduction (two heterozygotes) in a susceptible population)	4		✓	✓
6RR	Crossing with 6 homozygote resistant (Seed multiplication for further experiments)	1	✓	✓	✓
5R	Crossing with 5 heterozygotes resistant (F2 crossing)	1	✓	✓	✓
Allogamy	One plant enclosed alone in a pollen-proof cloth	1	✓		

Photo 1 : Crossings installation into a parterre, enclosured with pollen-proof cloth placed on a metal frame.

CROSSINGS IN OUTDOOR CONTROLLED CONDITIONS

Photo 2 : Petri Dishes from the 6RR population with no herbicide (Left Side) (Nil), and with 20 times the dose (Right Side).

NO DOSE EFFECT IN PETRI DISHES



PETRI DISHES TEST

Resistance

A bioassay in Petri dishes (Hull & Moss, 2007) has been conducted in order to evaluate the resistance level of different F1 populations and some other open-field populations. Petri dishes were prepared with paper filters according to the protocol, containing each 20 seeds. Sulfometuron-methyl was used but at different rates (i.e. 0; 0,5; 1; 2,5; 5; 10; 20 ppm). Zero ppm corresponds to untreated (nil) and 1ppm corresponds to the discriminating dose that is used in Rothamsted protocol. After 21 days the number of grown seedlings has been made with a shoot length measurement. A "**growth score**" per Petri dish is obtained by multiplying the proportion of emerged shoots by the mean length of these shoots. Scores obtained for treated dishes are divided by the score of the untreated one, in order to obtain a growth percentage, which can easily be compared between populations.

Allogamy

In parallel with that Petri dish test, all the seeds collected on the only plant isolated in one pollen-proof cloth were also put in four Petri dishes with the nutrient solution. Black-grass ought to be principally allogamous, so none of these seeds should germinate, except for partial autogamy.

GLASSHOUSE SPRAYING TEST

A sample of seeds from five F1 populations has been grown in glasshouse for four weeks. Then ten plants of each population, which seemed to be well-developed were divided in five tillers (plus the mother plant if applicable), and transplanted into individual pots. We thus obtained 5 clones per individual. Three weeks later, the four most homogenous tillers have been selected to be sprayed with three herbicides (plus untreated-nil). Four weeks after treatment, leaves were cut and the foliage weight was measured. The values are compared with the untreated one of each population, and with the reference.

Herbicides used were ALS and ACCase inhibitors. :

- **mesosulfuron-iodosulfuron** (ATLANTIS : 500 g.a.i.ha^{-1}), to be close to usual agricultural practices in case of resistant black-grass and to highlight any NTSR;
- **Sulfometuron-methyl** (OUST : 200 g.a.i.ha^{-1}), to detect TSR;
- **pinoxaden** (AXIAL : 18 g.a.i.ha^{-1}), to notice any eventual multiple resistance and to control the efficacy of another herbicide mode of action, consequently the specificity of the mutation endowing resistance towards ALS inhibitors.

DNA ANALYSES

Derived Cleaved Amplified Polymorphic Sequence dCAPS

DNA analyses have been performed following dCAPS protocol (Délye & Boucansaud, 2008). DNA extraction was performed with a fast extraction kit manufactured by OmegaBiotek² (*E.Z.N.A. Plant DNA Extraction Mini Kit*). The foliage material was either fresh or Frozen.

Sequencing

Three DNA samples previously identified by dCAPS as resistant were sequenced so as to verify the accuracy of the method in our lab conditions and to confirm the obtained results. We performed these analyses at the Biotechnological Department of the Gembloux A gricultural Research Centre, equipped with a sequencer.

RESULTS

PETRI DISHES TEST

Resistance : **Shoot growth percentage** is presented in Table II. Value close to 0 means that nothing has grown, and over 100, that plants have grown better in the treated dishes than in the untreated ones.

Allogamy : None of the seed harvested on the **single plant, enclosed** in a pollen-proof bag, did germinate. Actually, those seeds did not contain any germ and seed hull was in fact empty. In this case and for this wild-type genotype, strict allogamy is verified. Further analyses areconducted with other genotype, with more individuals in order to confirm this result.

Table II : Shoot growth percentage in comparison with the untreated

Nil's Percentage (%)	Sulfometuron doses					
Populations	0,5ppm	1ppm	2,5ppm	5ppm	10ppm	20ppm
Emines	4	7	1	11	0	7
Vezin	5	5	6	3	7	1
Middelkerke 1	0	0	1	2	1	1
Middelkerke 2	1	2	3	3	6	5
Gembloux	21	12	5	5	13	15
6RR	89	87	81	92	79	87
5RS	64	107	83	65	63	*

OBVIOUS DIFFERENCES BETWEEN SUSCEPTIBLE AND RESISTANT BIOTYPES

GLASSHOUSE SPRAYING TEST

Measures of fresh weight at the end of the test, transformed in **growth percentage in comparison with the untreated object of each population** are presented in Table III. A value close to 0 corresponds to a complete growth inhibition (maximal treatment efficacy) and an over-100% value means that the treated pot grew more than the untreated one.

SLIGHT DIFFERENCES BETWEEN RESISTANT BIOTYPES MIGHT COME FROM DIFFERENT FITNESS

Table III : Growth percentage in comparison with the untreated (SS: homozygote susceptible – RS: heterozygote resistant – RR: homozygote resistant)

F1 Population	Sulfometuron (ALS)	Mesosulfuron+ Iodosulfuron (ALS)	Pinoxaden (ACCase)
2RR x 4SS	27	22	13
2RS x 2SS	98	90	41
5RS	87	87	7
6RR	120	130	12
ROTHAMSTED	20	19	18

DNA ANALYSES

dCAPS Analyses : The DNA of each of the **60 plants cloned for the glasshouse assay** was extracted and tested with dCAPS method.

Table IV : W574 mutation status of plants of five F1 populations tested in the glasshouse assay. (SS: homozygote susceptible – RS: heterozygote resistant – RR: homozygote resistant)

	SS	RS	RR
2RR x 4SS	8	2	0
2RS x 2SS	2	6	2
5RS	0	2	4
6RR	0	0	10
ROTHAMSTED	10	0	0

DNA RESULTS CORRESPOND INDEED TO THOSE OF THE GLASSHOUSE ASSAY

Sequencing

In order to validate the implementation of the dCAPS analysis method in our lab conditions, a sequencing was performed on four DNA samples previously analysed as:

- one homozygote resistant (population 169, Quevy (Belgium));
- one homozygote susceptible (population 174, Chastrès(B));
- two heterozygotes resistant (population 171, Blandain (B);

Results of the sequence comparison of the ALS gene, around the W574 position, corresponding here to the 1640th base, are presented in Figure 1. The first two lanes show resistant pattern (**G→T substitution, implying a Tryptophan to Leucine substitution**), corresponding to a homozygote resistant individual from population 169 (Quévy) and a heterozygote resistant allele of an individual from population 171 (Blandain). The third lane corresponds to the same individual's wild-type allele. The fourth lane fits to a susceptible individual from population 174 (Chastrès), whereas the last lane is the reference ALS gene sequence (Délye & Boucansaud, 2008). The modified codon (GTG → CCA) comes from the dCAPS method, with a modified primer so as to introduce the discriminating restriction point within the amplicons.

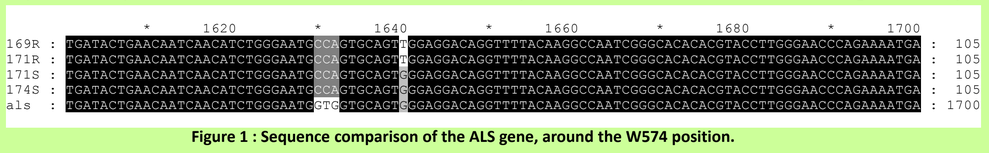


Figure 1 : Sequence comparison of the ALS gene, around the W574 position.

ALS SEQUENCE MUTATION CORRESPONDS

DISCUSSION

PETRI DISHES TEST

Populations issued from farmers' fields seem to be susceptible to sulfometuron, while they were tested as highly resistant to mesosulfuron+iodosulfuron in a previous glasshouse test, not presented here. So, these ones ought to be non target-site resistant. There is **quite no dose effect** in this assay, neither for susceptible populations nor for target-site resistant ones. Up to twenty times the recommended dose was implemented in that test with almost no effect on resistant seedlings growth. This confirms the postulate that increasing doses is useless in case of TSR.

GLASSHOUSE TEST

For Rothamsted population, the standard susceptible population, each herbicide achieved a growth reduction of 80 %. Scores for the 2RRx4SS progeny are really close to those of the standard population. This can be explained by the limited contribution of resistant alleles involved in the crossing. Considering growth plant per plant (results not presented in this paper), only one individual on ten of this progeny actually showed signs of resistance. **At the scale of one "population" and after only one generation (F1), the introduction of a few resistant individuals within a larger susceptible population appears to be difficult to quantify directly.**

Pinoxaden allows a good control of all TSR resistant F1 populations, since the mode of action is different, and so it is not affected by the mutation.

Scores for both ALS inhibiting herbicides are fairly equal for every F1 population, pointing out that the TSR is the main resistance mechanism involved in this case.

DNA ANALYSES

These DNA analyses allow to link glasshouse results with the exact genotype of each plant, which received three different herbicides. They also confirm the tendency of resistance level observed in glasshouse for each F1 population. Indeed, most individuals of the 2RRx4SS crossing are susceptible, while the progeny of the 2RSx2SS crossing is fairly resistant. Glasshouse results for these F1 populations showed for ALS inhibiting herbicides, growth of 80% and 20% respectively, which can be linked to 8 SS – 2 RS individuals and 2 SS – 6 RS – 2RR respectively. As expected, every individual from the 6RR F1 crossing are homozygote resistant, while those from Rothamsted are wild-type homozygote.

Results of the F1 crossing progeny (RRxSS) are not presented, neither for Petri Dish test nor for the glasshouse assay, because they are not reliable. **Some of the crossings did not work due to a lack of synchronization during flowering time between the plants.** This problem was mostly encountered for small groups of plants. It could be due to fitness difference, which could influence the development kinetics and thus accelerate (or delay) flowering stage for the heterozygote mutants. For instance, in the case of ACCase mutant alleles, fitness costs were highlighted for one mutation (Gly-2078), but not for other mutations (Menchari *et al.*, 2008). Further experiments have to be conducted in order to confirm this hypothesis. In the case of the 2RRx4SS crossing, the absence of resistant homozygotes in the tested progeny could come from a desynchronisation of the two RR during the crossing, preventing any matching between these two homozygotes. **The absence of RR might come from the weak probability of finding a RR individual within the progeny, according to the expected segregation proportions.**

CONCLUSION

The most reliable way to detect and to follow a mutation through generations is **DNA Analyses**. **Petri dishes** tests give coherent results and are advantageous for their convenience, their rapidity and the large amount of seeds that can be tested at a time. Their main drawback is that they do not reflect the behavior of the whole plant, once sprayed with herbicide, on the contrary of **glasshouse assay**. In this paper plants were previously cloned before getting sprayed with herbicide. These clones were used for **assessing the resistance of one plant towards several herbicides and linking this to one genotype**.

Experiments conducted for this paper aimed at assessing the heredity of the target-site mutation W574 endowing resistance towards ALS inhibiting herbicide. Globally, **these preliminary results do not highlight any major modifications of heredity rules, compared to expected Mendelean proportions.** The crossing involving two resistant individuals with four susceptible ones showed limited differences with a standard susceptible population, indicating that **after only one generation, it is rather difficult to detect any major effect of the mutation introduction within the population**. But it is impossible to draw definitive conclusions from the crossings that were implemented for these experiments, because of the **synchronization** problem between enclosed plants. This problem can come from **external factors**, such as biotic or abiotic stress, or obstacles to take roots down, but could also be due to **intrinsic characteristics, such as a different fitness**. Crossings have to be implemented again with more precautions to avoid these synchronization problems, and fitness study has to be conducted on these ALS TSR individuals.

The heredity of this ALS TSR consists in the ability of this mutation to spread within a susceptible population, represented in this test by a group of plant enclosed in a pollen-proof cloth, but that can be extended to a plot, to a field, or even at a regional-scale. Once this heredity will be characterized and linked with propagation abilities of resistant pollen (distance, viability), it will be possible to predict the resistance dispersal velocity. When combining these results with possible fitness costs associated with these mutations, it will be possible to refine present management strategies in order to **prevent ALS Target-Site Resistance wid-spreading**.

REFERENCES

Delye C & Boucansaud K (2008) A molecular assay for the proactive detection of target site-based resistance to herbicides inhibiting acetolactate synthase in *Alopecurus myosuroides*. *Weed Research* **48**, 97–101.

Hull, R. & Moss, S. R. (2007). The 'Rothamsted Rapid Resistance Test' for ALS inhibiting herbicides: black-grass *(Alopecurus myosuroides)*. *Rothamsted technical publication*, 7pp. Rothamsted Research, Harpenden, UK.

Marshall R & Moss SR (2008). Characterisation and molecular basis of ALS inhibitor resistance in the grass weed *Alopecurus myosuroides*. *Weed Research* **48**, 439–447.

Menchari Y., Chauvel B., Darmency H. & Délye C. (2008) Fitness costs associated with three mutant acetylcoenzyme A carboxylase alleles endowing herbicide resistance in black-grass *Alopecurus myosuroides, Journal of Applied Ecology,**45**, 939–947*

Moss, S. R. (1999). The 'Rothamsted Rapid Resistance Test' for detecting herbicide resistance in black-grass, wild-oats and Italian Rye-grass. *Rothamsted technical publication*, 16pp. Rothamsted Research, Harpenden, UK.

Petit C., Duhieu B., Boucansaud K., Délye C. (2010) Complex genetic control of non-target-site-based resistance to herbicides inhibiting acetyl-coenzyme A carboxylase and acetolactate-synthase in *Alopecurus myosuroides* Huds., *Plant Science* **178** 501–509